TTT ATA GAC CAG G-3′ and reverse 5′-AGA AAG CGT GCC ATA GGC AG-3′; $S1P_5$, forward 5′-GAG TGC CGG TTA CAG GAG ACT T-3′ and reverse 5′-CGC TGC TGT GTC CTG CC-3′; and glyceraldehyde-3-phosphate dehydrogenase, forward 5′-CTA CTG GCG CTG CCA AGG CAG T-3′ and reverse 5′-GCC ATG AGG TCC ACC ACC CTG-3′. PCR cycling conditions consisted of 95°C for 15 min followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s. Real-time PCR analysis of S1P receptors was expressed as the relative amount of the indicated mRNA normalized by that of glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis. Results are expressed as means ± SD. Groups of data were compared by the Mann-Whitney *U*-test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Treatment with FTY720 suppresses the development of colitis induced by adoptive transfer of naïve CD4+CD45RB^{high} T cells. We first tested whether FTY720 suppressed the development of colitis originally induced by the adoptive transfer of splenic CD4+CD45RB^{high} T cells from normal BALB/c mice into CB-17 SCID mice (Fig. 2*A*). Mice were administered daily with FTY720 (0.3 mg/kg) or DW orally starting 1 day before transfer. The control DW-treated mice manifested progressive weight loss from 2 wk after transfer (Fig. 2*B*) and clinical symptoms of colitis (Fig. 2*C*) such as diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 3–4 wk. In contrast, FTY720-treated mice appeared healthy with a gradual increase of body weight and without clinical symptoms during the whole period of observation (Fig. 2, *B* and *C*). In total, the assessment of colitis by clinical scores showed a clear difference between the control DW-treated mice and FTY720-treated mice (Fig. 2*D*). Histological examination showed a marked elongation of the villi with a massive infiltration of mononuclear cells in the LP of the colon from control DW-treated mice (Fig. 2*E*). In contrast, the elongation of the villi was mostly abrogated and only a few mononuclear cells were observed in the LP of the colon from FTY720-treated mice (Fig. 2*E*). This difference was also comfirmed by the histological scoring of multiple colon sections, which was 0.4 ± 0.8 in FTY720-treated mice versus 4.2 ± 0.8 in control DW-treated mice ($P < 0.05$; Fig. 2*F*).

A further quantitative evaluation of CD4+ T cell infiltration was made by isolating CD4+ T cells from each tissue or the peripheral blood. Only a few CD4+ T cells were recovered from the colonic tissue of FTY720-treated mice compared with control DW-treated mice (Fig. 3). The number of CD4+ cells recovered from the colon of control DW-treated mice (23.6 ± 18.4 × 10^5 cells) far exceeded the number of originally injected cells (3 × 10^5 cells), indicating extensive T cell migration and/or proliferation in the inflamed colon, which was mostly abrogated in FTY720-treated mice. Similarly, the numbers of CD4+ cells in the spleen, MLN, and peripheral blood from FTY720-treated mice was significantly decreased compared with those from control DW-treated mice (Fig. 3). In contrast, the number of CD4+ cells in the PLN from FTY720-treated mice was comparable with that in DW-treated mice (Fig. 3).

We next examined cytokine production by LP CD4+ T cells from control PBS-treated mice and FTY720-treated mice. As shown in Fig. 4, LP CD4+ cells from FTY720-treated mice produced significantly less TNF-α, IFN-γ, and IL-2 compared

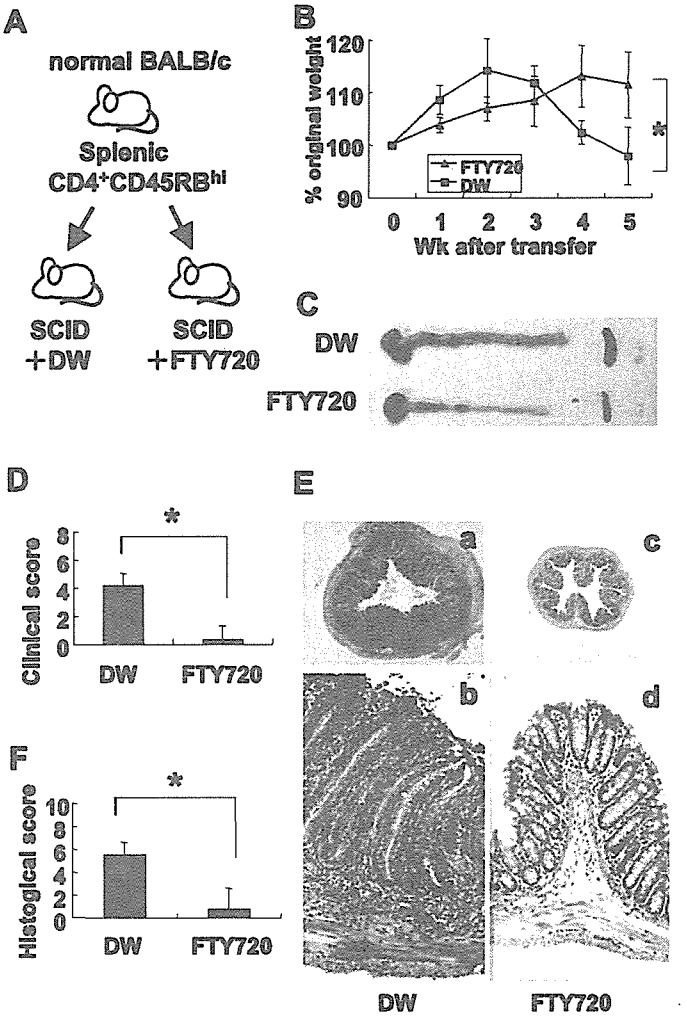

Fig. 2. Preventive effect of FTY720 on the development of CD4+CD45RB^{high} T cell-transferred colitis. *A*: 7 CB-17 SCID mice from each group were administered with FTY720 at a dose of 0.3 mg/kg or distilled water (DW) daily by a gavage for 5 wk starting from 1 day before CD4+CD45RB^{high} T cell transfer. *B*: change in body weight over time expressed as a percentage of the original weight. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice. *C*: gross appearance of the colon, spleen, and mesenteric lymph node (MLN) from CD4+CD45RB^{high} T cell-transferred severe combine immunodeficiency (SCID) mice treated with DW (*top*) or FTY720 (*bottom*) at 5 wk after transfer. *D*: clinical scores were determined at 24 days after transfer as described in MATERIALS AND METHODS. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice. *E*: histological examination of the colon from DW-treated (*left*) or FTY720-treated (*right*) mice at 24 days after transfer. Original magnification: ×40 in *a* and *c* and ×200 in *b* and *d*. *F*: histological scoring of colitis in DW-treated mice and FTY720-treated mice at 5 wk after transfer. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice.

with those from control DW-treated mice upon in vitro stimulation. In contrast, the production of IL-4, IL-5, or IL-10 was not significantly affected. These results suggested that FYT720 prevented the development of colitis primarily by promoting the sequestration of naïve CD4+ T cells and/or inhibiting the egress of colitogenic CD4+ T cells in the MLN, followed by inhibiting the development of pathogenic Th1 cells producing TNF-α, IFN-γ, and IL-2.

We further evaluated whether FTY720 affected cell differentiation of the transferred CD4+CD45RB^{high} T cells in vivo. As shown in Fig. 5, almost all CD4+ T cells in any tissue and

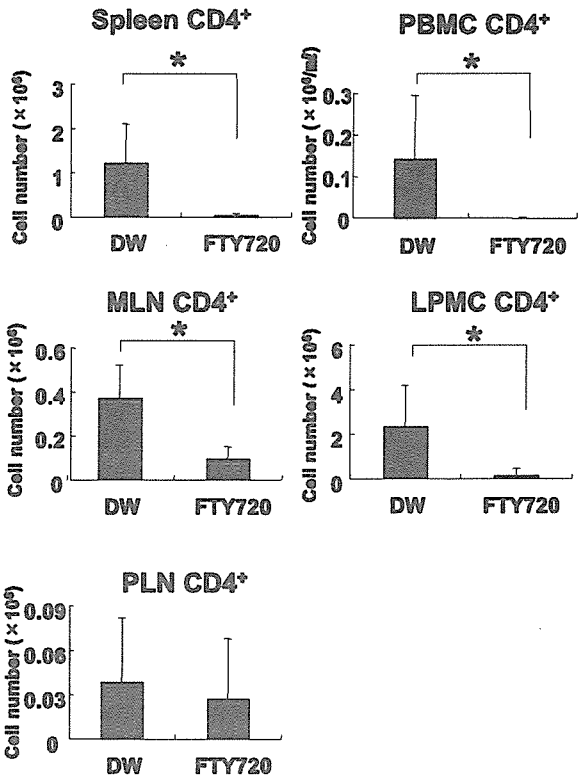

Fig. 3. Mononuclear cells from the spleen, PB, peripheral lymph node (PLN), MLN, and lamina propia (LP; LPMC) were isolated from the colon at 5 wk after transfer. Cells were stained with FITC-anti-CD3 and PE-anti-CD4, and the total number of CD4+ cells was determined by flow cytometry. Data are means ± SE of 7 mice/group. *$P < 0.01$. Data are indicated as the mean ± SD of seven mice in each group.

the blood from DW- or FTY720-treated SCID mice had a phenotype of T_{EM} cells, which express $CD62L^{-}CD44^{high}$, in contrast to the originally transferred $CD4^{+}CD45RB^{high}$ $CD62L^{+}CD44^{low}$ T cells (data not shown). The results indicated that cell activation/differentiation from naïve T cells to T_{EM} cells in this model was not impaired by FTY720 treatment.

Treatment with FTY720 suppresses the development of colitogenic CD4+ T_{EM} cell-mediated colitis. Although we found that FTY720 suppressed the development of chronic colitis induced by the adoptive transfer of naïve $CD4^{+}CD45RB^{high}$ T cells into SCID mice (Figs. 2–5), it was still unclear how FTY720 controlled the migration property of colitogenic memory T cells in memory T cell-mediated chronic colitis. Because it was possible that the effect of FTY720 in the $CD4^{+}CD45RB^{high}$ T cell transfer model was owing to the promotion of the sequestration of naïve $CD4^{+}$ T cells and/or to the inhibition of the egress of colitogenic $CD4^{+}$ memory T cells in the MLN, as previously demonstrated by others (3), we used our recently established colitogenic $CD4^{+}$ T_{EM} cell-mediated chronic colitis model (29) under the condition without impact of naïve T cells. CB-17 SCID mice were injected intraperitoneally with LP $CD4^{+}$ T_{EM} ($CD44^{high}$ $CD62L^{-}$; as shown in the *inset* in Fig. 6*A*) cells obtained from colitic SCID mice originally induced by $CD4^{+}CD45RB^{high}$ T cells and were treated with DW or FTY720 (0.3 mg/kg) daily starting 1 day before transfer over a period of 4 wk (Fig. 6*A*). As shown in Fig. 6*B*, control DW-administered mice manifested progressive weight loss from 2–4 wk after the transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 4 wk. In contrast, FTY720-treated mice appeared healthy without any clinical signs during the whole period of observation (Fig. 6*B*). At 4 wk after the transfer, the colon from control DW-treated mice, but not from FTY720-treated mice, was enlarged and had a greatly thickened wall (Fig. 6*C*). In total, the assessment of clinical scores showed a clear difference between control DW-treated mice and FTY720-treated mice (Fig. 6*D*). Histological examination showed a marked inflammation in the LP of the colon from control DW-treated mice (Fig. 6*E*). In contrast, it was mostly abrogated in the LP of the colon from FTY720-treated mice (Fig. 6, *E* and *F*).

Furthermore, a few $CD4^{+}$ T cells were recovered from the colonic tissue of FTY720-treated mice compared with control DW-treated mice (Fig. 7). The numbers of $CD4^{+}$ cells recovered from the colon of control DW-treated mice ($91.4 \pm 63.7 \times 10^5$ cells) far exceeded the number of originally injected memory cells (5×10^5 cells), indicating extensive T cell migration and/or proliferation in the inflamed colon, which was mostly abrogated in FTY720-treated mice. Similarly, the numbers of $CD4^{+}$ cells in the spleen and peripheral blood from FTY720-treated mice was significantly decreased compared with control DW-treated mice (Fig. 7). Somewhat at odds, the numbers of $CD4^{+}$ cells in the MLN from FTY720-treated mice was comparable with that from DW-treated mice (Fig. 7). Unlike the $CD4^{+}CD45RB^{high}$ T cell-transferred model, transferred colitogenic LP $CD4^{+}$ T_{EM} cells could not be detected in the PLN.

