

Figure 5. LT α expression is not required for the development of colitogenic Th17 and Th17/Th1 cells. (A) Scheme of the adoptive transfer experiment. (B) Histopathology of distal colon at 6 weeks after transfer. Original magnification, $\times 40$. (C) Histologic scores of distal colon. (D) Clinical score at 6 weeks after transfer. (E) Absolute cell number of LP CD3⁺CD4⁺ T cells from colon at 6 weeks after transfer. (F) Expression of IL-17A and IFN- γ on colonic LP CD3⁺CD4⁺ T cells of the indicated mice (i). Mean percentages of Th17, Th17/Th1, and Th1 cells in LP CD3⁺CD4⁺ T cells from each group (ii). (C, D, E, and Fii) Data show mean \pm standard error of the mean (n = 5/group). *P < .05. Gr, group.

Intestinal Naturally Occurring Th17 Cells Are Not Enterocolitogenic Cells

Because it is well known that naturally occurring Th17 cells reside in the ROR γ t (GFP)^{intermediate} subpopulation, whereas LTI-like cells reside in the ROR γ t (GFP)^{high} subpopulation,¹ we strictly isolated CD3⁺CD4⁺GFP^{intermediate} cells

from the SI of Rorc(γ t)^{sfp/+} mice to exclude LTI-like cells for further study (Supplementary Figure 6A and B). We previously showed that almost all the LTI-like cells did not express CD4 (Figure 2B). However, to further exclude the possibility that the isolated naturally occurring GFP⁺ Th17 cells contaminate a small number of LTI-like cells that can respond to IL-23, we stimulated the sorted GFP⁺ SI LP CD4⁺ T cells from Rorc(γ t)^{sfp/+} mice with IL-23. As depicted in Supplementary Figure 6C, although the ratio of IFN- γ expression in colitic LP CD3⁺CD4⁺ T cells without IL-23 stimulation was significantly higher than that in isolated GFP⁺ CD4⁺ T cells, GFP⁺ CD4⁺ T cells as well as colitic CD3⁺CD4⁺ T cells did not up-regulate the expression of IFN- γ in response to IL-23 as compared with the paired cells without IL-23. Collectively, we concluded that GFP^{intermediate} cells were CD3⁺CD4⁺ Th17 cells (hereafter called *naturally occurring Th17 cells*), whereas GFP^{high} cells were CD3⁻ LTI-like cells.

To clarify the difference between naturally occurring Th17 cells and colitogenic LP ROR γ t⁺ (GFP⁺) CD4⁺ T cells obtained from colitic RAG-2^{-/-} mice previously transferred with Rorc(γ t)^{sfp/+} CD4⁺CD45RB^{high} T cells (colitogenic Th17 cells), we assessed phenotypical differences in parallel with in vitro-stimulated ROR γ t⁺ Th17 cells differentiated with TGF- β and IL-6 (in vitro-stimulated Th17 cells) (Figure 6A). Naturally occurring ROR γ t⁺ cells and in vitro-stimulated ROR γ t⁺ cells expressed IL-17A, but not IFN- γ , whereas colitogenic Th17 cells included mainly IL-17A⁻IFN- γ ⁺ Th1-like cells, and some IL-17A⁺IFN- γ ⁺ Th17/Th1 and IL-17A⁺IFN- γ ⁻ Th17 cells as previously described.¹⁴ Colitogenic Th17 cells did not express IL-17F, but a very small portion of naturally occurring Th17 cells expressed IL-17F as only IL-17A⁺IL-17F⁺ cells. In contrast, approximately one-third of in vitro-stimulated Th17 cells expressed IL-17F as IL-17A⁺IL-17F⁺ or IL-17A⁻IL-17F⁺ cells. IL-22-expressing cells were detected only in colitogenic Th17 cells as IL-17A⁻IL-22⁺ cells, but not in the other 2 ROR γ t⁺ populations. Distinct expression of TNF- α among the 3 groups was intriguing because only colitogenic Th17 cells abundantly included an IL-17A⁻TNF- α ⁺ subpopulation, although all 3 ROR γ t⁺ populations equally included an IL-17A⁺TNF- α ⁺ subpopulation.

Regarding regulatory molecules, Foxp3-expressing cells were detected in naturally occurring Th17 cells and in vitro-stimulated Th17 cells, but not in colitogenic Th17 cells, as IL-17A⁻Foxp3⁺ cells. Intracellular CTLA-4¹⁵ was expressed in naturally occurring Th17 cells, whereas it was expressed at a low level in colitogenic Th17 cells and in vitro-stimulated Th17 cells as IL-17A⁺CTLA-4⁺ or IL-17A⁻CTLA-4⁺ cells. Glucocorticoid-induced tumour necrosis factor receptor (GITR), another regulatory molecule,¹⁶ was expressed exclusively on colitogenic Th17 cells and in vitro-stimulated Th17 cells as IL-17A⁺GITR⁺ or IL-17A⁻GITR⁺ cells, but very slightly expressed on naturally occurring Th17 cells. Last, we addressed homing receptors: Th17 cell-associated CCR6¹⁷ and gut homing-associated CD103.

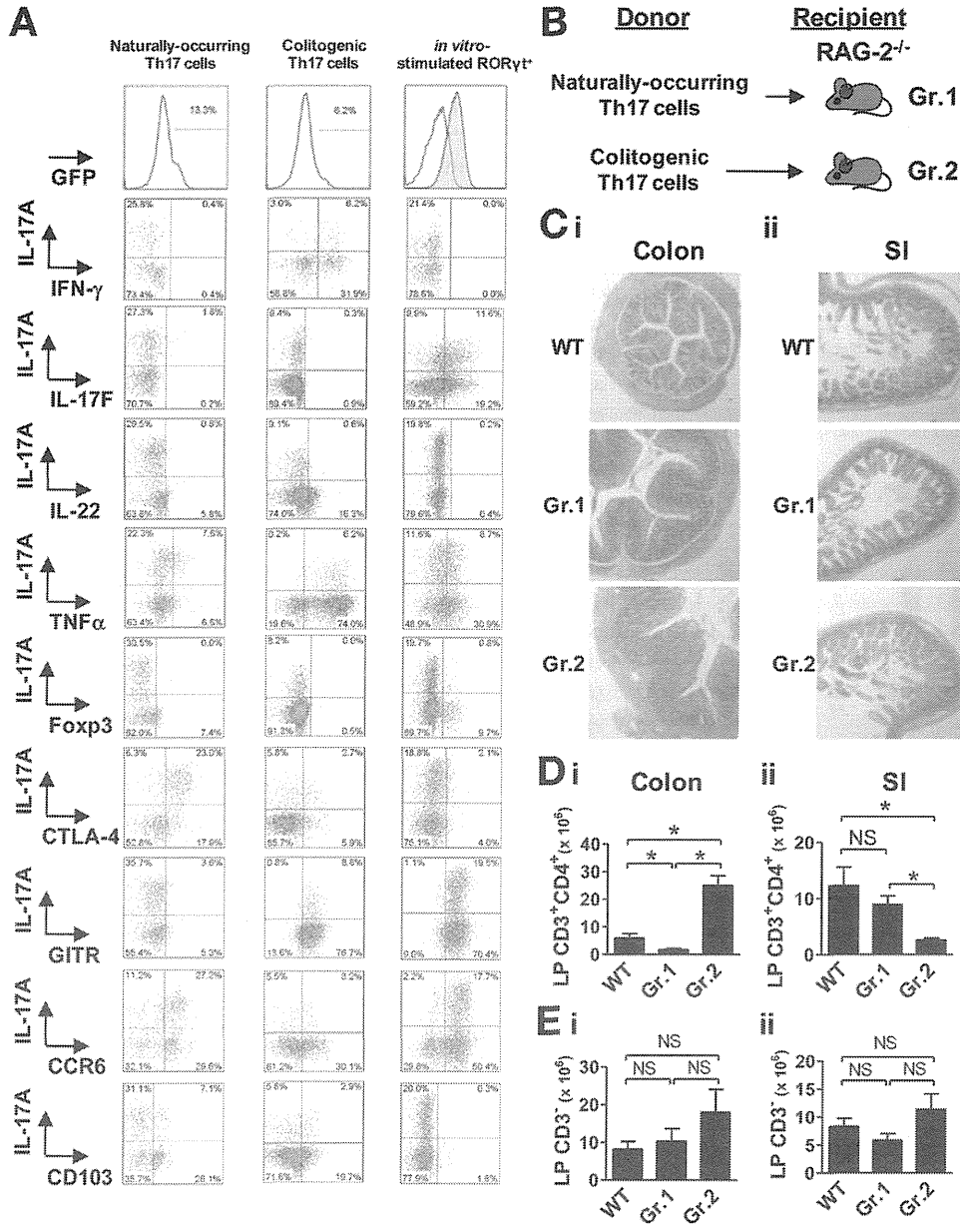


Figure 6. Naturally occurring ROR γ ⁺ Th17 cells are not enterocolitogenic cells. (A) Expression of Th17-associated cytokines, regulatory molecules, and homing receptors on/in naturally occurring ROR γ ⁺ Th17 cells, colitogenic ROR γ ⁺ CD4⁺ T cells, and *in vitro*-stimulated ROR γ ⁺ Th17 cells. (B) Scheme of the adoptive transfer experiment. (C and D) Histopathology of (C) distal colon and (D) terminal ileum of the indicated mice 8 weeks after transfer. Original magnification, $\times 40$. (E and F) Absolute cell number of (E) colonic and (F) SI LP CD3⁺CD4⁺ T cells of the indicated mice at 6 weeks after transfer. (D and E) Data are indicated as the mean \pm standard error of the mean (n = 5/group). *P < .05. Gr, group.

Naturally occurring Th17 cells and *in vitro*-stimulated Th17 cells included substantial CCR6⁺ cells as IL-17A⁺CCR6⁺ or IL-17A⁻CCR6⁺ cells, although almost all CCR6⁺ cells in colitogenic Th17 cells resided in the IL-17A⁻CCR6⁺ subpopulation. In contrast, almost all *in vitro*-stimulated Th17 cells did not express CD103, but naturally occurring Th17 cells and colitogenic Th17 cells substantially expressed CD103 as IL-17A⁻CD103⁺ cells, and some IL-17A⁺CD103⁺ cells. Together, these 3 types of ROR γ ⁺ cells were quite distinct at least in view of phenotypical characterization; naturally occurring Th17 cells, resting Th17 cells; colitogenic Th17 (ROR γ ⁺) cells, a mixture of Th17, Th17/Th1, and Th1-like cells with activation; and *in vitro*-stimulated Th17 cells, activated Th17 cells.