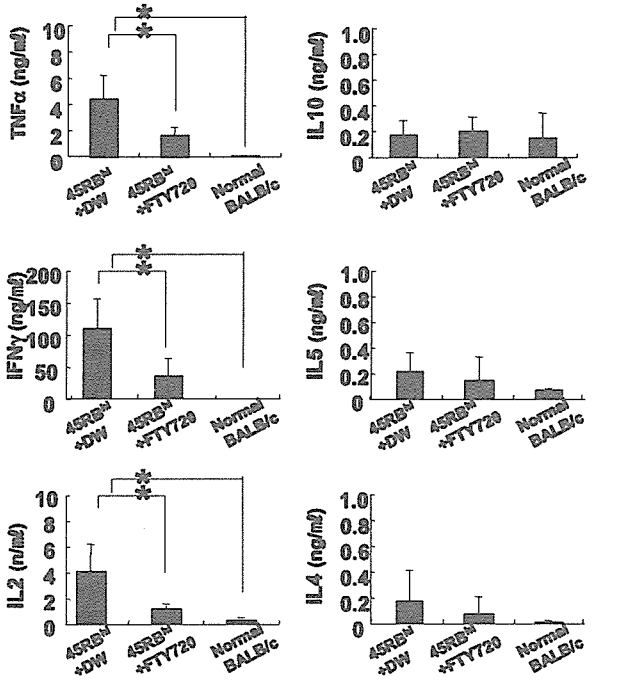

Fig. 4. Cytokine production by LP $CD4^{+}$ T cells. LP $CD4^{+}$ T cells were isolated from PBS- or FTY720-treated mice 5 wk after $CD4^{+}CD45RB^{high}$ T cell transfer or normal BALB/c mice (12 wk old) and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies for 48 h. Interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10, and tumor necrosis factor (TNF)-α concentrations in culture supernatants were measured by a specific ELISA (IL-10) or a mouse T helper (Th)1/Th2 cytokine bead array kit (IL-2, IL-4, IL-5, TNF-α, and IFN-γ). Data are means ± SD of 6 mice/group. *$P < 0.05$.

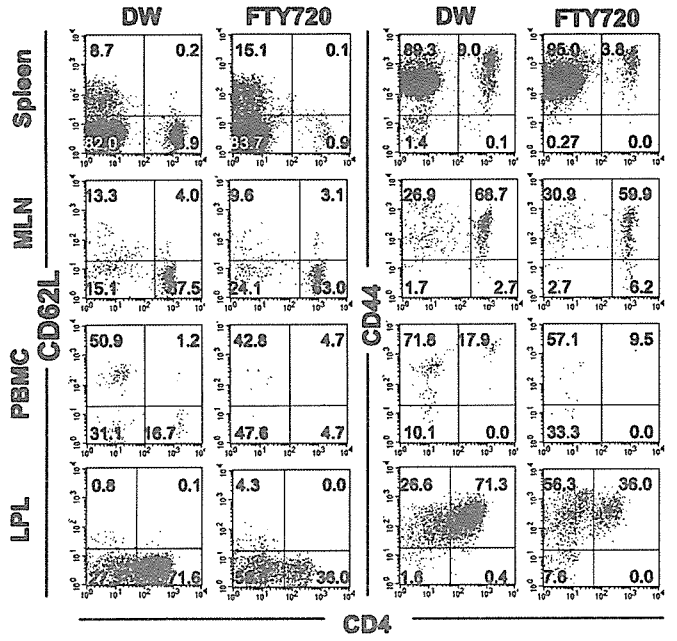

Fig. 5. Expression of CD62L and CD44 on CD4+ T cells in various organs and the blood of DW-treated or FTY720-treated mice transferred with CD4+CD45RB^{high} T cells. Freshly isolated cells from DW-treated or FTY720-treated mice transferred with CD4+CD45RB^{high} T cells at 5 wk after transfer were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD62L or PE-labeled anti-CD44 monoclonal antibodies. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and sidescatter profiles. LPL, LP lymphocytes. Data are displayed as a dotted plot (4-decade log scale), and quadrant markers were positioned to include >98% of control Ig-stained cells in the *bottom left*. Percentages in each quadrant are indicated. Results shown are representative of 3 mice/group.

Cytokine production by LP CD4+ T cells from control PBS- or FTY720-treated mice transferred with colitogenic LP CD4+ T_{EM} cells was assessed. As shown in Fig. 8, LP CD4+ cells from FTY720-treated mice produced significantly less TNF-α, IFN-γ, and IL-2 compared with control DW-treated mice upon in vitro stimulation. In contrast, the production of IL-4, IL-5, or IL-10 was not significantly affected.

We further determined whether FTY720 affected the cell differentiation of transferred CD4+CD62L−CD44^{high} T_{EM} cells in vivo, because we initially speculated that memory T cells residing in lymph nodes preferentially express CD62L, which is termed as central memory T (T_{CM}) cells. As shown in Fig. 9, however, almost all CD4+ T cells in any organ and the blood from DW-treated or FTY720-treated SCID mice retained the characteristics of T_{EM} cells, including the MLN, indicating that FTY720 did not affect the T_{EM}-to-T_{CM} conversion in this model.

Colitogenic CD4+ T_{EM} cells express receptors for S1P. Because it has been reported FTY720-phosphate acts via S1P receptors (5), we finally evaluated the expression pattern of S1P receptors in sorted normal splenic CD4+, CD4+ CD45RB^{high}, and colitic LP CD4+ T_{EM} cells using quantitative PCR analysis. As depicted in Fig. 10, colitogenic LP T_{EM} cells contained mRNA encoding $S1P_1$, $S1P_2$, and $S1P_4$ receptors, albeit to a lesser extent compared with naïve CD4+ CD45RB^{high} T cells, with only a minimal representation of $S1P_3$ and $S1P_5$ receptors.

DISCUSSION

In the present study, we demonstrated that FTY720 suppressed the development of colitis induced by the adoptive transfer of colitogenic LP T_{EM} cells into SCID mice under the condition without the impact of naïve T cell migration. In addition, we also found that FTY720 ameliorates the development of colitis induced by the adoptive transfer of naïve

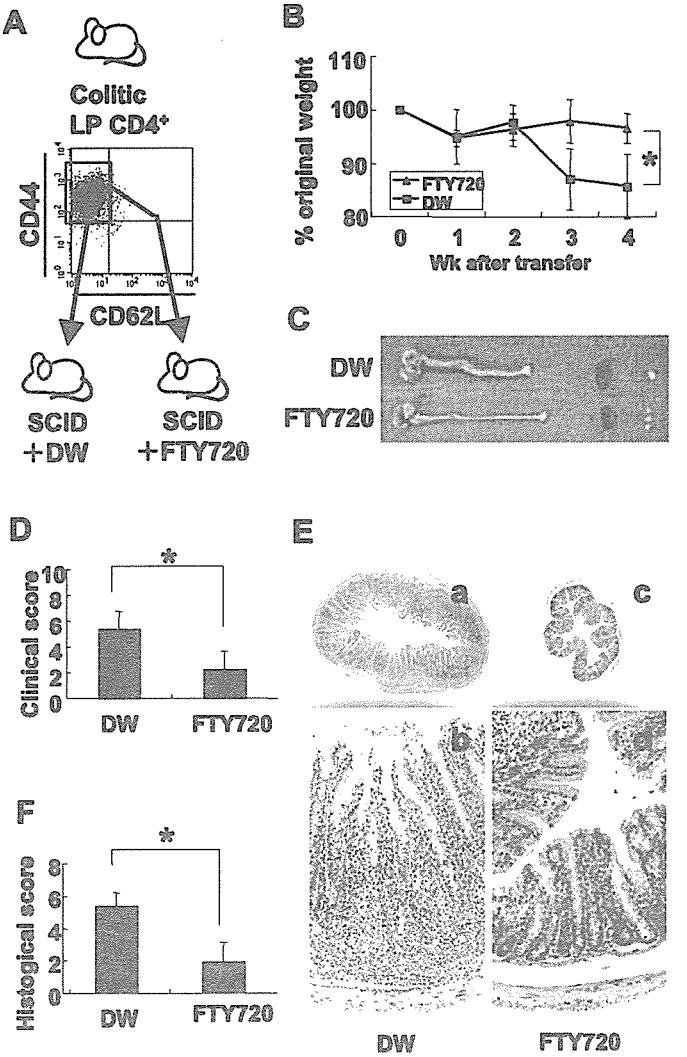

Fig. 6. Effect of FTY720 on the development of colitogenic CD4+ effector memory T cell (T_{EM} cell)-mediated colitis. *A*: colitogenic CD4+ T_{EM} cells (CD44^{high}CD62L−; as shown in the *inset* by fluorescence-activated cell sorting) were isolated from inflamed mucosa of colitic mice transferred with CD4+CD45RB^{high} T cells. Seven CB-17 SCID mice from each group were administered with FTY720 at a dose of 0.3 mg/kg or DW daily by a gavage for 4 wk starting from 1 day before colitogenic CD4+ T_{EM} cell transfer. *B*: changes in body weight over time expressed as a percentage of the original weight. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice. *C*: gross appearance of the colon, spleen, and MLN from colitogenic CD4+ T_{EM} cell-transferred SCID mice treated with DW (*top*) or FTY720 (*bottom*) at 4 wk after transfer. *D*: clinical scores were determined at 4 wk after transfer as described in MATERIALS AND METHODS. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice. *E*: histological examination of the colon from DW-treated (*left*) or FTY720-treated (*right*) mice at 4 wk after transfer. Original magnification: ×40 in *a* and *c* and ×200 *b* and *d*. *F*: histological scoring of colitis in DW- and FTY720-treated mice at 4 wk after transfer. Data are means ± SE of 7 mice/group. *$P < 0.05$ compared with DW-treated mice.

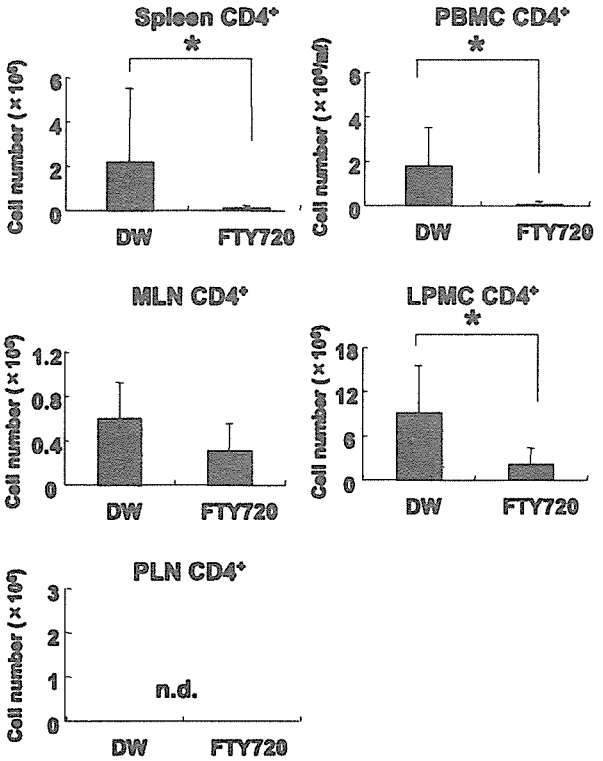

Fig. 7. Mononuclear cells from the spleen, peripheral blood, PLN, MLN, and LP were isolated from the colon at 4 wk after transfer. Cells were stained with FITC-anti-CD3 and PE-anti-CD4, and the total number of CD4+ cells was determined by flow cytometry. Data are means ± SE of 7 mice/group. ND, not determined. *$P < 0.05$.

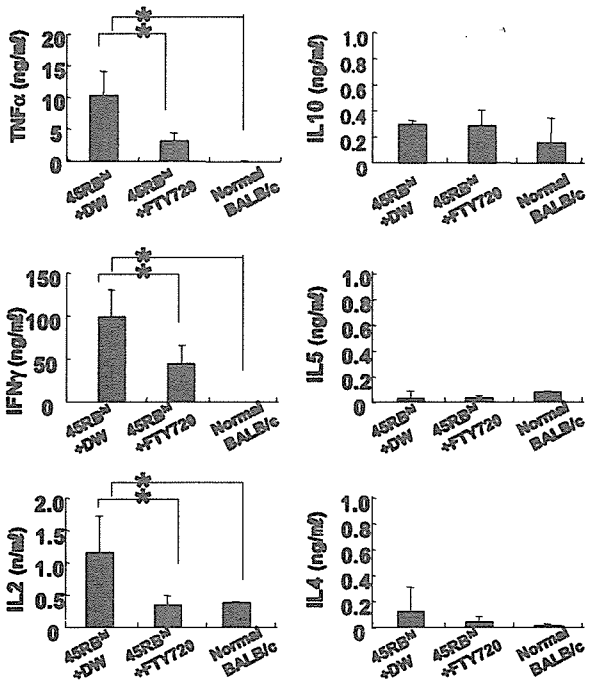

Fig. 8. Cytokine production by LP CD4+ T cells. LP CD4+ T cells were isolated from control PBS- or FTY720-treated mice 4 wk after the transfer of colitogenic LP CD4+ TEM cells and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies for 48 h. Cytokine production was measured by the same methods as those described in Fig. 4. Data are means ± SD of 6 mice/group. *$P < 0.05$.

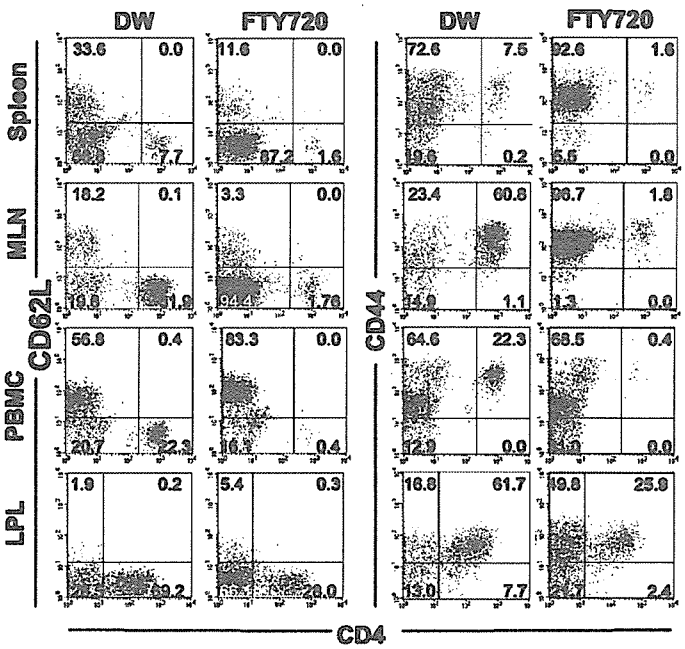

Fig. 9. Expression of CD62L and CD44 on CD4+ T cells in various organs and the blood of DW- or FTY720-treated mice transferred with colitogenic CD4+ TEM cells. Freshly isolated cells from DW- or FTY720-treated mice transferred with colitogenic CD4+ TEM cells at 24 days after transfer were analyzed by flow cytometry. Percentages in each quadrant are indicated. Results shown are representative of 3 mice/group.

CD4+CD45RB^{high} T cells into SCID mice, in which all processes responsible for the development of colitis, such as the initiation of naïve T cells by antigen-bearing dendritic cells and the elicitation to effector or TEM cells in secondary lymphoid organs, are involved. Because we demonstrated that colitogenic LP TEM cells as well as normal splenic CD4+CD45RB^{high} T cells expressed several S1P receptors that are targets for FTY720-phosphate, the present study provides the suggestion that FTY720 can directly control memory CD4+ T cell-mediated immune diseases in addition to the impact of naïve T cells.

Because it has been largely thought that the effect of FTY720 treatment on autoimmune diseases in the previous preventive protocols is owing to the sequestration of naïve T cells rather than pathogenic memory T cells, it was unclear

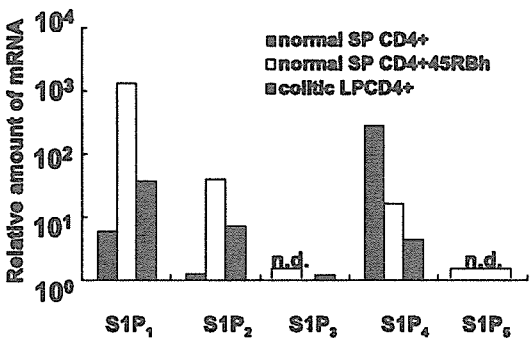

Fig. 10. Quantitative PCR analysis of spingonsine-1-phosphate (S1P) receptors in sorted normal splenic CD4+, CD4+CD45RB^{high}, and colitic LP CD4+ TEM cells expressed as the relative amounts of indicated mRNA normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Results shown are representative of 3 experiments.

whether FTY720 directly affects the migration property of memory T cells in autoimmune established stages. To overcome this issue, we conducted the adoptive transfer of only colitogenic LP CD4$^+$ T_{EM} cells into SCID mice in the present study. As there were no naïve T cells in this transfer system because almost all isolated LP CD4$^+$ T cells from colitic mice had a characteristic of CD44^{high}CD62L$^-$CD45RB^{low} T_{EM} cells, we could directly evaluate the effect of FTY720 on colitogenic LP CD4$^+$ T_{EM} cells. Surprisingly, we found that FTY720 suppressed the development of colitogenic memory T_{EM} cell-mediated colitis, indicating that FTY720 affects the migration of colitogenic LP CD4$^+$ T_{EM} cells. Consistent with this, we demonstrated that colitogenic LP T_{EM} cells as well as normal splenic CD4$^+$CD45RB^{high} T cells expressed several S1P receptors ($S1P_1$, $S1P_2$, and $S1P_4$). However, we found that the numbers of CD4$^+$ T cells in the MLN were also significantly decreased in FTY720-treated mice transferred with colitogenic LP T_{EM} cells compared with DW-treated mice. Although the result might indicate that FTY720 could not sequester colitogenic CD4$^+$ T_{EM} cells in FTY720-treated mice, it is more likely that colitogenic CD4$^+$ T_{EM} cells in PBS-treated mice had a greater chance to repeat the migration into the MLN and the egress from the MLN to sites of inflammation and could expand their number, which exceeded the original transferred cell number (3×10^5 cells). However, it is possible that the finding that there were decreased numbers of CD4$^+$ T cells in the various compartments may be a result of the lack of the colitis in FTY720-treated mice and not necessarily a direct effect of FTY720 on egress or sequestration, because we could not detect any differences of cell numbers in the MLN between T_{EM} cell-transferred DW-treated mice and FTY720-treated mice. Nonetheless, we found that FTY720 caused a significant loss of CD4$^+$ T_{EM} cells from the blood (lymphopenia) and spleen, reducing numbers by ~100- to 1,000-fold compared with DW-treated mice, indicating that FTY720 induced a smaller chance to recirculate into the MLN repeatedly, followed by no development of colitis. Further studies will be needed to address this issue using another short-term in vivo transfer system.

The present study would provide another impact in terms of a characteristic of memory T cell trafficking for the maintenance of autoimmune diseases. Although some investigators have suggested that LP T cells do not migrate out of the gut (12), our results indicated that LP CD4$^+$ T_{EM} cells are needed to constitutively recirculate into the MLN and are restimulated by antigen-bearing dendritic cells in the MLN to maintain colitogenic CD4$^+$ T_{EM} cells for sustaining chronic colitis. An earlier study (32) has demonstrated that the thoracic duct lymph (TDL), which empties into the blood, contains many lymphocytes. In a landmark experiment, Gowans and Knight (7) transferred radiolabeled thoracic duct lymphocytes intravenously into naïve syngeneic recipients and detected donor memory lymphocytes in their TDL again, providing the first formal demonstration that memory lymphocytes recirculate continuously between blood and lymph. Consistent with this notion, we also found that LP CD4$^+$ T cells obtained from FTY720-treated SCID mice transferred with colitogenic CD4$^+$ T_{EM} cells in FTY720-treated mice produced less amounts of Th1 cytokines compared with DW-treated SCID mice, indicating that the recirculation and restimulation of Th1 cells in lymph nodes, in this case, the MLN, are essential for the maintenance of Th1-mediated autoimmune disorders.

In nohuman primates treated with FTY720 for over 100 days, however, ~10% of peripheral blood CD4$^+$ T cells were refractory to depletion by FTY720 (23). It is likely that these cells represent long-lived T_{EM} cells. Because T_{EM} cells lack the lymph node homing receptors CCR7 and CD62L (28) and express high levels of inflammatory chemokine receptors (28), and they therefore could preferentially reside in nonlymphoid tissues (16) in contrast to naïve and T_{CM} cells. Thus it is possible that T_{EM} cells might not be trapped by FTY720 treatment in the lymph node but be controlled by the unidentified mechanism of action of FTY720 in our model. Conversely, Henning and colleagues (9) recently reported that FTY720 rescues the homing defect in both CCR7$^{-/-}$ mice and plt mice, which lack expression of CCL19 and CCL21-ser, both ligands for CCR7 on high endothelial cells (9), suggesting that FTY720 enables T_{EM} cells to sequester into lymph nodes in a CCR7/CD62L-independent mechanism for lymphocyte homing through endothelial venules, which is strongly augmented in the presence of FTY720. Further studies will be needed to address this issue.

The ultimate goal of any treatment for autoimmune diseases, including IBDs, is antigen-specific suppression of pathology. Autoaggressive antigen-specific lymphocytes need to be eliminated or controlled to prevent tissue damage and halt the progression of clinical disease. Although it is so far poorly understood regarding the self-antigens and commensal bacteria flora responsible for the pathogenesis of IBDs, colitogenic memory T cells rather than naïve T cells should be the targets for the treatment of IBDs, even if the primary, initiating self-antigens/commensal bacteria flora are unknown and inflammation is progressive (19, 25). Although Mizushima and colleagues (18) recently demonstrated that FTY720 treatment of established colitis in IL-10$^{-/-}$ mice ameliorated colitis, it was still unclear whether FTY720 affected the migration of naïve T cells or colitogenic memory T cells. In light of this, the present SCID colitis model induced by the adoptive transfer of colitogenic CD4$^+$ T_{EM} cells is a useful model, because we can assess a character of memory T cells without the impact of naïve T cells. Because we demonstrated that FTY720 could control and suppress colitogenic CD4$^+$ T_{EM} cell-mediated colitis, FTY720 might be beneficial for the treatment of ongoing or established IBDs as well as their prevention.

In conclusion, we demonstrated here that FTY720 was useful to suppress memory T cell-mediated chronic colitis without the impact of naïve T cell activation using memory CD4$^+$ T_{EM} cell adoptive transfer experiments. Although we have to pass many critical checkpoints, this study indicates that FTY720 offers a hope for the treatment of human IBDs.

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Naturally arising CD4+CD25+ regulatory T cells suppress the expansion of colitogenic CD4 +CD44high CD62L– effector memory T cells

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Naturally arising $CD4^+CD25^+$ regulatory T cells suppress the expansion of colitogenic $CD4^+CD44^{high}CD62L^-$ effector memory T cells

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Kanai, T., K. Tanimoto, Y. Nemoto, R. Fujii, S. Makita, T. Totsuka, and M. Watanabe. Naturally arising $CD4^+CD25^+$ regulatory T cells suppress the expansion of colitogenic $CD4^+CD44^{high}CD62L^-$ effector-memory T cells. *Am J Physiol Gastrointest Liver Physiol* 290: G1051–G1058, 2006. First published December 22, 2005; doi:10.1152/ajpgi.00429.2005.—Naturally arising $CD4^+CD25^+$ regulatory T (T_R) cells have been shown to prevent and cure murine T cell-mediated colitis. However, their exact mechanism of controlling colitogenic memory $CD4^+$ T cells in in vivo systems excluding the initial process of naive T cell activation and differentiation has not been examined to date. Using the colitogenic effector memory (T_{EM}) $CD4^+$ cell-mediated colitis model induced by adoptive transfer of colitogenic $CD4^+CD44^{high}CD62L^-$ lamina propria (LP) T cells obtained from colitic $CD4^+CD45RB^{high}$ T cell-transferred mice, we have shown in the present study that $CD4^+CD25^+$ T_R cells are able not only to suppress the development of colitis, Th1 cytokine production, and the expansion of colitogenic LP $CD4^+$ T_{EM} cells but also to expand these cells by themselves extensively in vivo. An in vitro coculture assay revealed that $CD4^+CD25^+$ T_R cells proliferated in the presence of IL-2-producing colitogenic LP $CD4^+$ T_{EM} cells at the early time point (48 h after culture), followed by the acquisition of suppressive activity at the late time point (96 h after culture). Collectively, these data suggest the distinct timing of the IL-2-dependent expansion of $CD4^+CD25^+$ T_R cells and the their suppressive activity on colitogenic LP $CD4^+$ T_{EM} cells.

murine colitis model; interleukin-2

THE ULTIMATE GOAL OF ANY TREATMENT for autoimmune diseases, including inflammatory bowel diseases (IBDs), is antigen- and/or site (including regional lymph node)-specific suppression of pathology. Autoaggressive lymphocytes need to be eliminated or controlled to prevent tissue damage and halt the progression of clinical disease. Although the self-antigens and commensal bacterial flora responsible for the pathogenesis of IBDs is poorly understood to date, strong evidence is emerging that the induction of regulatory T (T_R) cells can suppress disease, even if the primary initiating self-antigens and/or commensal bacterial flora are unknown and inflammation is progressive (6, 8, 15).

$CD4^+CD25^+$ T cells have been shown to be potent T_R cells in a number of murine models as well as in rats and humans (12). Functional analysis of murine $CD4^+CD25^+$ T_R cells has shown that those cells, which constitutively express inhibitory cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), and Foxp3 transcription factor (Forkhead box Foxp3 transcriptional isoform) (10, 12, 17), failed to proliferate or secret cytokines in response to polyclonal or antigen-specific stimulation in the in vitro system (13, 18). $CD4^+CD25^+$ T_R cells suppress the proliferation of responder $CD4^+CD25^-$ T cells in a cell contact-dependent manner (13, 18). However, their effect in vivo appears to depend in some but not all systems on IL-10 and/or TGF-β expression (1, 14). An advantage of these T_R cells is their ability to act as bystander suppressors and dampen the inflammation (19), which was recently followed by the demonstration that naturally arising $CD4^+CD25^+$ T_R not only prevented the development of colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ naive T cells into severe combined immunodeficient (SCID) mice (10) but also cured the established colitis (6, 8). Although these studies suggest that manipulation of $CD4^+CD25^+$ T_R cells may be beneficial in the treatment of patients with IBD, it is still unclear whether the late administration of $CD4^+CD25^+$ T_R cells (10 days–4.5 wk after transfer of $CD4^+CD45RB^{high}$ cells) suppresses the pathogenic effector memory T (T_{EM}) cells or the residual $CD4^+CD45RB^{high}$ naive T cells in the gastrointestinal system.

In this study, we have attempted to clarify the exact nature of the suppressive activity of $CD4^+CD25^+$ T_R cells against pathogenic/colitogenic memory $CD4^+$ T cells using the colitogenic lamina propria (LP) $CD4^+$ effector T_{EM}-mediated colitis model induced by adoptive transfer of the colitogenic $CD4^+CD62L^-CD44^{high}$ T cells obtained from established colitic $CD4^+CD45RB^{high}$ T-cell-transferred mice.