We next assessed whether naturally occurring Th17

cells in the SI and colitogenic Th17 cells induced enteritis and/or colitis if transferred into RAG-2^{-/-} mice. RAG-2^{-/-} mice were transferred with naturally occurring Th17 cells (group 1) or colitogenic Th17 cells (group 2) (Figure 6B). Group 2, but not group 1, mice developed severe colitis 6 weeks after transfer, whereas neither group 1 nor group 2 mice developed ileitis (Figure 6Ci and ii). Consistently, the absolute cell number of colonic LP CD4⁺ T cells obtained from group 2 mice was significantly higher than group 1 mice and WT mice, whereas the SI LP CD4⁺ T cells obtained from group 1 mice was comparable with WT mice (Figure 6Di and ii). The number of SI LP CD4⁺ T cells obtained from group 2 mice was significantly lower than from WT mice (Figure 6Dii), suggesting that colitogenic Th17 cells, but not naturally occurring Th17 cells, are pathogenic.

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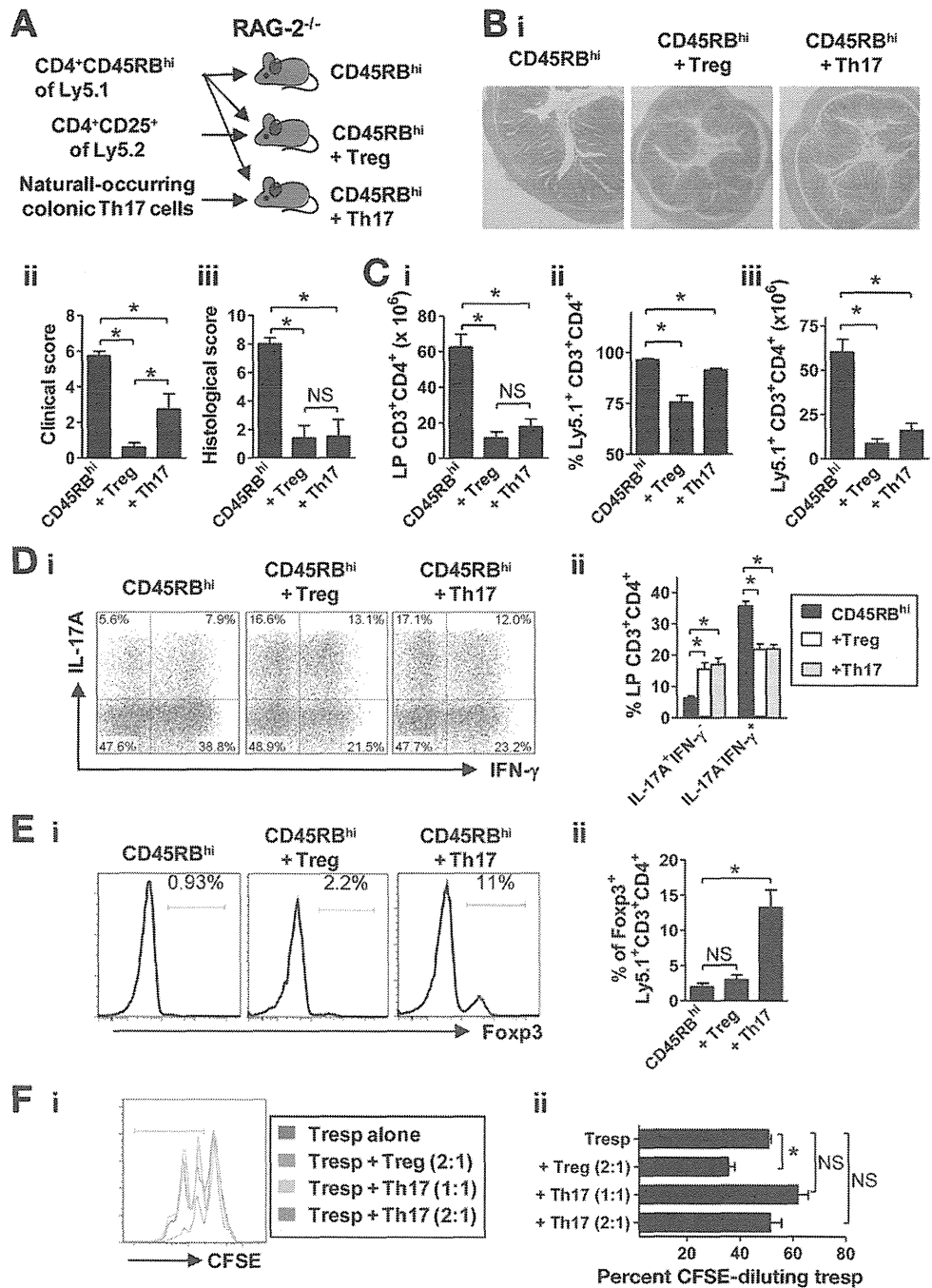


Figure 7. Naturally occurring Th17 cells in the colon have regulatory activity to inhibit the development of colitis. (A) Scheme of the adoptive transfer experiment. (B) Histopathology of distal colon at 6 weeks after transfer (i). Original magnification, $\times 40$. Clinical score at 6 weeks after transfer (ii). Histologic scores of distal colon (iii). (C) Absolute cell number of LP CD3⁺CD4⁺ T cells from colon (i). Mean percentages of Ly5.1⁺ cells in LP CD3⁺CD4⁺ T cells (ii). Absolute cell number of LP Ly5.1⁺CD3⁺CD4⁺ T cells (iii). (D) Expression of IL-17A and IFN- γ on colonic LP CD3⁺CD4⁺ T cells of the indicated mice (i). Mean percentages of IL-17A⁺IFN- γ ⁻ and IL-17A⁺IFN- γ ⁺ cells in LP CD3⁺CD4⁺ T cells (ii). (E) Expression of Foxp3 in Ly5.1⁺ T cells (i). Mean percentages of Foxp3⁺ cells in LP Ly5.1⁺CD3⁺CD4⁺ T cells (ii). (F) Carboxy Fluorescein Succinimidyl Ester (CFSE) dilution of CFSE-labeled CD4⁺ T cells after coculture with or without CD4⁺CD25⁺ Treg cells or naturally occurring Th17 cells (i). Mean percentages of CFSE-diluting Tresp at least one time in CFSE-labeled CD4⁺ T cells after coculture (n = 3/group) (ii). (C, D, Eii, and Fii) Data are indicated as the mean \pm standard error of the mean. *P < .05.

Naturally Occurring Th17 Cells in the Colon Have Regulatory Activity to Inhibit the Development of CD4⁺CD45RB^{high} T-Cell-Induced Colitis

Given that naturally occurring SI Th17 cells were not colitogenic, we lastly addressed the possibility that naturally occurring Th17 cells could suppress the development of colitis because a substantial subpopulation of these cells expressed CTLA-4 (Figure 6A). In this setting, we used colonic naturally occurring Th17 cells to inhibit the development of colitis because colonic, but not SI, naturally occurring Th17 cells would preferentially mi-

grate to the colon because of their tissue-specific trafficking properties. Therefore, we divided the mice into 3 groups as depicted in Figure 7A. The results clearly showed that control of intestinal inflammation was caused by the colonic naturally occurring Th17 cells (Figure 7B), although the clinical scores of mice co-transferred with CD4⁺CD45RB^{high} T cells and colonic naturally occurring Th17 cells was more than that of mice co-transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD25⁺ Treg cells (Figure 7Bii). Consistently, co-transfer in RAG-2^{-/-} mice significantly inhibited the development of colitis (Figure 7Biii) as compared with mice transferred with

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CD4⁺CD45RB^{high} T cells alone. We consistently observed significantly fewer numbers of colonic CD4⁺ T cells in the mice co-transferred with CD4⁺CD45RB^{high} T cells and Treg cells or naturally occurring Th17 cells compared with mice transferred with CD4⁺CD45RB^{high} T cells alone (Figure 7C*i*). Although it was possible that naturally occurring Th17 cells expanded and thereby prevented colitis, this was not the case because a major population in co-transferred mice was cells derived from Ly5.1⁺ CD4⁺CD45RB^{high} T cells (Figure 7C*ii* and *iii*). Furthermore, the proportion of IFN- γ ⁺IL-17A⁻ Th1 cells in the colon of mice transferred with CD4⁺CD45RB^{high} and Treg cells or naturally occurring ROR γ t⁺ cells was reduced significantly as compared with that in mice reconstituted with CD4⁺CD45RB^{high} alone. The percentage of IFN- γ ⁻IL-17A⁺ Th17 cells in mice reconstituted with CD4⁺CD45RB^{high} and Treg cells or naturally occurring Th17 cells was increased significantly as compared with that in mice reconstituted with CD4⁺CD45RB^{high} alone (Figure 7D*i* and *ii*). Of note, cotransfer of naturally occurring Th17 cells, but not Treg cells, resulted in the significant increase of Foxp3⁺ cells derived from Ly5.1⁺ CD4⁺CD45RB^{high} T cells (Figure 7E*i* and *ii*). However, unlike Treg cells, naturally occurring Th17 cells did not suppress the proliferation of naive CD4⁺ T cells (Figure 7F*i* and *ii*). These data suggest that naturally occurring Th17 cells function as another type of Treg cells in the colon.

Discussion

In the current study, we showed that the existence of an LT α -dependent GALT structure is essential for the generation of intestinal naturally occurring Th17 cells in the steady state, but the lack of LT α molecules results in a marked increase in IL-17-producing LTi-like cells. In sharp contrast to physiological conditions, the generation of colitogenic Th17 cells during inflammation is not dependent on LT α . Consistent with the distinct developmental mechanisms of naturally occurring and colitogenic Th17 cells, naturally occurring Th17 cells have regulatory activity to suppress the development of colitis. Our current immunologic scenario is depicted in Supplementary Figure 7.

Th17 cell differentiation in vitro from naive T cells requires the coordinated action of multiple cytokines.¹⁸⁻²⁰ In vitro stimulation with TGF- β and IL-6 can induce Th17 differentiation dependent on ROR γ t and STAT3.¹⁹ Recently, however, it has been shown that Th17 differentiation can occur in the absence of TGF- β signaling.²¹ Th17 cells in vitro generated with IL-6 and IL-23 but in the absence of TGF- β were encephalitogenic, whereas Th17 cells generated with IL-6 in the presence of TGF- β were not.²¹ Here, we showed that the proportion of naturally occurring Th17 cells markedly decreased in the SI of LT α ^{-/-} mice despite their intact ROR γ t gene, although in vitro-manipulated Th17 cells normally develop from LT α ^{-/-} naive T cells.