MATERIALS AND METHODS

Mice. Female BALB/c, C.B-17 scid/scid (SCID), C57BL/6-Ly5.2, and C57/BL6 recombinase-activating gene (*RAG*)-2-knockout mice (*RAG-2*-KO; Ly5.2) were purchased from Japan Clear (Tokyo, Japan). C57BL/6-Ly5.1 mice were obtained from The Jackson laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used at 6–12 wk of age according to the guidelines of the Institutional Committee on Animal Research in Tokyo Medical and Dental University and were approved by the committee.

Antibodies. The following MAbs were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: Fc-γ (CD16/CD32)-blocking MAb (2.4G2); FITC-, phenylephrine (PE)-, and CyChrome-conjugated anti-mouse CD4 (RM4-5); FITC- and PE-conjugated anti-mouse CD25 (7D4); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); purified anti-murine CD3e (145-2C11); purified anti-murine CD28 (37.51); purified anti-murine IL-2 (JES6-5H4); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG.

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Induction of colitis induced by the adoptive transfer of CD4+CD45RB^{high} T cells. Colitis was induced in SCID mice by adoptive transfer of CD4+CD45RB^{high} T cells as described previously (9). Briefly, CD4+ T cells were isolated from splenocytes from BALB/c mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched CD4+ T cells were labeled with PE-conjugated anti-mouse CD4 MAb and FITC-conjugated anti-CD45RB MAb and sorted into CD45RB^{high} (highest staining 30%) and CD45RB^{low} (lowest staining 30%) fractions on a FACSVantage (Becton Dickinson, Sunnyvale, CA). Each SCID mouse underwent intraperitoneal injection with 3×10^5 CD4+CD45RB^{high} T cells. The colitic CD4+CD45RB^{high} T-cell-transferred SCID mice were killed 6–8 wk after transfer to isolate the colitogenic LP CD4+ T_{EM} cells (20). The entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}- and Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington Biochemical) for 2 h. The cells were pelleted twice through a 40% isotonic Percoll gradient solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%-75%). Enriched CD4+ LP T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells analyzed using FACSCalibur contained >96% CD4+ cells.

Flow cytometry. Flow cytometric two-color analysis was performed as described previously (4). Isolated LP mononuclear cells (LPMCs) from colitic CD4+CD45RB^{high} cell-transferred SCID mice or age-matched normal BALB/c mice were preincubated with Fc-γ receptor-blocking MAb for 20 min, followed by incubation with CyChrome-conjugated anti-mouse CD4 MAb and PE-conjugated anti-CD45RB, anti-CD44, anti-CD62L, or anti-CD69 MAb for 30 min on ice. After cells were stained, flow cytometry and data analysis were performed using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

In vitro proliferation assay. As antigen-presenting cells (APCs), CD4− cells were prepared from BALB/c splenocytes by depleting CD4+ cells with anti-CD4 MACS beads and treated with 50 μg/ml mitomycin C (MMC) for 45 min at 37°C. To obtain splenic CD4+CD25+ T cells, enriched CD4+ splenocytes were stained with PE-conjugated anti-mouse CD25 MAb and FITC-conjugated anti-CD4 MAb and sorted into CD4+CD25+ and CD4+CD25− subpopulations on a FACSVantage. In coculture experiments, splenic CD4+CD25+ or CD4+CD25− T cells (0, 1.0, 2.0, or 4.0×10^4 as T_R or control non-T_R cells) were cultured with splenic CD4+CD25− T cells (1×10^4 as control responders) or colitogenic memory CD4+ T cells (1×10^4 as responders) and MMC-treated CD4− cells (1×10^5 as APCs) in round-bottomed 96-well plates in RPMI 1640 medium containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (Complete medium) supplemented with soluble anti-CD3 MAb (145-2C11, 50 ng/ml colitogenic memory LP CD4+ cells, 5 μg/ml CD4+CD25− cells; BD Pharmingen). In some experiments, blocking MAb against IL-2 was added at 0, 2, or 10 μg/ml from the beginning of culture. To determine proliferation, each well was pulsed with 1.0 μCi of [^{3}H]thymidine (NEN, Boston, MA) for the last 9 h of 48- or 96-h culture. For cytokine assay, the supernatants, which were removed before addition of [^{3}H]thymidine at proliferation assays, were collected and analyzed by a specific ELISA (R&D Systems, Minneapolis, MN). In some experiments, cells were labeled with 1.0 ml of PBS-1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37°C, followed by addition of 1.0 ml of FCS for 2 min, and then were washed three times in PBS. CFSE-labeled Ly5.1+CD4+ responder cells (1×10^5) were cocultured with CFSE-labeled Ly5.2+CD4+CD25+ T_R cells (1×10^5) with APC (5×10^5) and anti-CD3 (50 ng/ml for LP CD4+ responders) in 96-well round-bottomed plates for 120 h in triplicate experiments. After incubation, cells were collected, stained for Ly5.1 or Ly5.2, and analyzed using FACS. Propidium iodide was added to exclude dead cells. Proliferation analysis was based on the division times of CFSE+CD4+ T cells.

Induction of colitis induced by adoptive transfer of colitogenic LP CD4+CD44^{high}CD62L− T_{EM} cells. SCID mice underwent intraperitoneal injection with sorted CD4+ T cell subpopulations in PBS. These mice were administered 4×10^5 colitogenic LP CD4+CD44^{high}CD62L− T_{EM} cells obtained from inflamed mucosa of CD4+CD45RB^{high} cell-transferred SCID mice alone or in combination with splenic 4×10^5 CD4+CD25+ T_R cells. As controls, mice were administered 4×10^5 splenic or mesenteric lymph node CD4+CD44^{high}CD62L− T cells obtained from CD4+CD45RB^{high} cell-transferred SCID mice. After this cell transfer was performed, the recipient SCID mice were weighed initially and then three times per week afterward. They also were observed for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. Mice were killed 4 wk after T cell transfer and assessed for clinical scores (20) representing 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; and 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and 3, bloody stool).

Tissue samples were fixed in PBS containing 6% neutral buffered formalin. Paraffin-embedded sections (5 μm thick) were stained with hematoxylin and eosin. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. The most affected area was graded according to 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 (4). After lipoprotein lipases (LPLs) and splenocytes were isolated using the above-mentioned method, these cells were stained with FITC-conjugated anti-CD3 and PE-conjugated anti-CD4+ MAbs and the percentage of CD3+CD4+ T cells were analyzed using FACSCalibur, followed by calculation of the total number of CD4+ T-cells.

To measure cytokine production, 1×10^5 freshly isolated LP CD4+ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates precoated with 5 μg/ml anti-mouse CD3 MAb (145-2C11) and 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 performing specific ELISA (IL-10) (R&D Systems, Minneapolis, MN) or mouse a Th1/Th2 cytokine CBA kit (IL-2, IL-4, IL-5, TNF-α, and IFN-γ; BD Biosciences, San Jose, CA) according to the manufacturer's recommendations.

Statistical analysis. The results are expressed as means ± SD. Groups of data were compared using the Mann-Whitney *U*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Interaction between CD4+CD25+ T_R cells and colitogenic T_{EM} cells. Efforts to delineate T_R cell population have revealed that CD4+CD25+ T cell populations in mice and humans retain T_R function (12). However, the mechanism by which naturally arising CD4+CD25+ T_R cells control pathogenic CD4+ T_{EM} cells in autoimmune diseases is not fully understood. To clarify this mechanism, we assessed the in vivo T_R activity of CD4+CD25+ T_R cells from normal BALB/c spleen against the isolated colitogenic memory CD4+ T_{EM} cells. To study CD4+ T_{EM} cells, we first isolated LP CD4+ T cells of Th1-mediated colitic C.B-17 SCID mice by inducing the adoptive transfer of BALB/c splenic CD4+CD45RB^{high} T cells (9).

As shown in Fig. 1, flow cytometric analysis revealed that the colitic LP CD4+ T_{EM} cells were $CD44^{high}CD69^+CD62L^-$, indicating that they were activated T_{EM} cells. In contrast, normal splenic CD4+ T cells express $CD44^{high-low}CD69^-CD62L^{+/-}$.

We next sought to determine whether splenic $CD4^+CD25^+$ T_R cells suppress the proliferation of colitic LP CD4+ T_{EM} cells in vitro. In a 48-h coculture assay, $CD4^+CD25^+$ cells significantly suppressed the proliferation of normal splenic $CD4^+CD25^-$ responders at 0.5-to-4.0 T_R-to-responder ratios, but they did not suppress that of colitic LP CD4+ T_{EM} cells at any ratio (Fig. 2*A*). Interestingly, [^{3}H]thymidine uptake of coculture with $CD4^+CD25^+$ T cells and the colitic LP CD4+ T_{EM} cells was conversely increased in parallel with the increased numbers of splenic $CD4^+CD25^+$ T_R cells. In the late assay performed 96 h after culture, however, $CD4^+CD25^+$ cells significantly suppressed both the proliferation of splenic $CD4^+CD25^-$ cells at 0.5-to-4.0 responder-to-T_R ratio and the colitic LP CD4+ cells at 1.0-to-4.0 T_R-to-responder ratio (Fig. 2*B*).

To determine which type of cocultured cells proliferate and which type is suppressed, we next conducted a per-cell proliferation assay using CFSE labeling. After normal splenic C57/BL6J-Ly5.2 $CD4^+CD25^+$ T_R cells and the colitic LP memory CD4+ T_{EM} cells obtained from colitic C57/BL6J-Ly5.1 $CD4^+CD45RB^{high}$ T cell-transferred mice were labeled with CFSE, one or two subpopulations were stimulated for 120 h with anti-CD3 MAb and unlabeled, MMC-treated C57/BL6J-Ly5.2 $CD4^-$ APCs. Colitic LP CD4+ T_{EM} cells did proliferate in the absence of $CD4^+CD25^+$ T_R cells (Fig. 3*A*, *top*). In contrast, $CD4^+CD25^+$ T_R cells did not proliferate in the absence of colitic CD4+ T_{EM} cells (Fig. 3*A*, *middle*). Importantly, $CD4^+CD25^+$ T_R cells expanded in the presence of colitic CD4+ T_{EM} cells, whereas the proliferation of colitic LP CD4+ T cells conversely was suppressed in the presence of $CD4^+CD25^+$ T_R cells compared with colitic LP CD4+ T_{EM} cells cultured without $CD4^+CD25^+$ cells (Fig. 3*A*, *bottom*). These data indicate that $CD4^+CD25^+$ T_R cells not only suppressed the expansion of colitic LP CD4+ T_{EM} cells but also expanded by themselves via factors produced by the colitic LP CD4+ T cells or through intercellular interaction between $CD4^+CD25^+$ T_R cells and colitic LP CD4+ T_{EM} cells.

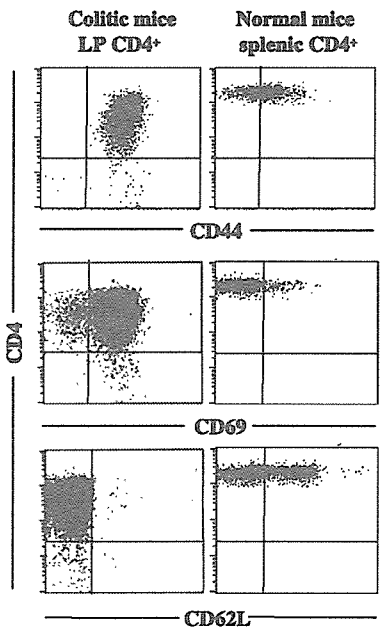

Fig. 1. Scatterplots showing the phenotypic characterization of normal splenic CD4+ T cells and colitic lamina propria (LP) CD4+ T cells induced by adoptive transfer of normal splenic $CD4^+CD45RB^{high}$ T cells.

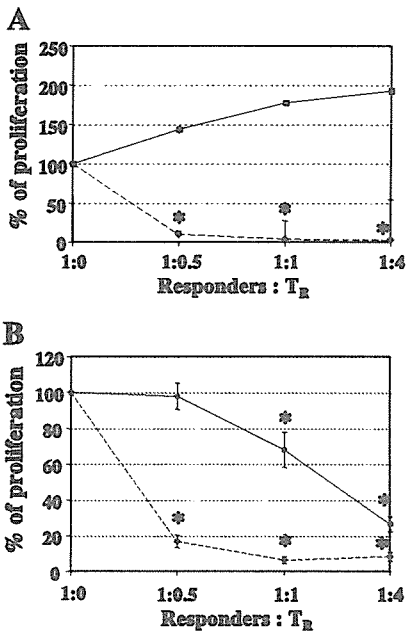

Fig. 2. Different timing of the suppressive activity of $CD4^+CD25^+$ regulatory T (T_R) cells plotted against colitogenic memory LP CD4+ colitogenic effector memory (T_{EM}) cells. $CD4^+CD25^+$ cells suppressed the proliferation of LP CD4+ T_{EM} cells in 96-h coculture assay (*B*) but not in 48-h coculture assay (*A*), whereas $CD4^+CD25^+$ T_R cells suppressed the proliferation of normal splenic $CD4^+CD25^-$ responders in both 48-h (*A*) and 96-h (*B*) coculture assays. Colitogenic LP CD4+ T_{EM} cells or $CD4^+CD25^-$ responders were incubated with soluble anti-CD3 MAb (50 ng/ml concentration for colitogenic LP CD4+ T_{EM} cells and 5 μg/ml concentration for normal splenic $CD4^+CD25^-$ T cells) in the presence of antigen-presenting cells (APCs) and the indicated ratio of $CD4^+CD25^+$ T_R cells. Results are expressed as means ± SD of triplicate cultures. $^*P < 0.05$.