Knowledge of the origin and relationship among various innate lymphocyte populations is admittedly incomplete at present. Because LTi-like cells differentiate into NKp46⁺ cells at a point when ROR γ t levels have de-

creased, and begin to produce IFN- γ to have a colitogenic potential, LTi-like cells do express NKp46 at a certain point.⁹ However, a contrary theory has been put forward by Sawa et al¹³; LTi cells and NKp46⁺ (IL-22-producing) innate NK22 cells are derived from separate lineages so that the latter cells are not LTi-like cells. Given the fact that the IL-17-producing CD3⁻ cells arising in mice lacking LT α do not bear NKp46, we would conclude that our IL-17-producing CD3⁻ cells are LTi-like cells, but are nevertheless distinct from those LTi-like cells that have been implicated as colitogenic cells by other groups.^{8,9} Furthermore, the present study showed that the proportion of NK22 cells also was increased in the SI of LT α ^{-/-} mice in parallel with the increase of LTi-like cells, suggesting the sequential development of NK22 cells from IL-17-producing LTi-like cells even in the absence of LT α /lymphotoxin β receptor (LT β R) signaling.

The possible role of LTi-like cells on the pathogenesis of colitis should be discussed. We showed that IL-17A-expressing LTi-like cells were increased significantly in LT α ^{-/-} mice, and those cells produced a large amount of IL-22. Furthermore, LT α ^{-/-} × RAG-2^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells did develop colitis, but the severity of those mice was significantly less than that of the control RAG-2^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells. Although it is possible that the decreased severity in LT α ^{-/-} × RAG-2^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells is the result of another possibility, that LT α ^{-/-} × RAG-2^{-/-} mice lack GALT including mesenteric lymph nodes, LTi-like cells may function as protective cells that produce IL-22. Further studies are needed to address this issue.

To investigate whether LT α expression on CD4⁺ T cells is required for the development of Th17 cells, we adopted a parabiosis system between Ly5.1⁺ WT and Ly5.2⁺ LT α ^{-/-} mice. Our data showed that LT α expression on CD4⁺ T cells is dispensable for the development of Th17 cells in the steady state. In contrast, LTi-like cells were restricted to the original SI as resident cells of the SI even after parabiosis surgery. These results clearly show that LT α ^{-/-} CD4⁺ T cells migrate to the SI of WT mice that retain normal GALT residing LT α -expressing LTi-like cells, and thereafter become Th17 cells in the GALT. By using BM chimeras, we observed the expression of LT α on BM-derived cells was dispensable for the development of naturally occurring Th17 cells, but that it was dependent on LT α expression in recipient mice. This suggested the development of naturally occurring Th17 cells required the existence of GALT as the induction site. In this regard, Atarashi et al² showed that naturally occurring Th17 cells develop normally in PP-null mice that are generated by injection with anti-IL-7R α monoclonal antibody (A7R34) during the gestational period. Importantly, because PP-null mice develop normal cryptopatches and isolated lymphoid follicles after birth, we concluded that naturally occurring Th17 cells are generated in the GALTs, except for PP.

To investigate whether the LT α 1 β 2/LT β R signaling pathway was essential for the development of colitogenic Th17 cells during inflammation, we performed adoptive

transfer using a colitis model induced by transfer of CD4⁺CD45RB^{high} T cells. We found that LT α ^{-/-}×RAG-2^{-/-} recipients developed colitis 6 weeks after transfer, despite the lack of GALT, although the clinical and histologic scores in the groups using LT α ^{-/-}×RAG-2^{-/-} recipients was significantly milder than those in the groups using RAG-2^{-/-} recipients. This is not surprising because this difference may be caused by the absence of GALT, including mesenteric lymph nodes that are the main places for priming naive CD4⁺ T cells in LT α ^{-/-}×RAG-2^{-/-} recipients.¹⁵ Notably, comparable proportions of Th17 and Th17/Th1 cells emerged in colonic LP of all 4 groups whether in RAG-2^{-/-} or LT α ^{-/-}×RAG-2^{-/-} recipients. These results indicated that the LT α /LT β R signaling pathway is not required for the development of colitogenic Th17 and Th17/Th1 cells that were hardly detected in physiological conditions. This may be consistent with a recent report showing that LTI-like cells in inflammatory conditions lose the expression of ROR γ t with an ability to produce IFN- γ production by those cells.⁹ Notably, these inflammatory ROR γ t-losing LTI-like cells down-modulated the expression of surface LT α molecules, suggesting a possibility that other molecules rather than LT α may be involved in the induction of pathologic (colitogenic) Th17 and Th17/Th1 cells in inflammatory conditions.

This study indicates that LT α are essential for the development of naturally occurring Th17 cells in the steady-state condition, but colitogenic Th17 and Th17/Th1 cells develop by different mechanisms independent of GALT and LT α -expressing LTI-like cells, suggesting a link between the current topics regarding protective and pathogenic Th17 cells in vivo.

Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2012.07.108>.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Luminal CD4⁺ T Cells Penetrate Gut Epithelial Monolayers and Egress From Lamina Propria to Blood Circulation

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BACKGROUND & AIMS: The egress of memory T cells from peripheral tissues, such as lung and skin, into the draining lymph nodes requires their expression of CC chemokine receptor 7 (CCR7). In the intestine, resident memory T cells in the intestinal lamina propria (LP) do not express CCR7, indicating that they are tissue bound and do not exit the intestine. **METHODS:** We developed a cell transfer system, using rectal administration of lymphocytes to C57BL/6 mice. Lymphotoxin α -deficient mice were crossed with RAG-2^{-/-} (recombination-activating gene-2) mice to generate lymphotoxin α -deficient \times RAG-2^{-/-} mice. **RESULTS:** Severe combined immunodeficient (SCID) or RAG-2^{-/-} mice given rectal administration of splenic CD4⁺ T cells from normal mice developed colitis; the cells proliferated not only in the LP but also in spleen. SCID or RAG-2^{-/-} mice given rectal administrations of CD4⁺ T cells that expressed green fluorescent protein (GFP⁺CD4⁺ T cells) localized to the LP within 6 hours but were not found in the spleen until 24 hours after administration. Immunohistochemical and electron microscopic analyses detected CD4⁺ T cells in the intraepithelial space just 3 hours after intrarectal administration. However, neither CCR7 deficiency nor the sphingosine-1-phosphate receptor agonist Fingolimod impaired the egress of CD4⁺ T cells from LP to systemic circulation. **CONCLUSIONS:** CD4⁺ T cells not only penetrate from the luminal side of the intestine to the LP but also actively egress from the LP into the circulation. We developed a rectal administration system that might be used to further investigate cell trafficking in intestinal mucosa and to develop enema-based therapeutics for intestinal diseases.

Keywords: T-Cell Migration; Localization; Mouse Model; Chemokine; Treatment.

Although accumulated evidence has revealed how effector/memory T cells migrate to peripheral tissues, there are still many enigmas about how these cells egress from peripheral tissues to blood circulation. Egress of immune cells from nonlymphoid peripheral tissues is a critical step in lymphocyte migration as well as lymphocyte homing to these tissues.^{1–4} It has been reported that the draining lymphatics of tissues contain substantial numbers of CD4⁺ and CD8⁺ lymphocytes, some of which

are of memory phenotype,^{5,6} but it remains unclear whether these memory lymphocytes are derived from cells that have egressed from peripheral tissues or from the blood via lymph nodes located at closer sites to peripheral tissues.

Recent reports suggest that the egress of effector or memory CD4⁺ and CD8⁺ T cells into the draining lymph nodes from the lung⁷ and of B cells and naive or memory CD4⁺ and CD8⁺ T cells into the popliteal lymph nodes from the footpad of skin⁸ requires the expression of CCR7 on these cells. In the intestine, however, the unique phenotype (CCR9 or integrin $\alpha_4\beta_7$ or $\alpha_E\beta_7$ -expressing cells) of the resident memory T cells and the lack of such cells elsewhere suggest that memory T cells in the intestinal lamina propria (LP) and intraepithelial space are tissue bound and do not exit the intestine,¹ but this theory remains unproven experimentally.

The intrarectal administration of cells employed in this study was suggested by the fact that intratracheal instillation of cells in mice can induce their cell migration to the lung and thereafter to the blood systemically.⁶ In this paper, we demonstrate that living CD4⁺ T cells can not only penetrate intestinal barriers from the lumen to the LP but also constantly egress from the LP to the bloodstream constantly in a CCR7- and sphingosine 1-phosphate 1 (S1P₁)-independent manner.

Materials and Methods

Please see Supplementary Materials and Methods for more details.

Animals

C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). C57BL/6-Ly5.1 and C57BL/6-RAG-2^{-/-} mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). CCR7^{-/-} mice were previously generated by M. Lipp (Max-Delbruck Cen-

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cells; GFP, green fluorescent protein; LP, lamina propria; MLN, mesenteric lymph node; NK, natural killer; PB, peripheral blood; SCID, severe combined immunodeficient; SP, spleen; S1P, sphingosine 1-phosphate; T_{EM}, effector-memory T; T_g, transgenic; T_R, regulatory T; UC, ulcerative colitis; WT, wild type.

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ter for Molecular Medicine, Berlin, Germany).⁹ Green mice (CAG-green fluorescent protein [GFP] transgenic [Tg] mice) were originally generated by M. Okabe (Osaka University, Japan).¹⁰ Lymphotoxin α (LT α)-deficient (LT $\alpha^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and intercrossed into RAG-2^{-/-} mice to generate LT $\alpha^{-/-}$ \times RAG-2^{-/-} mice. 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 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Results

Substantial Numbers of Lymphocytes Reside in Crypt Abscesses of Inflamed Mucosa in Patients With Ulcerative Colitis

Accumulating evidence suggests an active moving state of immune compartments between intestinal epithelial cell barriers that separate the inside and outside of the body. For instance, recent reports demonstrated that murine CX3CR1⁺ dendritic cells (DC) beneath intestinal epithelial cells in small intestine penetrate epithelial barriers by extending dendrites into the luminal side through intraepithelial gaps to capture antigens including commensal bacteria.¹¹ In addition, it is well-known that large amounts of granulocytes accumulate in the crypt of inflamed mucosa of patients with human inflammatory bowel diseases, suggesting that granulocytes actively penetrate intestinal barriers into the luminal side under inflammatory conditions.¹² This accumulation is pathologically defined as a “crypt abscess,” which is one feature of chronic inflammatory diseases of the intestine, such as ulcerative colitis (UC).¹² However, it remains largely unknown whether this is a case with other immune compartments, such as CD4⁺ T lymphocytes. We therefore re-evaluated immune cell compartments in crypt abscesses in colonic inflamed mucosa of patients with severe UC (Supplementary Table 1). As shown in Figure 1A and B, in addition to a major compartment, myeloperoxidase-positive granulocytes, we found that substantial numbers of CD4⁺ and CD8⁺ T cells and CD20⁺ B cells, but not CD56⁺ natural killer (NK) cells, reside in cell aggregates of crypt abscess, suggesting that these lymphocytes can actively commute across the intestinal epithelial barriers.

SCID Mice Intrarectally Administered With Splenic CD4⁺ T Cells Developed Severe Colitis

Given the above results from human samples of crypt abscesses in inflamed mucosa of UC, we hypothesized that lymphocytes can actively move across intestinal epithelial barriers and therefore that intrarectally administered CD4⁺ T cells should, conversely, penetrate them from the lumen side. If so, this approach seems to be an ideal strategy to experimentally determine whether intestinal LP CD4⁺ T cells can thereafter egress LP to the systemic circulation. However, because a huge number of intestinal bacteria, over 10³ million per mouse constantly

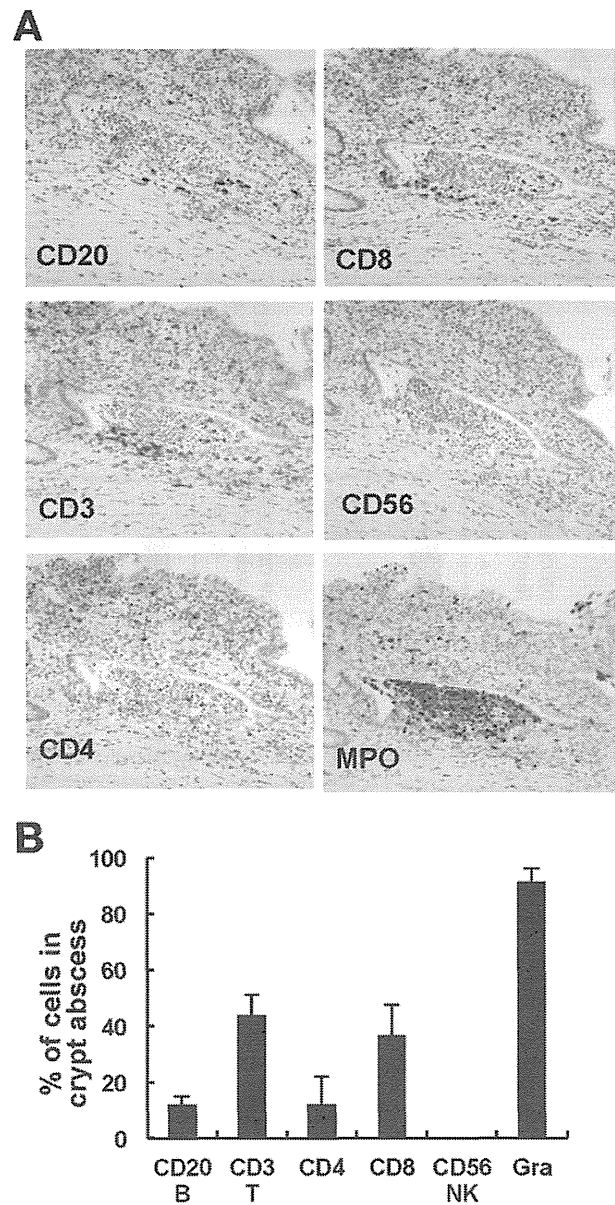


Figure 1. CD4⁺ T cells reside in crypt abscesses of inflamed mucosa of patients with ulcerative colitis (UC). (A) Immunohistochemistry of CD20, CD3, CD4, CD8, CD56, and myeloperoxidase (MPO) using samples of patients with severe UC. Four patients with UC (Supplementary Table 1) were examined. Sections were stained brown with a given antibody and counterstained with Mayer's hematoxylin. Representative section of crypt abscess with each stain. Original magnification, $\times 100$. (B) Proportion of each compartment per total stained cell number (CD3⁺ + CD20⁺ + CD56⁺ + MPO⁺) in each crypt abscess.

occupy the intestinal lumen,^{13–15} no attempts to intrarectally administer the cells have yet been reported. To test our hypothesis, we administered splenic CD4⁺ T cells from normal BALB/c mice intrarectally or intraperitoneally (as a control) into C.B-17 SCID mice (Figure 2A). This adoptive transfer system into immunodeficient recipients was adopted because it can be easily assessed whether intrarectally administered cells can penetrate intestinal barriers to the LP by amplifying the cell number of pen-

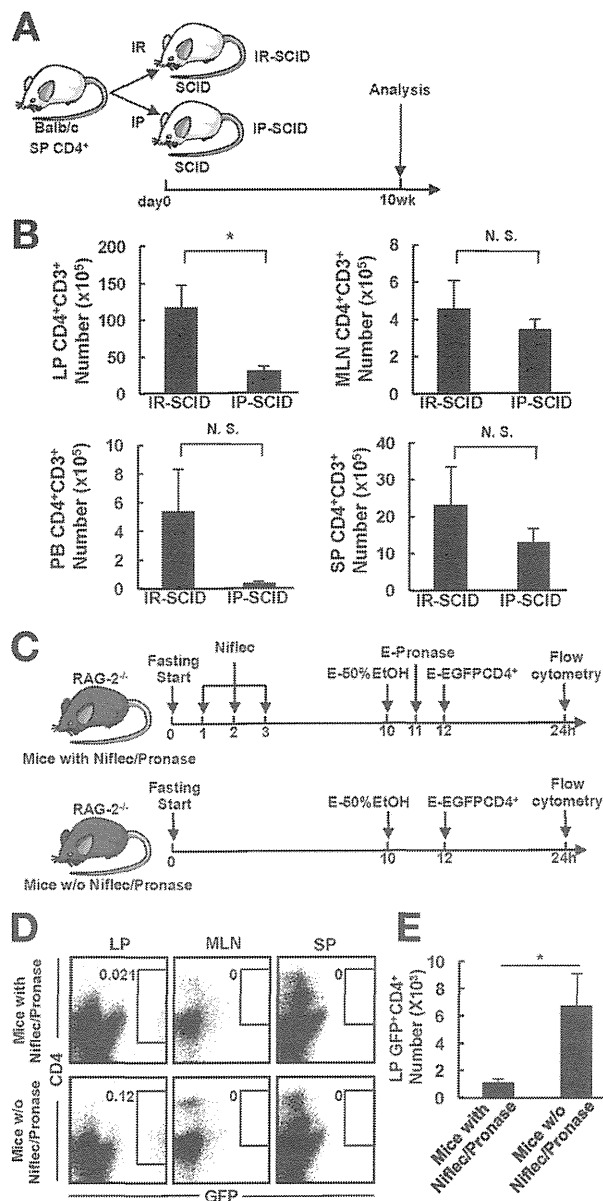


Figure 2. Intrarectally administered splenic CD4⁺ T cells can penetrate gut epithelial monolayers and expand not only in colonic lamina propria but also outside the intestine. (A) C.B-17 SCID recipient mice were administered with splenic CD4⁺ T cells from normal BALB/c mice intrarectally (5×10^6 , IR-SCID mice, $n = 9$) or intraperitoneally (5×10^5 , IP-SCID mice, $n = 9$). (B) LP, MLN, and SP CD3⁺CD4⁺ T cells were isolated from the colon at 10 weeks after T-cell administration, and the number of CD3⁺CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean \pm standard error (SEM) of mean of 9 mice in each group. * $P < .01$. (C) RAG-2^{-/-} mice were pretreated just like protocol of Supplementary Figure 1 (Mice with Niflec/Pronase) or without the pretreatments of Niflec and pronase (Mice w/o Niflec/Pronase) before cell administration. RAG-2^{-/-} mice in each group were administered with 5×10^6 CD4⁺ T cells from EGFP-Tg mice ($n = 10$ in each group). (D) LP, MLN, and SP GFP⁺CD4⁺ cells were isolated from the colon at 24 hours after cell administration, and the absolute number of GFP⁺CD4⁺ cells was determined by FACS. Dot plot analysis data of FACS is representative one of each group. Representatives of 10 separate samples in each group. (E) The absolute number of LP GFP⁺CD4⁺ cells. Data are indicated as the mean \pm SEM of 9 mice in each group. * $P = .034$.

etrating CD4⁺ T cells in a mechanism of lymphopenia-driven rapid proliferation in a long-term observation.¹⁶ As a protocol of intestinal preparation, mice were maintained without feeding for 3 hours before cell administration and were given 1 mL of Niflec water (Ajinomoto Pharma Co, Tokyo, Japan) 3 times at intervals of 1 hour by oral catheter to remove the resident stool. Mice were then pretreated with or without 50% ethanol enema and thereafter with 5% pronase enema at 1 hour before cell administration (Supplementary Figure 1).

Consistent with previous reports,¹⁷ SCID mice intraperitoneally administered with splenic CD4⁺ T cells (hereafter called IP-SCID mice) were healthy throughout the observation period and showed no clinical symptoms of colitis as estimated by clinical score, possibly because of the presence of regulatory T (T_R) cells in the donor CD4⁺ T-cell preparation (Supplementary Figure 2A and B). Surprisingly, SCID mice intrarectally administered with CD4⁺ T cells (IR-SCID mice) developed severe colitis with diarrhea, anorectal prolapses, hunched posture, and weight loss in spite of the presence of T_R cells in the donor CD4⁺ T cells (data not shown). Ten weeks after cell administration, the colon from IR-SCID mice, but not from IP-SCID mice, was enlarged and had a greatly thickened wall (Supplementary Figure 2A). The difference in clinical scores at 10 weeks after administration between the 2 groups was significant (Supplementary Figure 2B). Histologic examination showed prominent epithelial hyperplasia and loss of goblet cells with massive infiltration of mononuclear cells in the LP of colon from IR-SCID mice but not from IP-SCID mice (Supplementary Figure 2C). The difference in histologic scores between the 2 groups was also significant (Supplementary Figure 2D).

A further evaluation of CD4⁺ T-cell infiltration was made by assessing the absolute number of LP CD3⁺CD4⁺ T cells. No CD3⁺CD4⁺ T cells were recovered from the colonic tissue of SCID mice without cell administration (data not shown). The absolute number of colonic LP CD4⁺ T cells recovered from IR-SCID mice, but not from IP-SCID mice, far exceeded the number of originally injected cells (Figure 2B), indicating penetration of CD4⁺ T cells from the lumen side to the LP and extensive expansion in the colon in IR-SCID mice during 10 weeks after administration. Furthermore, it was of note that substantial numbers of CD3⁺CD4⁺ T cells were also recovered from mesenteric lymph node (MLN), peripheral blood (PB), and spleen (SP) of IR-SCID mice (Figure 2B), suggesting that some of the LP CD3⁺CD4⁺ T cells in the colon of IR-SCID mice could not only penetrate the LP but also egress from LP to systemic circulation. Flow cytometric analysis revealed that CD3⁺CD4⁺ T cells recovered from the LP of IR-SCID or IP-SCID mice are mostly of CD44⁺CD62L⁻ effector-memory T (T_{EM}) phenotype irrespective of the presence or absence of colitis (Supplementary Figure 2E). To physiologically adopt this cell transfer system in vivo, we also performed it without ethanol preadministration and recovered a small but substantial number of CD3⁺CD4⁺ T cells from the LP,

MLN, PB, SP, and bone marrow (BM) of IR-SCID mice 10 weeks after administration (Supplementary Figure 3). We also developed simpler, but surprisingly, more efficient intrarectal cell transfer protocol without the pretreatments of Niflec and pronase as demonstrated in Figure 2C–E.