IL-2 produced by colitic CD4+ T_{EM} cells at early phase extensively promoted proliferation of $CD4^+CD25^+$ T_R cells. We further attempted to investigate why $CD4^+CD25^+$ T_R cells proliferated in the presence of the colitic LP CD4+ T cells. We measured IL-2 concentrations in culture supernatants at the different time points, because it is well known that *1*) $CD4^+CD25^+$ T_R cells themselves are anergic to in vitro T cell receptor (TCR) stimulation, *2*) the anergic state of $CD4^+CD25^+$ T_R cells by in vitro TCR stimulation is broken by exogenously added IL-2, and *3*) abrogation of the anergic state in the presence of IL-2 results in simultaneous loss of T_R suppressive activity (12). As shown in Fig. 3*B*, IL-2 concentrations in supernatants cultured with colitic LP CD4+ T_{EM} cells alone, normal splenic $CD4^+CD25^-$ T cells alone, or colitic LP CD4+ T_{EM} cells with $CD4^+CD25^+$ T_R cells in the same culture conditions shown in Fig. 2 were high at 48 h after culture but gradually decreased at 72 h and significantly decreased at 96 h, respectively. In contrast, IL-2 concentrations in supernatants cultured with normal splenic $CD4^+CD25^-$ T cells with $CD4^+CD25^+$ T_R cells were undetectable at any time point (48, 72, or 96 h after coculture), indicating that $CD4^+CD25^+$ T_R cells suppressed both the proliferation (Fig.

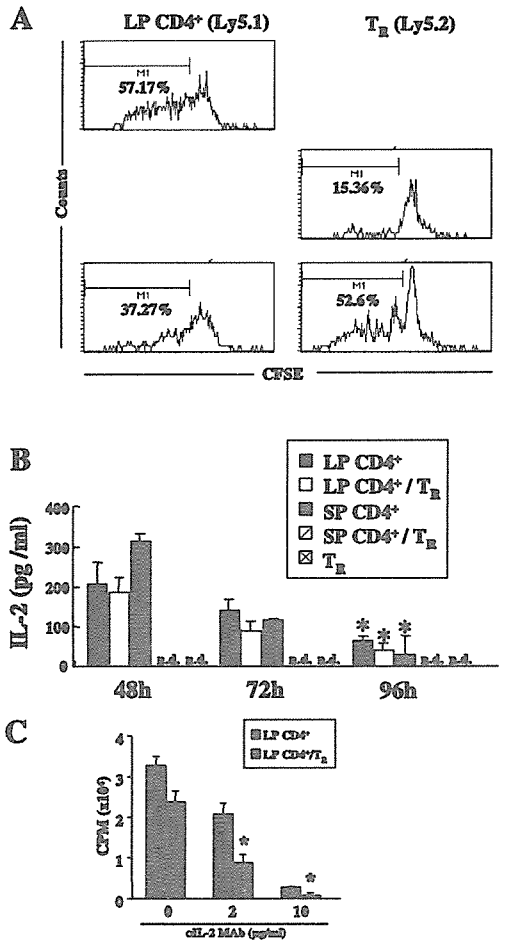

Fig. 3. CD4+CD25+ T_R cells extensively proliferated in coculture with colitogenic LP CD4+ T_{EM} cells. *A*: 1 × 10^5 total normal splenic CD4+CD25− or colitogenic LP CD4+ T_{EM} cells (Ly5.2) and 1 × 10^5 CD4+CD25+ T_R cells (Ly5.1) were cultured alone or were cocultured with 5 × 10^5 APCs in presence of anti-CD3 MAb. Proliferation of the CD4+ cells was measured by dilution with carboxyfluorescein diacetate succinimidyl ester (CFSE) 96 h after culture. *B*: colitogenic LP CD4+ T_{EM} cells produced a large amount of IL-2, whereas the counterpart CD4+CD25+ T_R cells did not. SP, spleen. *C*: effects of blocking MAb against IL-2 on colitogenic LP CD4+ T_{EM} cell-mediated proliferation of CD4+CD25+ T_R cells. An equal number (1 × 10^4) of colitogenic LP CD4+ T_{EM} cells and normal CD4+CD25+ T_R cells were cocultured and stimulated with anti-CD3 (50 ng/ml) and 5 × 10^4 APCs in the presence or absence of indicated MAbs. *$P < 0.05$.

2) and the IL-2 production of normal splenic CD4+CD25− T cells (Fig. 3*B*). Importantly, CD4+CD25+ T_R cells never produced IL-2 at any time point (Fig. 3*B*). To assess the role of IL-2 in the proliferation of colitic LP CD4+ T_{EM} cells alone or of colitic LP CD4+ cells with CD4+CD25+ T_R cells in 96-h culture, we used a neutralizing MAb against IL-2 in an in vitro proliferation assay. As shown in Fig. 3*C*, the proliferative responses of colitic LP CD4+ T_{EM} cells alone or colitic LP CD4+ T_{EM} cells with CD4+CD25+ T_R cells at 96 h in culture were significantly decreased in an IL-2 dose-dependent manner. In addition, the percentage of suppression of LP CD4+ T_{EM} cells by T_R cells with a neutralizing MAb against IL-2 (at both 2 and 10 μg/ml concentrations) was significantly increased compared with suppression without the use of MAb (Fig. 3*C*), indicating decreased IL-2.

CD4+CD25+ T cells inhibit development of T_{EM} cell-mediated colitis. To investigate the suppressor activity of splenic CD4+CD25+ T_R cells in a T_{EM}-cell-mediated chronic colitis model, we first transferred CD4+ T_{EM} cells obtained from various tissues of colitic CD4+CD45RB^{high} T cell-transferred SCID mice into new SCID mice (Fig. 4*A*). SCID mice that underwent transfer of colitic spleen (SP), mesenteric lymph node (MLN), or LP CD4+ T cells developed severe wasting diseases (Fig. 4, *B* and *C*) 5 wk after transfer, in contrast to normal SCID mice that were administered PBS. Histological examination showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells in the LP of the colon in SCID mice that had undergone transfer with colitic SP, MLN, or LP CD4+ T cells, but not in SCID mice that did not undergo cell transfer (Fig. 4*D*). This difference was confirmed by histological scoring of multiple colon sections (Fig. 4*E*). A further quantitative evaluation of CD4+ T cell infiltration was performed by isolating LPL from resected bowels. Only a few CD4+ T cells were recovered from colonic tissue of SCID mice that had not undergone transfer compared with SCID mice that had undergone transfer with colitic SP, MLN, or LP CD4+ T cells (Fig. 4*F*).

We next assessed whether naturally arising CD4+CD25+ T_R cells suppress the development of colitogenic LP CD4+ T_{EM} cell-mediated colitis (Fig. 5*A*). Because the mice that underwent transfer with colitogenic LP CD4+ T_{EM} cells developed rapid wasting disease and their percentage decrease in original body weight reached 10% (Fig. 5*B*), the mice were killed 4 wk after undergoing transfer. In contrast, mice that were coinjected with CD4+CD25+ T_R cells appeared to remain healthy, with gradual increases in body weight and without diarrhea during the entire observation period (Fig. 5*B*). Four weeks after being transferred, the colons from mice that had undergone transfer with colitogenic LP CD4+ T_{EM} cells alone, but not with both LP CD4+ T_{EM} cells and CD4+CD25+ T_R cells, were enlarged and had greatly thickened walls (Fig. 5*C*). Histological examination showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells in LP of the colons obtained from mice that had undergone transfer with LP CD4+ T_{EM} cells alone (Fig. 5*D*). In contrast, glandular elongation was mostly abrogated and only a few mononuclear cells were observed in LP of the colons from mice that had undergone transfer with both LP CD4+ T_{EM} cells and CD4+CD25+ T_R cells (Fig. 5*D*). This finding was confirmed on the basis of histological scores (Fig. 5*E*). As shown in Fig. 5*F*, the cotransfer of CD4+CD25+ T_R and LP CD4+ T_{EM} cells significantly inhibited the expansion and infiltration of LP CD4+ T_{EM} cells compared with those of LP CD4+ T_{EM} cells alone.

We also examined the cytokine production by CD4+ LPL in mice that underwent transfer with LP CD4+ T_{EM} cells or with both LP memory CD4+ T_{EM} cells and CD4+CD25+ T_R cells. As shown in Fig. 5*G*, LP CD4+ T cells from mice that underwent transfer with both LP CD4+ T_{EM} cells and CD4+CD25+ T_R cells produced significantly less IFN-γ, IL-2, and TNF-α upon in vitro anti-CD3/CD28 MAb stimulation compared with production of these cytokines in mice that underwent transfer with LP CD4+ T_{EM} cells alone. In contrast, the production of IL-4 or IL-10 was not significantly affected (Fig. 5*G*). These results suggest that naturally arising CD4+CD25+ T_R cells prevented the development of colitis

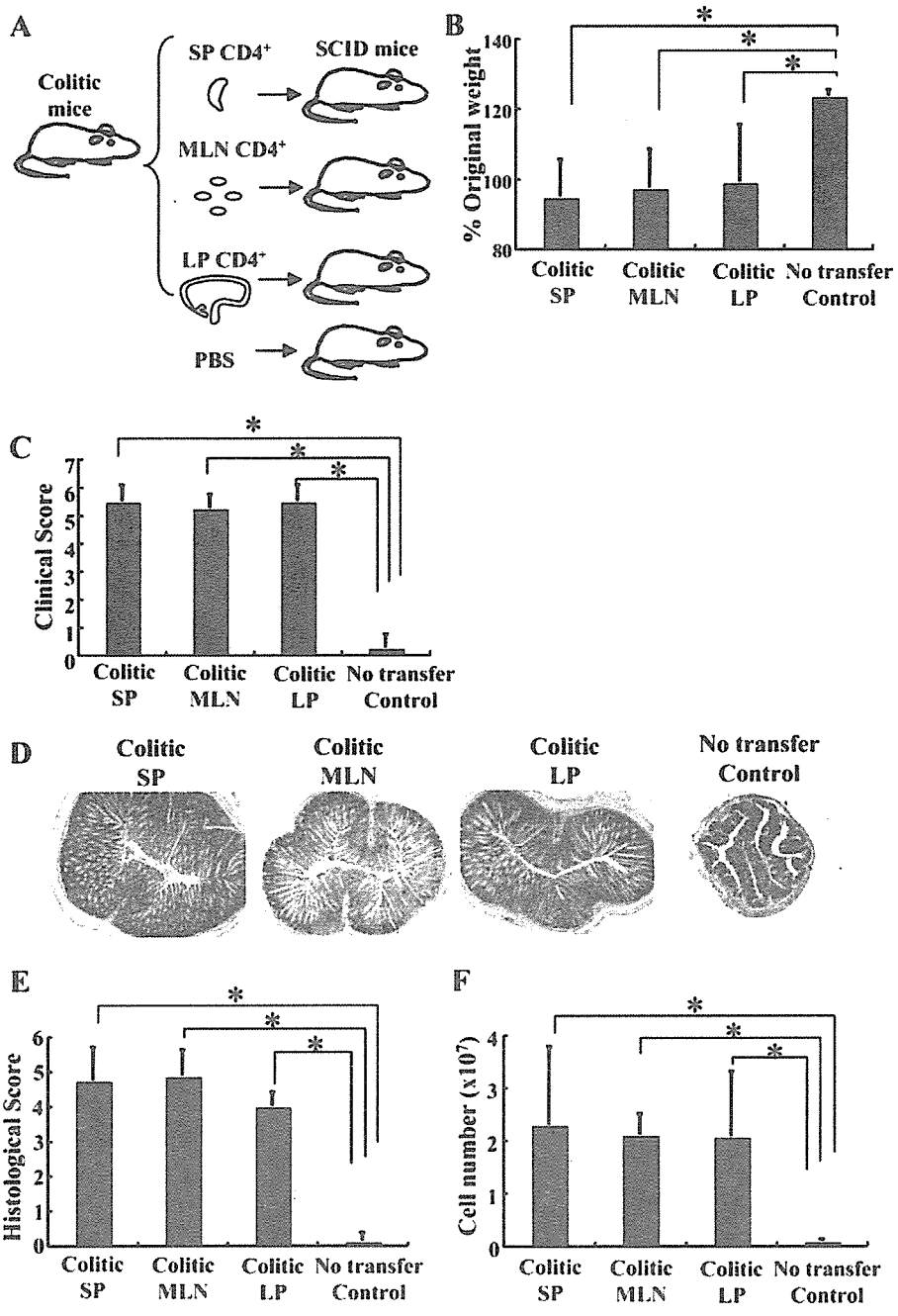

Fig. 4. Severe combined immunodeficient (SCID) mice that underwent transfer of SP, mesenteric lymph node (MLN), and LP $CD4^+$ T cells obtained from mice that had undergone classic $CD4^+$-$CD45RB^{high}$ colitic cell transfer developed colitis. *A*: C.B-17 SCID mice underwent intraperitoneal injection with normal splenic $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer, $CD4^+$ T cells were isolated from SP, MLN, and LP and were injected into new SCID mice (1.5×10^5 cells/mouse). As a negative control, PBS without cells was injected into SCID mice. $n = 5$ mice in each group. All mice were killed 5 wk after undergoing transfer. *B*: mice that underwent transfer with the colitic SP, MLN, and LP $CD4^+$ T cells did not gain weight. $^*P <$ 0.05. *C*: mice that underwent transfer with colitic SP, MLN, and LP $CD4^+$ T cells showed signs of severe clinical colitis. $^*P < 0.05$. Data are means ± SD of 5 mice in each group. $^*P < 0.05$. *D*: histopathogical comparison of distal colon from the mice injected with the colitic SP, MLN, or LP $CD4^+$ T cells, or PBS. Original magnification, ×10. *E*: histograms were obtained to calculate histological scores determined 5 wk after mice underwent transfer as described in MATERIALS AND METHODS. Data are means ± SD from 4 mice in each group. $^*P <$ 0.05. *F*: LP $CD4^+$ T cells were isolated from SCID mice that underwent injection with colitic SP, MLN, or LP $CD4^+$ T cells or with PBS 5 wk after transfer, and the number of LP $CD4^+$ cells was determined using flow cytometry. Data are means ± SD of 4 mice in each group. $^*P < 0.05$.

primarily by inhibiting the expansion and infiltration of colitogenic LP $CD4^+$ T_{EM} cells in the colon and secondarily by inhibiting the development of pathogenic Th1 cells producing IFN-γ, IL-2, and TNF-α.