The evidence that intrarectal, but not intraperitoneal, administration of splenic CD4⁺ T cells induced colitis was surprising (Figure 2), although this donor population contains a substantial number of T_R cells (approximately 10% in total CD4⁺ T cells, data not shown). To assess this issue, we next administered the same number of whole splenic CD4⁺ T cells or T_R cell-depleted CD4⁺CD25⁻ T cells into SCID mice (Supplementary Figure 4A). As expected, the 2 groups of mice similarly developed colitis (data not shown) and had comparable absolute numbers of CD3⁺CD4⁺ T cells in the LP, MLN, PB, SP, and BM at 7 weeks after cell administration (Supplementary Figure 4B), suggesting 3 possibilities: (1) naïve CD4⁺ T, but not T_R cells, penetrate epithelial barriers; (2) both T_R cells and naïve CD4⁺ T cells penetrate epithelial barriers, but T_R cells cannot suppress the expansion of naïve CD4⁺ T cells to become colitogenic CD4⁺ T cells in the LP; and (3) both T_R cells and naïve CD4⁺ T cells penetrate epithelial barriers, but naïve CD4⁺ T cells alone egress the LP and are instructed in MLN to become gut-homing receptor-expressing colitogenic CD4⁺ T cells (see the following section).

We also investigated whether other lymphocytes can penetrate intestinal epithelial monolayer and egress from LP or not. For this purpose, 1×10^8 splenocytes from Ly5.1⁺ C57BL/6 mice were intrarectally administered to Ly5.2⁺ RAG-2^{-/-} recipients (Ly5.1⁺ IR), and 1×10^7 splenocytes from C57BL/6 mice were intravenously administered to RAG-2 recipients (Ly5.1⁺ IV). Splenocytes were recovered from mice in each group at 4 weeks after cell administration, and the number of Ly5.1⁺CD3⁺CD4⁺ (CD4⁺ T), Ly5.1⁺CD3⁺CD8⁺ (CD8⁺ T), Ly5.1⁺CD3⁺NK1.1⁺ (NKT), Ly5.1⁺CD3⁻NK1.1⁺ (NK), B220⁺ (B) cells were determined by fluorescence-activated cell sorter (Supplementary Figure 5A). We confirmed that other lymphocytes such as CD8⁺ T, B, NK, and NKT cells were also detected in the SP of transferred mice, although the numbers of these cells are lower than that of CD4⁺ T cells (Supplementary Figure 5B).

SCID Mice Intrarectally Transferred With Colitogenic CD4⁺ T_{EM} Cells Developed Severe Colitis

To characterize further the penetration between epithelial barriers and egress from the LP of CD4⁺ T cells without the impact of the initiation phase of naïve CD4⁺ T cells, we next intrarectally administered the same number of colitogenic CD4⁺ T cells obtained from colitic mice previously transferred with CD4⁺CD45RB^{high} T cells or splenic CD4⁺ T cells from normal mice into SCID mice (Figure 3A). Both groups developed colitis as evidenced by clinical and histologic scores, and the recovered number of LP CD4⁺ T cells (Figure 3B–D), suggesting that colito-

genic memory CD4⁺ T cells also can penetrate from the intestinal lumen to the LP following intrarectal administration. However, it was of note that the numbers of CD4⁺ T cells recovered from MLN, PB, and BM of mice intrarectally administered with colitogenic CD4⁺ T cells were significantly lower than those of mice administered with splenic CD4⁺ T cells (Figure 3D), suggesting that the former cells have a tendency to remain in the LP and not easily egress to afferent lymphatics. Regarding the role of CD4⁺CD25⁺Foxp3⁺ T_R cells in their penetration between epithelial barriers and egress from the LP, it was notable that approximately normal proportion of CD4⁺Foxp3⁺ T_R cells resided in the LP and SP of mice administered with splenic CD4⁺ T cells, but not with colitogenic CD4⁺ T cells (Figure 3E), suggesting that intrarectally administered T_R cells not only penetrate epithelial barriers but also egress the LP.

Short Time Course Analysis of Intrarectally Administered CD4⁺ T Cells

To clarify that intrarectally administered CD4⁺ T cells continue to reside in the intraepithelial space or LP of mice without the impact of cell proliferation in the lymphopenic condition, we next administered splenic CD4⁺ T cells into RAG-2^{-/-} mice (hereafter referred to as IR-RAG mice) and visualized the localization of CD4⁺ T cells by immunohistochemistry at the early time points, 3, 12, and 24 hours after administration. As shown in Figure 4A, (1) CD4⁺ T cells (red) adhering to epithelial cells (green) from the lumen side were detected at 3 hours after administration; (2) many CD4⁺ T cells were found to reside in the intraepithelial space, and a few CD4⁺ T cells were detected in the LP at 12 hours after administration; and (3), thereafter, CD4⁺ T cells were as abundant in the LP as in the epithelial space at 24 hours after administration. To determine further whether CD4⁺ T cells could penetrate to intestinal barriers from the lumen side, we next conducted an electron microscopic analysis using intestinal samples at 6 hours after administration. As expected, we found that small lymphocytes with round nuclei and smooth surfaces resided in the intraepithelial space with normal epithelial structure in IR-RAG mice (Figure 4B).

To rule out the possibility that intrarectally administered cells might have directly entered the small blood vessels that were exposed to the damaged intestinal lumen by the ethanol treatment, we next checked the time course of the first emergence of CD4⁺ T cells from the LP, MLN, and SP. To this end, splenic CD4⁺ T cells from CAG-GFP Tg mice were administered intravenously or intrarectally to RAG-2^{-/-} mice to precisely determine the absolute number of CD4⁺ T cells. In RAG-2^{-/-} mice intravenously administered with CD4⁺ T cells (GFP-IV mice), GFP⁺ cells were detected in MLN and SP, but not the LP, at 6 hours or 24 hours after administration and in the LP as well as MLN and SP at 168 hours after administration (Figure 5). In contrast, in RAG-2^{-/-} mice intrarectally administered with CD4⁺ T cells (GFP-IR mice), GFP⁺ cells were found only in the LP or MLN, but not in the SP, at 6 hours after admin-

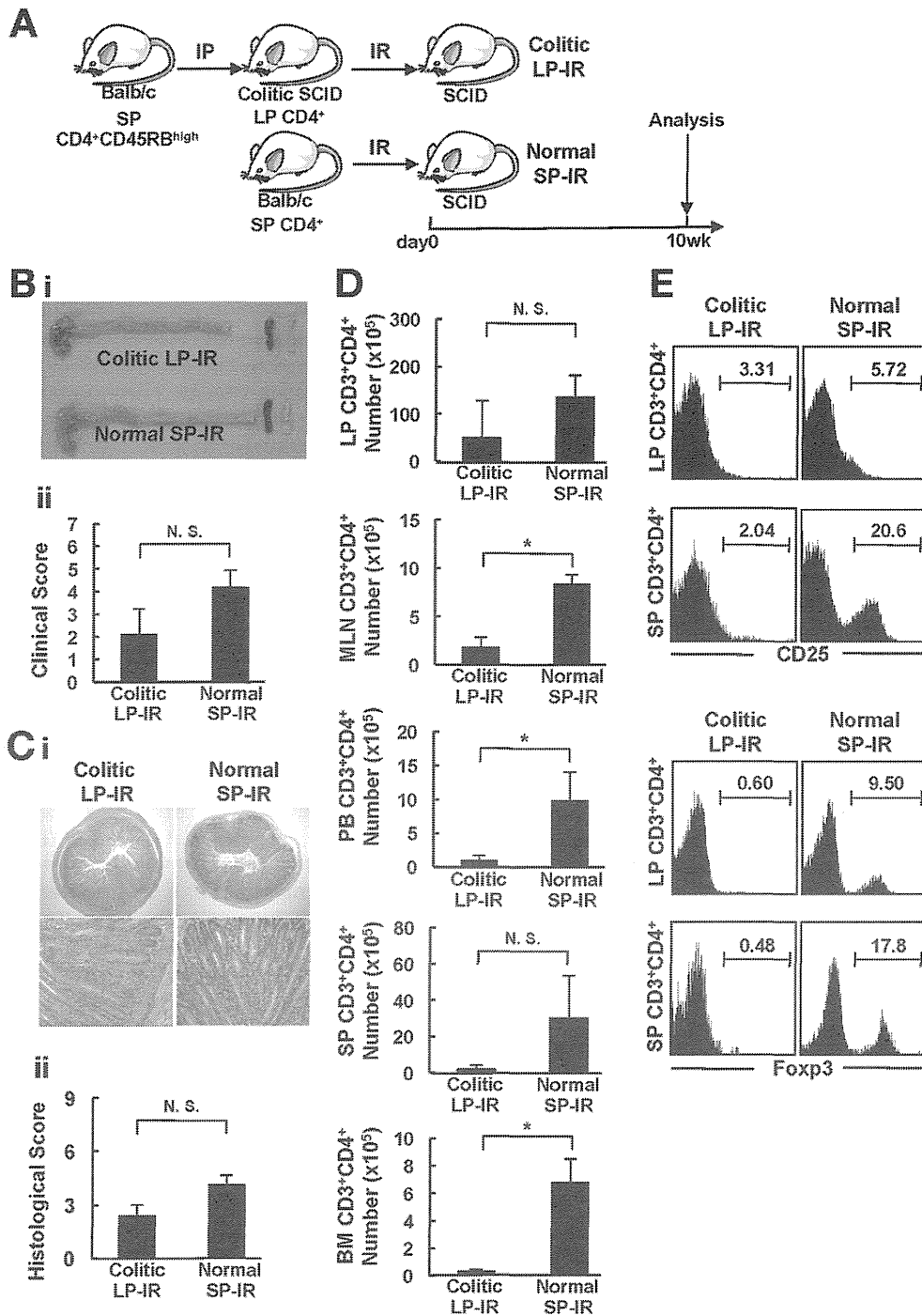


Figure 3. Intrarectally administered colitogenic memory CD4⁺ T cells not only penetrate intestinal epithelial barriers and reside in the LP but also migrate to MLN and SP. (A) SCID recipient mice were intrarectally administered CD4⁺ T cells from normal BALB/c mice (5×10^6 , *Normal SP-IR mice*, $n = 7$) or CD4⁺ T cells from colitic SCID mice previously transferred with CD4⁺CD45RB^{high} T cells (5×10^6 , *Colitic LP-IR mice*, $n = 7$). (B-a) Gross appearance of the colon from *Normal SP-IR* mice and *Colitic LP-IR* mice at 10 weeks after cell administration. (B-b) Clinical scores were determined at 10 weeks after administration as described in the Materials and Methods section. Data are indicated as the mean \pm standard error of mean (SEM) of 7 mice in each group. * $P < .01$. (C-a) Histologic examination of the colon at 10 weeks after administration. Original magnification, $\times 40$ (upper panels) and $\times 100$ (lower panels). (C-b) Histologic scores were determined at 10 weeks after transfer as described in the Materials and Methods section. Data are indicated as mean \pm SEM of 7 mice in each group. * $P < .05$. (D) LP, MLN, and spleen CD3⁺CD4⁺ T cells were isolated from the colon at 10 weeks after cell administration, and the number of CD3⁺CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean \pm SEM of 7 mice in each group. * $P < .01$. (E) Phenotypic characterization of CD3⁺CD4⁺-gated T cells expressing CD25⁺ or Foxp3⁺ T_H cells in SP and LP of the mice in each group by FACS. Representatives of 7 separate samples in each group.