DISCUSSION

In the present study, we have demonstrated that naturally arising $CD4^+CD25^+$ T_R cells suppressed the development of colitogenic $CD4^+$ T_{EM} cell-mediated chronic colitis. Furthermore, in vitro suppression assay revealed that $CD4^+CD25^+$ T_R cells proliferated extensively in response to a high dose of IL-2 produced by colitogenic LP $CD4^+$ counterpart T_{EM} cells at the early time point (48 h after coculture) but suppressed the proliferation of colitogenic memory $CD4^+$ T_{EM} cells at the late time point (96 h) in accordance with the decreased production of IL-2. These results may indicate the exact strategy of $CD4^+CD25^+$ T_R cells against $CD4^+$ T_{EM} cells in autoimmune disorders.

IL-2 is required for in vivo and in vitro activation of $CD4^+CD25^+$ T_R cells and to sustain their CD25 expression (3). Because $CD4^+CD25^+$ T_R cells do not produce IL-2 (12, 18) (Fig. 3*B*), their proliferation seems to depend on IL-2 produced by their target T cells or activated dendritic cells. Until recently, $CD4^+CD25^+$ T_R cells were described as refractory to stimulation through the TCR and as nonproliferative in in vitro culture (13). In addition, under circumstances in which T_R cells were made to proliferate in vitro by the addition of exogenous IL-2, they lost their suppressive capacity (12). How

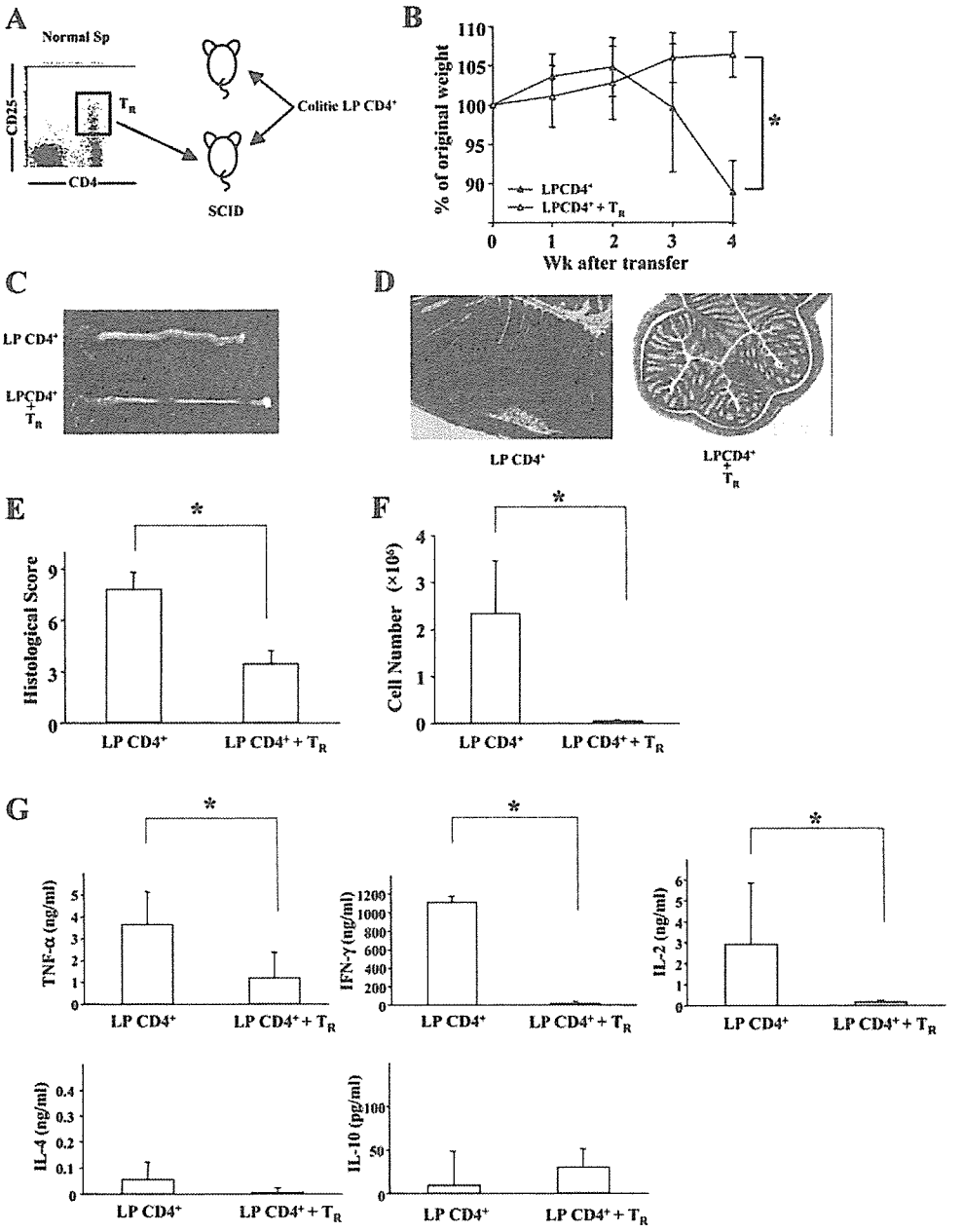

Fig. 5. CD4+CD25+ T_R cells inhibited development of T_{EM} cell-mediated colitis induced by adoptive transfer of colitogenic memory LP CD4+ T cells into SCID mice as well as that induced by CD4+CD45RB^{high} T cells. *A*: schematic of transfer protocols. Seven SCID mice in each group underwent intraperitoneal injection with the following T-cell subpopulations: *1*) colitogenic LP T_{EM} CD4+ cells alone (4×10^5 cells) or *2*) colitogenic LP T_{EM} CD4+ (4×10^5 cells) + CD4+CD25+ cells (4×10^5 cells). *B*: CD4+CD25+ T_R cells inhibited a wasting disease. SCID mice administered CD4+-CD25+ T_R cells were weighed on the day of T-cell transfer and 3 times/wk thereafter. Data regarding colitogenic LP T_{EM} CD4+ cells (−/−) and colitogenic LP T_{EM} CD4+ + CD4+CD25+ T_R cells (▲) are shown. Statistical analysis was performed to compare slopes of weight changes between groups for colitogenic LP T_{EM} CD4+ cells vs. colitogenic LP T_{EM} CD4+ + CD4+-CD25+ T_R cells. *$P < 0.05$. *C*: gross appearance of colon from SCID mice transferred with colitogenic LP CD4+ T_{EM} cells (*top lane*) and colitogenic LP CD4+ T_{EM} + CD4+CD25+ T_R cells (*bottom lane*). *D*: histopathological image showing distal colon 4 wk after transfer. Original magnification, ×100. *E*: histological score. *$P < 0.05$. *F*: number of LP CD4+ T cells. Data are means ± SD of 7 mice in each group. *$P < 0.05$. *G*: cytokine production by LP CD4+ T cells. Isolated LP CD4+ T cells were stimulated with plate-coated anti-CD3 MAb and soluble anti-CD28 MAb for 72 h. Cytokines in supernatants were measured using ELISA. Data are means ± SD of 7 mice in each group. *$P < 0.05$.

is IL-2 involved in suppressing IL-2-producing responder T cells by CD4+CD25+ T_R cells that cannot produce IL-2 in the intestine under inflammatory conditions?

At the early time point (48 h after in vitro coculture), CD4+CD25+ T_R cells proliferated vigorously in response to a high amount of IL-2 produced by the colitogenic LP CD4+ T_{EM} cells. Indeed, anergy-breaking CD4+CD25+ T_R cells cannot suppress colitogenic LP CD4+ T_{EM} cells directly by intercellular interaction at this stage; however, CD4+CD25+ T_R cells may compete for IL-2 with LP CD4+ T_{EM} cells. The constitutive expression of all three chains of the high-affinity IL-2 receptor might be enable CD4+CD25+ T_R cells to take up IL-2 preferentially rather than LP CD4+ T_{EM} cells, which rarely can express high level of IL-2R-α (CD25). Consistent with this hypothesis, it has been demonstrated in vitro and in vivo that competition for and/or consumption of IL-2 indeed occurs (5). During coculture of CD4+CD25+ T_R cells with responder cells, CD4+CD25+ T_R cells upregulate CD25, whereas induction of CD25 expression in responder T cells is conversely suppressed (5). Furthermore, upregulation of CD25 on CD4+CD25+ T_R cells is inhibited by the addition of anti-IL-2 antibody, whereas the addition of IL-2 conversely restores CD25 expression in responder cells, demonstrating that the differential expression of CD25 is regulated by IL-2 (2). Thus competition for and/or consumption of IL-2 by CD4+CD25+ T_R cells may be boosted by the positive feedback loop of IL-2 uptake and CD25 upregulation. The same inverse regulation of CD25 expression on CD4+CD25+ T_R cells vs. responder T cells has been observed in vivo upon adoptive cotransfer of T_R cells and responder cells (5). Although CD4+CD25+ T_R cells do not suppress the proliferation of the LP CD4+ T_{EM} cells directly, IL-2 consumption by

$CD4^+CD25^+$ T_R cells might lead to an increased ratio of T_R to responder cells and inactivation of LP $CD4^+$ T_{EM} cells because of the lack of IL-2 followed by decreased IL-2 production by LP $CD4^+$ T_{EM} cells.

In contrast, $CD4^+CD25^+$ T_R cells might gain original suppressive activity to responder T cells in accordance with the decreased IL-2 concentrations at the late stage. In fact, [^{3}H]thymidine uptake was significantly suppressed in a culture with both $CD4^+CD25^+$ T_R cells and colitogenic LP $CD4^+$ T_{EM} cells at 96 h but not at 48 h after culture. This suppression might depend on intercellular contact but would also require IL-2 competition and/or consumption. Altogether, we hypothesize that the suppression of IL-2-producing LP $CD4^+$ T_{EM} cells by $CD4^+CD25^+$ T_R cells occurs via the following two steps: *1*) IL-2 consumption followed by the expansion of $CD4^+CD25^+$ T_R cells and the adjustment of the T_R-to-responder ratio and *2*) regained suppressive activity via intercellular contact.

Most recently, two groups of researchers have demonstrated that naturally arising $CD4^+CD25^+$ T_R cells cured the established colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells (6, 8). Although these reports suggest that manipulation of $CD4^+CD25^+$ T_R cells may be beneficial in view of clinical checkpoints for the treatment of IBDs, whether delayed administration of $CD4^+CD25^+$ T_R cells suppress pathogenic $CD4^+$ T_{EM} cells or residual $CD4^+CD45RB^{high}$ naive T cells in their systems because of relatively early administration of $CD4^+CD25^+$ T cells (10 days–4.5 wk after transfer of $CD4^+CD45RB^{high}$ cells) is still unclear. Importantly, using T_{EM}-cell adoptive transfer experiments in the present study, we have demonstrated that $CD4^+CD25^+$ T_R cells suppress the expansion of pathogenic $CD4^+$ T_{EM} cells as well as the development of chronic colitis without having any impact on naive T cells.

However, the question arises where $CD4^+CD25^+$ T_R cells suppress the pathogenic $CD4^+$ T_{EM} cells in vivo. Because approximately two-thirds of $CD4^+CD25^+$ T_R cells express CD62L (12), which is essential for homing to lymph nodes, these $CD4^+CD25^+$ T_R cells appear to function in lymph nodes, mesenteric lymph nodes in this case, to suppress the activation and proliferation of naive T cells. However, it is also known not that one-third of $CD4^+CD25^+$ T_R cells obtained from normal spleen do not express CD62L (19) but also that $CD4^+CD25^+$ T_R cells can express integrin $\alpha_4\beta_7$ (16), which is a homing receptor for gut. Mottet et al. (8) recently demonstrated that $CD4^+CD25^+$ T_R cells acted in the intestine to regulate the effector function or the expansion of pathogenic T_{EM} cells as well as acting in the secondary lymph nodes in a murine colitis model induced by the adoptive transfer of normal $CD4^+CD45RB^{high}$ T cells into SCID mice. In fact, our group (7) has identified $CD4^+CD25^{bright}$ T cells with a regulatory phenotype in human colon obtained from normal individuals and from patients with IBD. Furthermore, LP $CD4^+$ T_{EM} cells have a characteristic of the T_{EM} phenotype and tend to recirculate through nonlymphoid tissues (11). Collectively, it is possible that $CD4^+CD25^+$ T_R cells act in the gut in this setting. However, we have demonstrated that the transfer of colitic SP and MLN $CD4^+$ T cells obtained from mice that underwent $CD4^+CD45RB^{high}$ T cell transfer into new SCID mice induced colitis as well as colitic LP $CD4^+$ T cells. The evidence suggests that the SP and the MLN may play an important role as a reservoir for colitogenic $CD4^+$ T_{EM} cells that can recirculate into the gut. Thus it might be possible that $CD4^+CD25^+$ T_R cells prevent the recruitment of recirculating colitogenic $CD4^+$ T_{EM} cells in the SP and the MLN. Further studies are needed to assess this issue using splenectomized lymph node-null mice to exclude the role of lymph nodes.

In summary, we have demonstrated herein that $CD4^+CD25^+$ T_R cells suppress the expansion of pathogenic $CD4^+$ T_{EM} cells as well as the development of chronic colitis without any impact on naive T cell activation on the basis of T_{EM} cell adoptive transfer experiments. Although many critical checkpoints remain to be passed, this study indicates that cell therapy using T_R cells as living immunosuppressants offers hope for the treatment of patients with autoimmune diseases such as IBDs.

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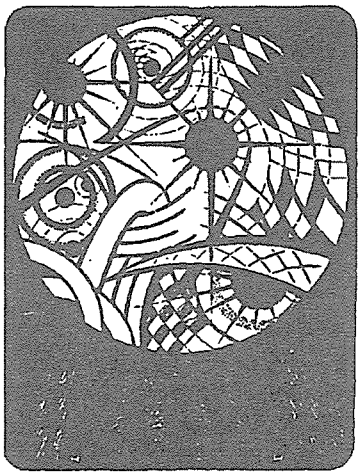

Repeated intravenous injection of recombinant human hepatocyte growth factor ameliorates liver cirrhosis but causes albuminuria in rats

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Abstract. Hepatocyte growth factor (HGF) is a promising agent for the treatment of liver cirrhosis because of its mitogenic and anti-fibrotic effects. We investigated the effect of recombinant human HGF (rh-HGF) on cirrhosis development; its pharmacokinetics and nephrotoxicity in rats with liver cirrhosis induced by 4-week treatment with dimethylnitrosamine (DMN). rh-HGF (0.3 mg/kg) was intravenously administered to rats once a day for 4 weeks in parallel with DMN treatment or twice a day for the last 2 weeks of DMN treatment. Repeated doses of rh-HGF increased the liver weight and serum albumin, and reduced serum ALT. The development of hepatic fibrosis was inhibited more efficiently by extended low-dose treatment with rh-HGF. In cirrhotic rats, serum levels of rh-HGF increased and clearance was decreased, leading to an increase in the area under the plasma-concentration time curve and a decrease in the steady-state volume of distribution. Repeated doses of rh-HGF led to increased urinary albumin excretion, but no rh-HGF-treated animals developed increased serum creatinine levels. Urinary albumin excretion returned to baseline after the cessation of rh-HGF. These results suggest that extended treatment with rh-HGF is required for the attenuation of cirrhosis, and repeated doses of rh-HGF cause adverse effects in extra-hepatic organs. These issues must be resolved before the widespread application of rh-HGF in the treatment of liver cirrhosis.

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Key words: hepatocyte growth factor, liver cirrhosis, albuminuria, pharmacokinetics

Introduction

Hepatocyte growth factor (HGF) was originally identified as a potent hepatocyte mitogen from the plasma of patients with fulminant hepatic failure (1,2). We have established an enzyme-linked immunosorbent assay (ELISA) to measure human HGF in the serum, and we identified increased serum HGF levels in patients with various liver diseases (3). Additionally, serum HGF levels are a valuable prognostic tool in fulminant hepatic failure (4).

HGF is a multifunctional growth factor that acts as a mitogen, motogen, and morphogen for a variety of cells, including epithelial and endothelial cells (5-8). It is a major agent promoting hepatocyte proliferation (9-11) and acts in concert with transforming growth factor (TGF)-α and heparin-binding epidermal growth factor during liver regeneration (12,13). Additionally, HGF ameliorates hepatic injury via anti-apoptotic effects in animal models of fulminant hepatic failure (14-20) and attenuates hepatic fibrosis in animals with liver cirrhosis (21-25). Consequently, HGF is considered to not only induce liver regeneration but also to inhibit disease progression and ameliorate hepatic fibrosis in patients suffering from intractable liver diseases. Therefore, the clinical uses of recombinant human HGF (rh-HGF) in the treatment of fatal liver diseases, including fulminant hepatic failure, small-for-size grafts in living donor liver transplantation, and liver cirrhosis, are currently under investigation in Japan.

We recently showed that bolus intravenous injection of rh-HGF led to increased serum levels of human HGF, and intravenously administered HGF was primarily distributed to the liver (26). Additionally, despite its short half-life, a single intravenous injection of rh-HGF induced tyrosine phosphorylation of c-Met, a specific receptor for HGF, in liver tissue. However, intravenously administered rh-HGF is thought to be primarily metabolized in the liver, but the effect of liver injury on the pharmacokinetics of rh-HGF is not well understood.

In the present study, we investigated the effect of intravenously administered rh-HGF on hepatic fibrosis and its

pharmacokinetics in a rat model of liver cirrhosis. We further examined changes in urinary albumin excretion, a known adverse effect in extra-hepatic organs caused by the repeated administration of rh-HGF.

Materials and methods

Animals. Seven-week-old male SD rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained under a constant room temperature of 25°C and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the Ethics Committee of University of Miyazaki, Faculty of Medicine (Miyazaki, Japan). All animal experiments were performed after a 1-week acclimation period on a standard diet.

To induce liver cirrhosis, 1% dimethylnitrosamine (DMN) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) dissolved in saline was given intraperitoneally at 1 ml per kg body weight for three consecutive days per week for 4 weeks. Rats were divided into four groups as follows: group C1 (n=7), rats treated with intravenous injection of phosphate-buffered saline (PBS) once a day for 4 weeks in parallel with DMN treatment; group H1 (n=8), rats treated with intravenous injection of rh-HGF (0.3 mg/kg) once a day for 4 weeks in parallel with DMN treatment; group C2 (n=9), rats treated with intravenous injection of PBS twice a day for the latter 2 weeks of DMN treatment; group H2 (n=10), rats treated with intravenous injection of rh-HGF (0.3 mg/kg) twice a day for the latter 2 weeks of DMN treatment.

After 4-week treatment with DMN, rats were sacrificed and serum albumin and alanine aminotransferase (ALT) were examined. The liver, spleen, and kidneys were immediately excised, and the wet weight of these organs was determined. Samples were subjected to histological analysis or frozen in liquid nitrogen and stored at -80°C until analysis. TGF-ß levels in liver tissue were measured by a commercially available ELISA kit (R&D Systems Co., MN, USA).

Seventy-percent partial hepatectomy was performed according to a modification of the method of Higgins and Anderson (27). The rats were anesthetized with ether and a two-thirds partial hepatectomy was performed.

Measurement of serum human HGF. A silicone-rubber catheter (0.5x1.0 mm o.d.) was inserted into the jugular vein of normal, partially hepatectomized, or DMN-induced cirrhotic rats, and saline was administered continuously via the catheter using an infusion pump (0.1 ml/h) to prevent obstruction. rh-HGF (0.1 mg/kg) was injected into the femoral vein in <10 sec, and sequential blood samples were obtained via the catheter 5, 10, 20, 30, 60, 90 and 120 min after the injection.

Serum levels of human HGF were determined using a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan), in which only human HGF and not rat HGF is detected (3,28). Pharmacokinetic parameters were calculated using WinNonlin (Pharsight Co., CA, USA).

Histological examination for liver cirrhosis. Two 5-mm thick slices from the two major liver lobes (left lateral and median lateral lobes) were fixed in 10% formalin. After routine processing of paraffin sections, 3-μm thick liver sections were made. To evaluate the degree of fibrosis, sections were stained with 0.1% (w/v) Sirius red F3BA (Sigma, St. Louis, MO, USA) for 1 h in a saturated aqueous solution (1.2% w/v) of picric acid (Wako); the final pH of the solution was 2.0. After staining, slides were rinsed for 2 min in 0.01 N HCl solution to remove unbound dye; following dehydration in alcohol, slides were mounted for observation. The Sirius red-positive areas were measured using an Olympus video micrometer, VM-30 (Olympus, Tokyo, Japan). The results were expressed as a fibrotic rate (%), which was calculated as the ratio of the Sirius red-positive area to the total area examined.

Measurement of urinary albumin excretion. Rats with DMN-induced liver cirrhosis were treated with PBS or rh-HGF as described above. Urine was periodically collected during PBS or DMN treatment, and urine albumin and creatinine were determined.

Repeated doses of rh-HGF (1.0 mg/kg/day) were intravenously administered to normal rats for 5 consecutive days per week for 2 weeks (days 1-5 and 8-12). Urinary levels of albumin and creatinine were examined during HGF treatment and for the following 2 weeks.

Statistical analysis. Unless otherwise specified, data are expressed as mean ± SD. Statistical parameters were ascertained using StatView J-4.5 software (Abacus Concepts Ind., Berkeley, CA). Differences between means were assessed by using the unpaired Student's t-test. The significance level was set at $p<0.05$.

Results

Repeated intravenous injection of rh-HGF stimulated liver regeneration and attenuated liver injury in DMN-induced cirrhotic rats. To examine the effects of repeated intravenous injection of rh-HGF on liver regeneration, we measured the liver weights and serum albumin levels in rats with DMN-induced cirrhosis treated with either saline or rh-HGF (Table I). Treatment with rh-HGF for both 2 and 4 weeks concurrent with DMN administration significantly increased both liver weights and serum albumin levels compared with PBS-treated animals. Additionally, compared to untreated rats, serum ALT levels were elevated in DMN-induced cirrhotic rats treated with PBS (groups C1 and C2) (Table I), and treatment with rh-HGF for 2 weeks significantly decreased ALT levels (C2 vs. H2). While ALT values for animals treated with rh-HGF for 4 weeks (group H1) were reduced compared to group C1, this difference was not significant. The reason for the differences in 2- and 4-week rh-HGF treatment is unclear. Nevertheless, repeated intravenous injection of rh-HGF not only stimulated liver regeneration but also attenuated liver injury in DMN-induced cirrhotic rats.

Prolonged treatment with rh-HGF efficiently attenuated hepatic fibrosis in DMN-induced cirrhotic rats. Liver weight and serum ALT are good markers for liver regeneration and injury, but the pathological hallmark of cirrhosis is extensive liver fibrosis. Thus, we wished to examine the effect of rh-HGF on hepatic fibrosis. When rh-HGF was intravenously

Table I. Effect of rh-HGF administration on body, liver and right kidney weight, and serum levels of albumin and ALT in rats with DMN-induced cirrhosis.

	Group			Group		
	C1 (n=7)	H1 (n=8)	p-value	C2 (n=9)	H2 (n=10)	p-value
Body weight (BW g)	270.1±64.6	319.0±32.5	0.105	305.3±38.2	311.0±22.9	0.705
Liver (g/100 g BW)	2.77±1.10	3.91±0.40	0.034	3.32±0.93	4.65±0.52	0.003
Kidney, right (g/100 g BW)	0.42±0.09	0.39±0.05	0.405	0.41±0.07	0.43±0.06	0.584
Albumin (g/dl)	1.81±0.50	2.34±0.19	0.031	2.06±0.43	2.92±0.18	0.0002
ALT (IU/l)	136.7±99.4	78.4±18.6	0.175	94.8±34.2	67.6±12.5	0.048

Group C1, rats treated with PBS once a day for 4 weeks in parallel with DMN administration; group H1, rats treated with 0.3 mg/kg of rh-HGF once a day for 4 weeks in parallel with DMN administration; group C2, rats administered PBS twice a day for the last 2 weeks of DMN treatment; group H2, rats administered 0.3 mg/kg of rh-HGF twice a day for the last 2 weeks of DMN treatment. Results are represented as the mean ± SD.

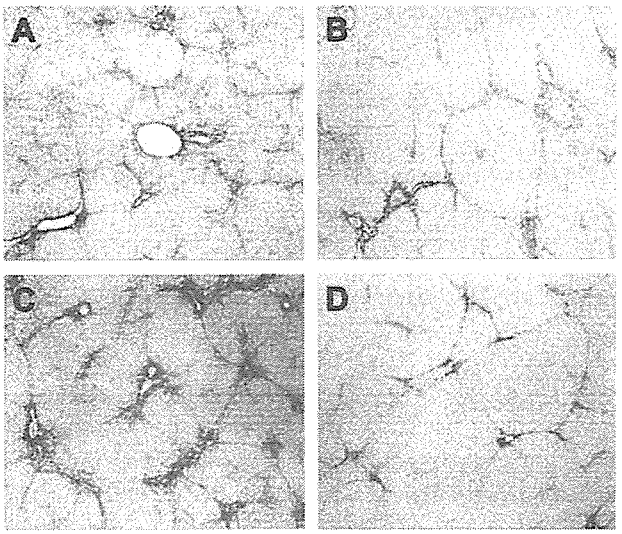

Figure 1. Representative microscopic appearance of liver tissue in DMN-induced cirrhotic rats treated with or without rh-HGF. Rats with DMN-induced cirrhosis were treated with rh-HGF for 4 or 2 weeks, and liver tissue was stained with Sirius red as described in Materials and methods. (A) Group C1; (B) Group H1; (C) Group C2; (D) Group H2 (magnification x40).

injected for 4 or 2 weeks in conjunction with DMN administration, the development of hepatic fibrosis was substantially inhibited (Fig. 1). Sirius red, a dye specific for fibrotic areas, was used to quantitate the extent of liver fibrosis in PBS- or rh-HGF-treated rats (Table II). Repeated injection of rh-HGF for 4 weeks concurrent with DMN treatment (group H1) significantly reduced the areas of hepatic fibrosis compared with PBS-treated rats (group C1), but there were no significant differences between the fibrotic areas of rats treated with PBS and those of rats treated with rh-HGF for 2 weeks (groups C2 and H2, respectively). We also examined the hepatic tissue levels of TGF-ß, a key mediator of cirrhosis development (Table II). Administration of rh-HGF reduced TGF-ß levels in liver tissue, regardless of the treatment period, but these differences did not achieve statistical significance.