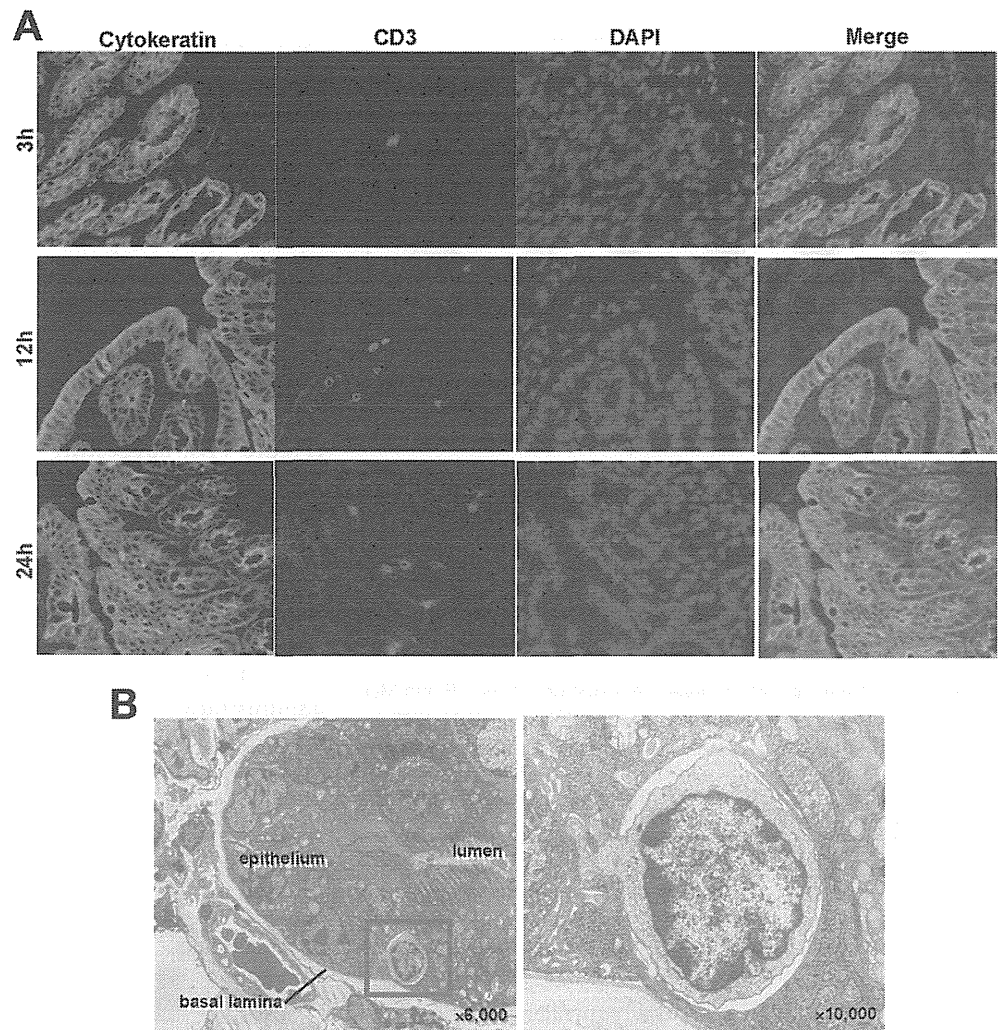


Figure 4. CD4⁺ T cells penetrate epithelial barriers in colon. Splenic CD4⁺ T cells from Ly5.1-background C57BL/6 mice were intrarectally administered to Ly5.2-background RAG-2^{-/-} mice. Mice were killed at 3, 12, or 24 hours after administration. (A) Immunostaining of cytokeratin (green), CD3 (red), and 4'6-diamidino-2-phenylindole (DAPI) (blue) counterstaining. Representative of 4 separate samples in each group. Original magnification, $\times 100$. (B) Electron microscopic analysis using intestinal samples at 3 hours after administration. Representative of 2 separate samples. Red square in the left panel was zoomed up to right panel. Original magnification, $\times 6000$ (left) and $\times 10,000$ (right).

istration and subsequently in the LP, MLN, and SP at 24 and 168 hours after administration (Figure 5). Similar emergence of intrarectally administered CD4⁺ T cells first in the LP and later in the MLN and SP was confirmed in a similar experiment using immunosufficient C57/BL6 recipient mice (Figure 6). These results suggest that cells administered intrarectally were transferred from the LP to SP via afferent lymphatics to MLN but not directly via blood vessels exposed by ethanol treatment.

We further performed long-term experiment with GFP⁺ donor/RAG-2^{-/-} recipient system (Supplementary Figure 6A) in addition to CD4⁺ donor/SCID recipient system (Figure 2) because it was possible that the existence of leaky CD4⁺ T cells in SCID recipient mice affected our previous results. Ten weeks after the transfer, intrarectally, but not intravenously, administered GFP⁺CD4⁺ T cells expanded not only in colonic LP but also in the MLN, SP and BM, which led to develop colitis in RAG-2^{-/-} recipient mice (Supplementary Figure 6B–F). We further checked cell surface markers of endogenous immune cells in addition to CD4 by fluorescence-activated cell sorter to clarify the accuracy of intrarectal administration of

GFP⁺CD4⁺ cells. Indeed, CD4⁺ cells contained both GFP⁺ and GFP⁻ cells, CD3-gated CD4⁺ T cells exclusively resided in GFP⁺ subpopulation, but not in GFP⁻ subpopulation (Supplementary Figure 6G), suggesting that GFP⁻CD4⁺ cells are non-T cells, such as CD4-expressing macrophages and lymphoid tissue inducer cells. In contrast, NK1.1⁺, Gr1⁺, CD11b⁺, and CD11c⁺ cells preferentially resided in GFP⁻ cells (Supplementary Figure 6H), concluding that GFP⁺ CD3⁺CD4⁺ T cells were all exogenous, but other immune compartments were all endogenous (Supplementary Figure 6G).

To further rule out the possibility that CD4⁺ T cells egress through the colonic lymphoid organ in the intestine such as isolated lymphoid follicle, we performed intrarectally administration of CD4⁺ T cells using LT α ^{-/-} \times RAG-2^{-/-} mice, which lack such lymphoid organ. LT α ^{-/-} \times RAG-2^{-/-} mice or RAG-2^{-/-} mice were intrarectally administered with splenic CD4⁺ T cells isolated from CAG-GFP Tg mice and were killed at 14 days after administration to assess the localization of CD4⁺ T cells (Supplementary Figure 7A). The absolute numbers of CD4⁺ T cells recovered from the LP, SP, and BM of

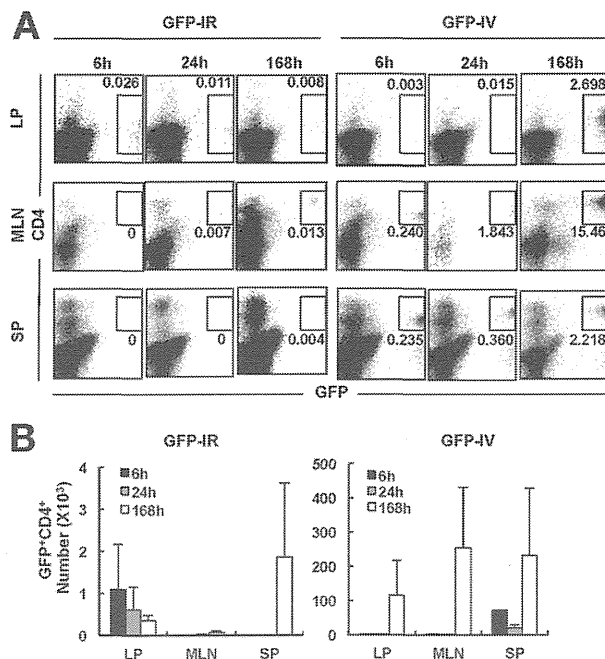


Figure 5. Intrarectally administered cells emerged in the LP and MLN before emerging in SP of recipient mice. (A) RAG-2^{-/-} mice were administered CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7 , GFP-IR mice, $n = 4$ at each time point) or intravenously (1×10^6 , GFP-IV mice, $n = 4$ at each time point) and were killed at 6, 24, or 168 hours after administration. Representatives of 4 separate samples in each group. (B) Cells were isolated from LP, MLN, and SP at 6, 24, or 168 hours after administration, and the number of GFP⁺ cells was determined by flow cytometry. Data are indicated as the mean \pm standard error of mean of 4 mice in each group.

LT α ^{-/-} \times RAG-2^{-/-} mice were equivalent to the paired absolute numbers from RAG-2^{-/-} mice, suggesting that egress of CD4⁺ T cells from LP is independent of gut-associated lymphoid tissue (GALT).