Pharmacokinetics of rh-HGF administered intravenously in a bolus in normal, partially hepatectomized, and cirrhotic rats. HGF is primarily metabolized by the liver, but patients most likely to receive rh-HGF have severe liver disease with possible impaired metabolism of a variety of compounds, including rh-HGF. Therefore, we examined changes in serum human HGF concentration sequentially after the single intravenous injection of rh-HGF in rats with normal or abnormal liver function, and we calculated pharmacokinetic parameters (Table III). When compared with normal rats, the serum levels of human HGF were elevated 5 min after injection and the terminal elimination half-life ($T_{1/2terminal}$) of rh-HGF was prolonged in rats with DMN-induced cirrhosis. Consequently, an increase in the area under the plasma-concentration time curve ($AUC_{0-\infty}$) and a decrease in clearance (CL) were observed in cirrhotic rats. Also, the steady-state volume of distribution (Vdss) was reduced to approximately 50%, and the mean resident time ($MRT_{0-\infty}$) was prolonged. The degree of pharmacokinetic change in rats with DMN-induced cirrhosis was virtually the same as that seen in rats with 70% partial hepatectomy.

Repeated administration of rh-HGF increases albuminuria. While most of the physiological effects of rh-HGF are confined to the liver, some changes in kidney function are seen in animals treated with rh-HGF. As a measure for kidney dysfunction, we examined urinary albumin excretion during the treatment with rh-HGF (Fig. 2). When rats were treated with rh-HGF (0.3 mg/kg, once a day) in parallel with DMN administration, the urinary albumin excretion gradually increased for 7 days from the beginning of HGF treatment, and moderate to high levels of albuminuria were observed during the following 14 days (Fig. 2A). Repeated injection of rh-HGF (0.3 mg/kg, twice a day) for days 15-28 of DMN administration rapidly increased the urinary excretion of albumin, and marked albuminuria continued throughout the experimental period. Conversely, kidney weight was not affected by either 2- or 4-week treatment with rh-HGF (Table I).

Table II. Effect of rh-HGF administration on hepatic fibrosis and hepatic levels of TGF-ß in DMN-induced cirrhotic rats.

	Group			Group		
	C1 (n=7)	H1 (n=8)	p-value	C2 (n=9)	H2 (n=10)	p-value
Fibrosis (%)	10.30±5.02	4.53±1.64	0.022	7.31±3.84	5.71±2.77	0.319
TGF-ß (ng/g tissue)	30.77±10.26	22.65±6.00	0.098	34.69±12.06	28.03±11.91	0.244

Results are represented as the mean ± SD.

Table III. Pharmacokinetics of rh-HGF in normal, partially hepatectomized, and cirrhotic rats.

	Normal (n=4)	DMN-LC (n=5)	70% PH (n=4)
C_5[a] (ng/ml)	89.71±20.57	255.4±181.4	341.2±55.1
$T_{1/2terminal}$ (min)	27.88±2.00	35.41±18.74	39.49±13.79
$AUC_{0-\infty}$ (μg·min/ml)	1.685±0.342	13.78±16.48	10.84±2.24
CL (ml/min/kg)	62.09±13.82	20.76±16.73	9.511±1.864
Vdss (ml/kg)	903.7±253.5	453.4±174.6	419.6±144.6
$MRT_{0-\infty}$ (min)	14.44±1.07	41.71±33.20	43.79±9.87

Results are represented as the mean ± SD. [a]Concentration of serum human HGF 5 min after injection. DMN-LC, rats with DMN-induced liver cirrhosis; 70% PH, rats with 70% partial hepatectomy; $T_{1/2terminal}$, terminal elimination half-life; $AUC_{0-\infty}$, the area under the plasma-concentration time curve; Vdss, steady-state volume of distribution; $MRT_{0-\infty}$, mean resident time.

To clarify whether HGF-induced albuminuria was reversible, we injected rh-HGF (1.0 mg/kg/day) via tail veins into normal rats for different periods of time, and monitored the urinary albumin excretion (Fig. 2B). Administration of rh-HGF for 5 days gradually increased albuminuria and, when rh-HGF injection was stopped on days 6 and 7, the albumin excretion reduced. When rh-HGF was subsequently administered on days 8-12, albuminuria once again worsened but, after complete cessation of rh-HGF injection, the albumin excretion gradually returned to baseline levels by days 21-28. On day 28, serum creatinine levels were unchanged compared to baseline indicating minimal permanent kidney damage (data not shown).

Discussion

HGF is produced by mesenchymal cells as an inactive precursor, pro-HGF (29-31), and the mature protein plays important roles in the regeneration and repair of various organs, including the liver, gastrointestinal tract, and kidney. Following tissue injury, pro-HGF is converted to an active heterodimer consisting of light and heavy chains by the specific serine protease HGF activator (HGFA) (32-35). Additionally, HGFA inhibitor (HAI)-1, which was first purified as an inhibitor of HGFA (36), regulates HGFA activity at sites of tissue injury (37). Recently, Yanagida *et al* reported that the administration of recombinant human HGFA stimulated the regeneration of cirrhotic liver after partial hepatectomy through the activation of endogenous pro-HGF (38). In the present study, we administered rh-HGF (0.3 mg/kg) in an already active heterodimeric form into cirrhotic rats once a day for 4 weeks (group H1) or twice a day for the last 2 weeks (group H2) of a 4-week DMN treatment. The total amount of rh-HGF administered throughout the experimental period was equivalent between both groups. Administration of rh-HGF stimulated liver regeneration and ameliorated hepatic fibrosis, and treatment with a lower dose for a longer period of time (group H1) was more effective at inhibiting fibrosis than a shorter therapeutic course with a higher dose (group H2). HGF decreases both extracellular matrix (ECM) deposition and mortality in various models of liver cirrhosis in rats (21,22,24,25), and hepatic expression of TGF-ß, a key mediator of the development of liver cirrhosis, is suppressed by HGF treatment (21,23). We examined hepatic TGF-ß levels but, although a trend toward decreased TGF-ß levels was seen in animals treated with rh-HGF, our results were not statistically significant. In contrast, HGF apparently enhances TGF-ß production and DNA synthesis in activated c-Met expressing hepatic stellate cells (HSCs) isolated from rats treated with carbon tetrachloride (39). These seemingly contradictory effects of HGF on TGF-ß production may be explained by *in vitro* or *in vivo* experiments and/or differences in animal models of liver cirrhosis.

Hepatic fibrosis is a long-term consequence of chronic or repeated liver injury. Two different cell populations play a key role in the development of liver cirrhosis. Once hepatic injury occurs, quiescent HSCs are activated and undergo phenotypic changes, developing into myofibroblast-like cells

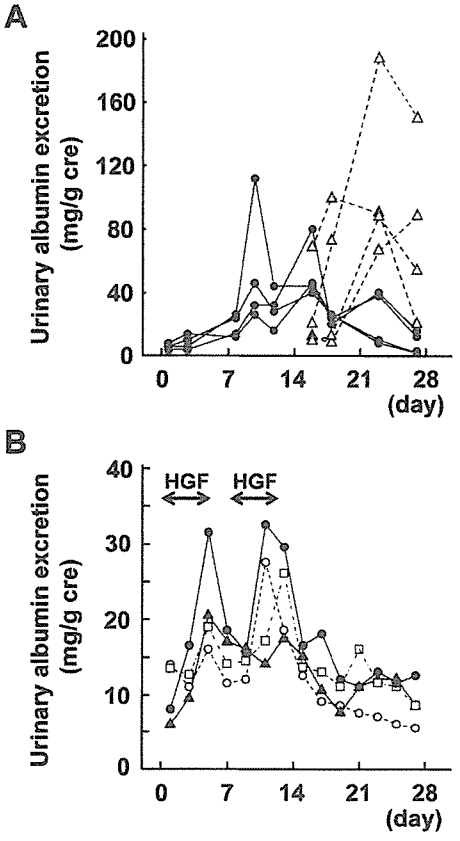

Figure 2. Sequential changes in urinary excretion of albumin in cirrhotic or normal rats treated with repeated intravenous injection of rh-HGF. (A) Administration of rh-HGF induced an increase in albuminuria in rats with DMN-induced liver cirrhosis. •, group H1 (n=4); △, group H2. (B) One mg/kg of rh-HGF was intravenously injected into normal rats (n=4) for days 1-5 and 8-12. Administration of rh-HGF increased urinary excretion of albumin. However, rh-HGF-induced albuminuria was a reversible change, which recovered after cessation of rh-HGF.

able to produce ECM proteins (40,41). In addition to activated HSCs, portal myofibroblasts also express ECM proteins involved in fibrotic changes of the liver (42,43). HGF stimulates the expression of matrix metalloproteinase-1 in human HSCs (44), and HGF inhibits cell growth and induces apoptosis in liver myofibroblasts, leading to the amelioration of hepatic fibrosis in cirrhotic rats (45). In the present study, we observed a more pronounced amelioration of increases in serum ALT levels in 2-week-treated rats (group H2) compared to 4-week-treated rats (group H1). Because the limited administration of HGF efficiently ameliorated experimental hepatic injury and rescued animals from lethal hepatic failure through anti-apoptotic effects (15-18), short-term administration and/or a high dose of rh-HGF appears sufficient to prevent tissue injury and resulting ALT release in DMN-induced cirrhotic rats. The ability of rh-HGF to prevent liver injury, maintain hepatic function and suppress the development of cirrhosis is probably mediated, at least in part, by the inhibition of HSC activation. Collectively, these data indicate that HGF prevents the development of cirrhosis through a variety of different mechanisms.

The liver is a primary target of intravenously administered rh-HGF in normal rats, and serum levels of rh-HGF were elevated in rats with partial hepatectomy or a choline-deficient L-amino acid-defined diet compared to normal rats (26). These findings suggest that rh-HGF administered intravenously is primarily metabolized by the liver. In this study, we further investigated the pharmacokinetics of intravenously injected rh-HGF in rats with DMN-induced cirrhosis. Serum levels of rh-HGF 5 min after injection were elevated, and the CL was reduced, resulting in an increase in the $AUC_{0-\infty}$ and $MRT_{0-\infty}$ and a decrease in the Vdss in cirrhotic rats. HGF in the circulating plasma is efficiently extracted by the liver compared with other HGF target organs, and the liver is responsible for approximately 70% of the overall elimination of rh-HGF (46,47). Furthermore, HGF uptake is three times higher by hepatocytes than other liver non-parenchymal cells (48). Additionally, HGF receptor expression is saturable (48), and elimination of HGF from the systemic circulation is affected by changes in HGF receptor density on the liver cell surface (49). Therefore, a decrease in the number of hepatocytes and a change in c-Met expression in cirrhotic rat livers possibly affected the pharmacokinetics of rh-HGF. Also, the administered dose of rh-HGF (0.1 mg/kg) may be in excess leading to the saturation of receptor-mediated endocytosis and significant increases in serum concentration of rh-HGF.

When rh-HGF was injected intravenously as a bolus, the kidneys, spleen and adrenal glands as well as the liver contained large amounts of rh-HGF (26), and higher concentrations of rh-HGF in the systemic circulation increase the potential of adverse effects. In the present study, an increase in urinary albumin excretion was observed in cirrhotic rats treated with rh-HGF (0.3 mg/kg, once or twice a day). Although repeated doses of rh-HGF (1.0 mg/kg/day) increased urinary albumin excretion in normal rats, the effect was reversible, and treatment with rh-HGF did not affect serum creatinine levels, indicating the absence of permanent kidney injury. Further studies are needed to clarify the renal involvement following repeated doses of rh-HGF. However, since an increase in urinary albumin excretion is more sensitive to changes in kidney function than proteinuria (data not shown), the measurement of urinary albumin may be a useful marker for the detection of HGF-induced nephrotoxicity in clinical applications of rh-HGF.

In the present study, we showed that, although short-term administration of rh-HGF stimulated liver regeneration and ameliorated hepatic injury, prolonged treatment with rh-HGF was more effective in preventing cirrhosis development. However, elevated serum concentrations of rh-HGF in cirrhotic rats have the potential to increase the incidence of adverse events. Therefore, the development of a tissue-specific and/or sustained release delivery system is required for clinical applications of rh-HGF in the treatment of liver cirrhosis. The development of these technologies may also allow the application of rh-HGF in the treatment of diseases of extra-hepatic organs, such as inflammatory bowel disease, pulmonary fibrosis, or neurodegenerative disease.

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