CD4⁺ T Cells Egress From the Intestinal LP in CCR7- and S1P₁-Independent Manner

Given the evidence that administered CD4⁺ T cells reside not only in the LP but also in MLN and SP of IR mice, we next attempted to investigate the molecular mechanism of egress of CD4⁺ T cells from the intestinal LP. Recent reports have demonstrated that CCR7 plays a key role in the return of T cells to lymph node from peripheral tissues, such as lung and skin.^{7,8} To assess whether this is the case with the egress of CD4⁺ T cells from the LP, RAG-2^{-/-} mice were intrarectally administered with splenic CD4⁺ T cells isolated from wild-type (WT) (WT-IR) or CCR7^{-/-} mice (CCR7-IR) and were killed at 14 days after administration (Figure 7A). Consistent with the above results, substantial numbers of CD4⁺ T cells were recovered from the LP of WT-IR and CCR7-IR mice (Figure 7B). Notably, the numbers of CD4⁺ T cells recovered from the LP, MLN, PB, SP, and BM of CCR7-IR mice were equivalent to paired numbers from WT-IR mice (Figure 7B), suggesting that egress from LP is mediated in a CCR7-

independent manner. In addition to the above experiment, we performed another experiment to assess the possibility that egress of CD4⁺ T cells from LP is mediated by S1P, which regulate the trafficking of lymphocytes in secondary lymphoid organs. RAG-2^{-/-} mice were pretreated with phosphate-buffered saline or sphingosine-1-phosphate receptor agonist (FTY720) (1.0 mg/kg), which is the agonist of S1P receptor, daily starting 1 day before the transfer over a period of 2 weeks and were administered 3×10^6 CD4⁺ T cells from WT C57BL/6. They were killed at 14 days after administration (Figure 7C). The number of CD4⁺ T cells recovered from each organ of FTY720-treated mice was equivalent to paired number from control mice (Figure 7D), suggesting that CD4⁺ T cells recovered from FTY720-treated mice could penetrate the gut wall and egress from the LP, and the egress of CD4⁺ T cells from LP to afferent lymphatics is not mediated by S1P₁-dependent manner either.

Discussion

In the present study, by a series of intrarectal administration of living CD4⁺ T cells into recipient mice, we demonstrated that CD4⁺ T cells can not only penetrate from the intestinal lumen side to the LP but also egress from

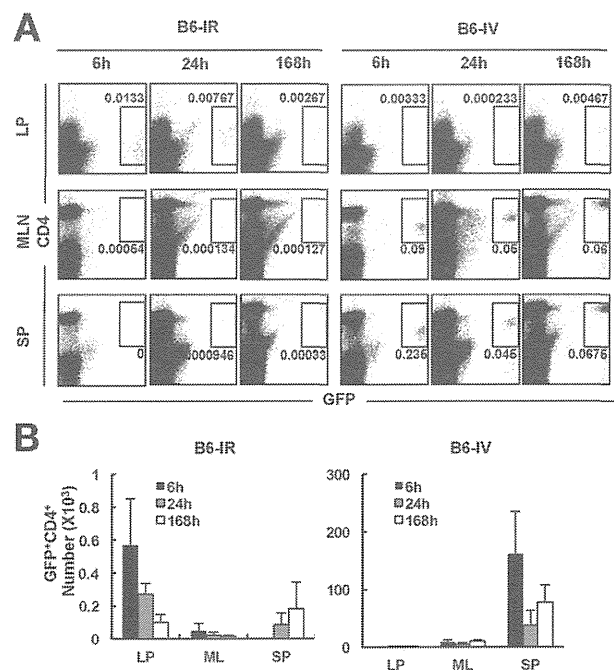


Figure 6. Intrarectally administered cells emerged in the LP and MLN before emerging in SP of immunosufficient CB57/BL6 recipient mice. (A) C57/BL6 mice were administered CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7 , B6-IR mice, $n = 4$ at each time point) or intravenously (1×10^6 , B6-IV mice, $n = 4$ at each time point) and were killed at 6, 24, or 168 hours after administration. (B) Cells were isolated from LP, MLN, and spleen at 6, 24, or 168 hours after administration, and the absolute number of GFP⁺ cells was determined by FACS. Data are indicated as the mean \pm standard error of mean of 7 mice in each group.

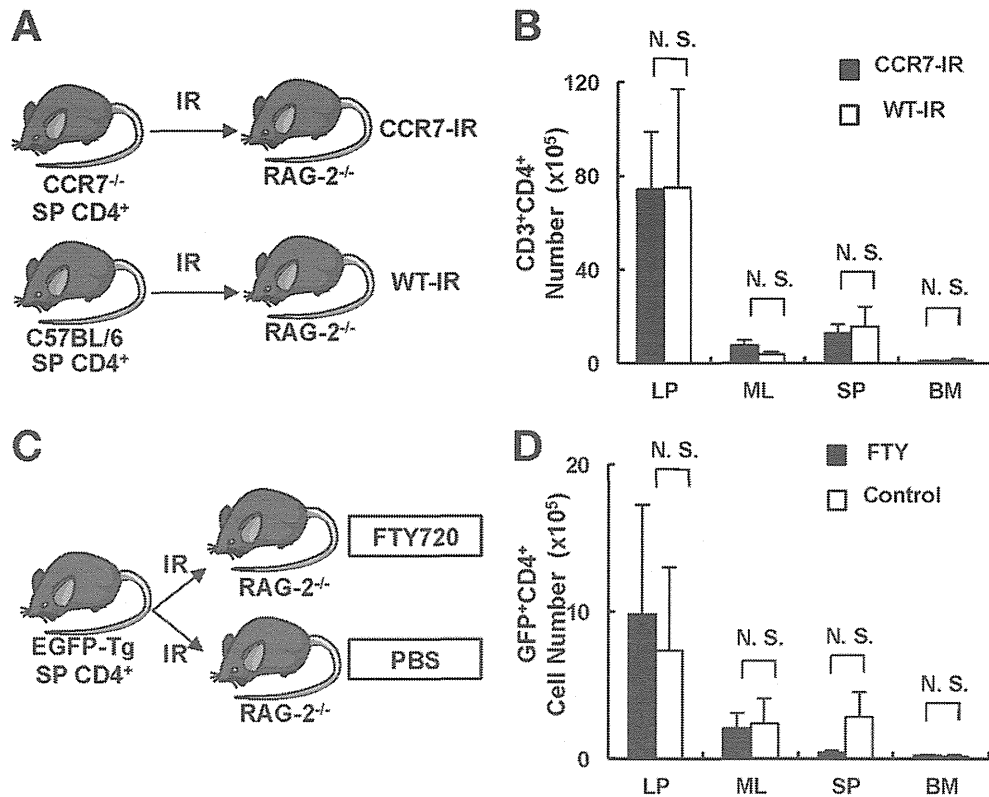


Figure 7. Intrarectally administered cells egress from the LP in a CCR7⁻ and a S1P₁⁻ independent manner. (A) RAG-2^{-/-} mice were intrarectally administered with 1×10^7 CD4⁺ T cells from WT C57BL/6 mice (WT-IR mice, $n = 5$) or age-matched CCR7^{-/-} mice (CCR7-IR mice, $n = 5$) and were killed at 14 days after administration. (B) Cells were isolated from LP, MLN, spleen, and BM at 14 days after administration, and the absolute number of CD3⁺CD4⁺ T cells recovered from each organ was determined by FACS. Data are indicated as the mean \pm standard error of mean (SEM) of 7 mice in each group. N. S., not significant. (C) RAG-2^{-/-} mice were pretreated with phosphate-buffered saline or FTY720 (1.0 mg/kg) daily starting 1 day before the transfer over a period of 2 weeks and were administered 1×10^7 CD4⁺ T cells from CAG-GFP Tg mice. Mice were killed at 14 days after administration. (D) Cells were isolated from LP, MLN, SP, and BM at 14 days after administration, and the number of CD3⁺CD4⁺ T cells was determined by flow cytometry. Data are indicated as the mean \pm SEM of 7 mice in each group. N. S., not significant.

the LP to the bloodstream in CCR7- and S1P-independent manners. Although the intestinal LP had been considered to be a “graveyard” of lymphocytes, which possesses a system that suppresses the egress of T cells from this tissue, we here showed experimentally that LP CD4⁺ T cells are actively returned to systemic circulation.

Pathologically, it is well-known that immune cells, such as granulocytes and macrophages, can penetrate from the intestinal LP to lumen side and accumulate in intestinal crypts to form “crypt abscesses,” which are often detected in active stage of UC.¹² In addition, microscopic examination of leukocytes in stool had long been recognized as an important diagnostic tool for patients with acute colitis.¹⁸ Of particular note, Harris et al demonstrated that the stool leukocytes depend on a break in the integrity of intestinal epithelial cells in patients with typhoid fever, in which more than 90% of the white cells were predominantly mononuclear,¹⁹ suggesting that lymphocytes are also able to transude to the intestinal lumen. Indeed, it is also well-known that T lymphocytes physiologically are able to reside in intraepithelial space as intraepithelial lym-

phocytes.^{1,20} Furthermore, recent elegant works demonstrated that CX3CR1⁺ DC beneath the epithelial cells in small intestine are able to open the tight junctions between adjacent epithelial cells and send dendrites out to sample luminal antigens directly.^{11,21} These findings may support the possibility that immune cells actively shuttle between the lumen and the LP side. If so, the present system would be applicable to cell therapy in which protective cells, such as CD4⁺CD25⁺Foxp3⁺ T_R cells, are administered intrarectally.

In this study, however, it was surprising that SCID mice intrarectally administered with splenic CD4⁺ T cells including T_R cells developed colitis. In this regard, it was possible that naïve CD4⁺ T, but not T_R cells, penetrate epithelial barriers and egress the LP, but this is not likely because we found both T_R and non-T_R cells in the LP and MLN and SP (Figure 3). Rather, it seems more likely that intrarectally administered T_R cells not only penetrate epithelial barriers but also egress the LP but that they cannot suppress the expansion of colitogenic CD4⁺ T cells in the first initiation site, LP, of naïve CD4⁺ T cells. In addition, it was also interesting

that the transepithelial migration of CD4⁺ T cells in the intestine may be a universal phenomenon, as shown in mice of multiple immunological background, such as C.B.-17 SCID, C57BL/6 RAG^{-/-}, and C57BL/6 mice, in sharp contrast to the dendrites of CX3CR1⁺ DC in small intestine are present in C57BL/6 but not in BALB/c mice.²²

So far, it has been thought difficult to determine whether T cells in intestinal LP can exit the gut to afferent lymphatics. Although an *in vivo* experiment using a direct injection of cells into the footpad has successfully demonstrated that lymphocytes in peripheral skin tissue can egress in a CCR7-dependent manner,⁸ in our hands, it was technically difficult to inject cells that can be distinguished by some molecular markers, such as GFP and Ly5.1, directly into the very thin intestinal wall of mice. In addition, even if it were possible to insert a catheter into afferent lymphatics to directly drain cells by using large animals such as sheep, it still remains unclear whether those cells would definitely be derived from the intestinal LP because they could be a mixture of cells from sites including adipose tissues around the intestine and could be drained from the sites of primary LNs that are located closer to the intestine than the draining site.²³ Therefore, we administered cells by the intrarectal route to clarify 2 possible steps: lymphocyte penetration between epithelial barriers and the egress of lymphocytes from the intestinal LP.

This approach may also be open to criticism that cells administered by intrarectal enema would directly enter the small blood vessels, which is exposed to the intestinal lumen via ulcer caused by the ethanol treatment. However, this possibility was not likely because of the following findings: first, a small, but substantial, number of cells administered intrarectally were also found in the LP of recipient mice without ethanol treatment (Supplementary Figure 3); and, second, the first emergent site of cells in recipient mice after intrarectal administration was the LP, but not MLN or SP, in the time course experiment (Figures 5 and 6). Although our results using CCR7^{-/-} mice could not demonstrate the CCR7 dependency of cell egress from the gut, this is not surprising because of the findings of 2 papers using CCR7^{-/-} mice and/or CCR7-Tg mice in skin⁸ and lung⁷ systems that also demonstrated a CCR7-independent mechanism of cell egress in addition to CCR7-dependent one. In other words, the lack of evidence for CCR7 dependency of cell egress from the gut may explain why it has been so far believed that LP lymphocytes are not able to egress from the gut and thereby die there as if in a "graveyard."²¹ Consistent with this, we also observed that intrarectally administered colitogenic CD4⁺CD44^{high}CD62L⁻ T_{EM} cells were subsequently detected in sites outside the intestine, such as MLN and SP (Figure 3).

In conclusion, we have here demonstrated 2 important findings: first, CD4⁺ T cells are able to migrate from the lumen to the LP side through intraepithelial space; and, second, LP CD4⁺ T cells are also able to

egress from the LP systemically via the bloodstream. This new method may provide a tool for investigation of cell trafficking of intestinal mucosa and also a concept of cell therapy by enema administration for intestinal diseases including inflammatory bowel diseases. Supplementary Figure 8.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.08.035.

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Conflicts of interest

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Supplementary Materials and Methods

Patients

Four patients with ulcerative colitis (UC) undergoing colectomy at the Tokyo Medical and Dental University Hospital between 1999 and 2006 were enrolled in the study for immunohistochemical study (Supplementary Table 1).

Antibodies

Biotin-conjugated anti-mouse interleukin 7 Receptor alpha chain (A7R34) was obtained from eBioscience (San Diego, CA). Fc gamma II/III receptor (CD16/CD32)-blocking monoclonal antibodies (mAb) (2.4G2), Phycocerythrin (PE)-, Peridinin chlorophyll protein (PerCP)-, and allophycocyanin (APC)-conjugated anti-mouse CD4 (RM4-5); fluorescein isothiocyanate (FITC)- and PerCP-conjugated anti-mouse CD3 (145-2C11); PE-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); PE-conjugated anti-mouse Ly5.1 (A20); FITC-conjugated anti-mouse B220 (RA3-6B2); FITC-conjugated anti-mouse NK1.1 (PK136); FITC-conjugated anti-mouse CD8a (53-6.7); and PE-conjugated streptavidin were obtained from BD Pharmingen (San Diego, CA).

Purification of T-Cell Subsets

CD4⁺ T cells were isolated from spleen and colon using the anti-CD4 (L3T4)- MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. To isolate normal lamina propria (LP) CD4⁺ T cells, the entire length of the colon was opened longitudinally, washed with phosphate-buffered saline (PBS), and cut into small pieces. The dissected mucosa was incubated with Ca²⁺, Mg²⁺-free Hank's balanced salt solution containing 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, MO) for 45 minutes to remove mucus then treated with 3.0 mg/mL collagenase (Roshe Diagnostics GmbH, Germany) and 0.01% DNase (Worthington Biomedical, Freehold, NJ) for 2 hours. The cells were pelleted 2 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, as estimated by fluorescence-activated cell sorter [FACS] Calibur [Becton Dickinson, Sunnyvale, CA]) were used as donor cells.

Intrarectal Administration of CD4⁺ T Cells

As a standard protocol of intestinal preparation, recipient mice were maintained without feeding for 1 hour and were given 1 mL of Niflec water (Ajinomoto Pharma Co, Tokyo, Japan) at the concentration of 69 g/L (standard concentration for human) 3 times at intervals of 1 hour by oral catheter. Thereafter, mice were pretreated with 1 mL of ethanol (50% concentration) or distilled water enema and subsequently with 5% pronase enema at 1 hour before cell administration. The method

is illustrated in detail in Supplementary Figure 1. Experiment 1 (Figure 2A and B, Supplementary Figure 2): C.B-17 severe combined immunodeficient (SCID) recipient mice were administered with splenic CD4⁺ T cells from normal BALB/c mice intrarectally (5×10^6) or intraperitoneally (5×10^5 , as a control). Mice were monitored for clinical manifestations. The mice were killed at 10 weeks after administration and assessed for a clinical score that is the sum of 4 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; soft stool; 2, diarrhea; and an additional point was added if gross blood was noted.^{1–3} Experiment 2 (Figure 2C–E): Recombination-activating gene-2 (RAG-2)^{-/-} mice were pretreated just like protocol of Supplementary Figure 1 (Mice with Niflec/Pronase) or without the pretreatments of Niflec and pronase (Mice w/o Niflec/Pronase) before cell administration. RAG-2^{-/-} recipient mice in each group were administered with 5×10^6 CD4⁺ T cells from CAG-GFP transgenic mice (n = 10 in each group). Experiment 3 (Supplementary Figure 3): SCID recipient mice were administered with splenic CD4⁺ T cells from normal BALB/c mice intrarectally (5×10^6) with ethanol or PBS pretreatment. Mice were monitored for clinical manifestations. The mice were killed at 10 weeks after administration. Experiment 4 (Supplementary Figure 4): SCID mice were intrarectally administered with splenic whole CD4⁺ T cells (5×10^6) or CD4⁺CD25⁻ T cells (5×10^6) from normal BALB/c mice intrarectally. Mice were monitored for clinical manifestations. Mice were killed at 7 weeks after administration and assessed for a clinical score as mentioned above. Experiment 5 (Supplementary Figure 5): Ly5.2-background RAG-2^{-/-} recipient mice were administered with Ly5.1⁺ splenocytes intrarectally (1×10^8) or intraperitoneally (1×10^7). Mice were killed at 4 weeks after administration, and the absolute cell numbers of Ly5.1⁺ donor-derived CD3⁺CD4⁺ T, CD3⁺CD8⁺ T, CD3⁺NK1.1⁺ NKT, CD3⁻NK1.1⁺ NK, and B220⁺ B cells in spleen of recipient mice were assessed. Experiment 6 (Figure 3): SCID mice were intrarectally administered the same number (5×10^6) of colitogenic CD4⁺ T cells obtained from colonic LP of colitic mice previously transferred with CD4⁺CD45RB^{high} T cells or CD4⁺ T cells from SP of normal mice. The mice were killed at 10 weeks after administration. Experiment 7 (Figure 4): C57BL/6-background RAG-2^{-/-} mice were administered with splenic CD4⁺ T cells from CAG-GFP transgenic mice intrarectally (1×10^7) or intravenously (1×10^6 , as a control). Mice were killed at 3, 12, or 24 hours after administration for immunohistochemical and electron microscopic analyses. Experiment 8 (Figure 5): C57BL/6-background RAG-2^{-/-} mice were administered with splenic CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7) or intravenously (1×10^6 , as a

control). Mice were killed at 6, 24, or 168 hours after administration for flow cytometric analysis. Experiment 9 (Figure 6): Immunosufficient C57BL/6 mice were administered with splenic CD4⁺ T cells from CAG-GFP Tg mice intrarectally (B6-IR mice; 1×10^7 cells) or intravenously (B6-IV mice; 1×10^6 cells, as a control). Mice were killed at 6, 24, or 168 hours after administration for flow cytometric analysis. Experiment 10 (Supplementary Figure 6): C57BL/6-background RAG-2^{-/-} mice were administered with splenic CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7) or intraperitoneally (1×10^6 , as a control). Mice were killed at 10 weeks after administration for flow cytometric analysis. Experiment 11 (Supplementary Figure 7): RAG-2^{-/-} or lymphotoxin α -deficient \times RAG-2^{-/-} mice were administered with splenic CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7). Mice were killed at 14 days after administration. Experiment 12 (Figure 7A and B): RAG-2^{-/-} mice were intrarectally administered with 1×10^7 splenic CD4⁺ T cells obtained from wild-type C57BL/6 or age-matched CCR7^{-/-} mice and were killed at 14 days after administration. Experiment 13 (Figure 7C and D): RAG-2^{-/-} mice were pretreated with PBS or FTY720 (1.0 mg/kg) daily starting 1 day before the transfer over a period of 2 weeks and were administered with 3×10^6 CD4⁺ T cells from CAG-GFP Tg mice intrarectally (1×10^7). Mice were killed at 14 days after administration.

Immunohistochemistry

For mice studies, tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 mm) were stained with H&E. The sections were analyzed without prior knowledge of the type of T-cell reconstitution or recipient. 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.¹⁻³ Consecutive cryostat colon sections were used for immunohistochemistry with purified hamster mAb against CD3e (BD PharMingen, San Diego, CA) and rabbit polyclonal Ab against cytokeratine (DAKO, Glostrup, Denmark). Briefly, Optimal cutting temperature (O.C.T.) compound-embedded tissue samples were cut into serial sections 6 mm thick, placed on coated slides, and fixed with 4% paraformaldehyde phosphate buffer solution for 30 minutes. Slides were then incubated with the primary antibody at 4°C for overnight then stained with Alexa Fluor 594 goat anti-hamster immunoglobulin G (Invitrogen, San Diego, CA) for CD3e detection and with Alexa Fluor 488 donkey anti-rabbit immunoglobulin G for cytokeratine detection at room temperature for 60 minutes. All slides were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and observed under a confocal microscope (LSM510; Carl Zeiss, Jena, Germany). For human studies, sections (4 mm thick) of the colons and rectums were fixed in 10% buffered formalin and processed for

routine pathologic examination. Two paraffin-embedded tissue blocks that included the most crypt abscesses were selected in each case and used for the study. The staining conditions are described in Supplementary Table 2.

Electron Microscopy

The colons of the mice were cut into 1-mm pieces and fixed in 2.5% glutaraldehyde and embedded in Epon. Ultrathin sections (95 nm thick) were cut on a Reichert Ultracut S (Leica Microsystems; Heidelberg GmbH, Mannheim, Germany) and collected on Maxtaform grids (Pyser-SGL Ltd, Kent, UK). The sections were double stained with uranyl acetate and lead citrate and examined with an H-7100 electron microscope (Hitachi High-Technologies Co, Tokyo, Japan).⁴

Flow Cytometry

To detect the surface expression of a variety of molecules, isolated spleen, mesenteric lymph node (MLN), or LP mononuclear cells were preincubated with an Fc gamma II/III receptor-blocking mAb (CD16/32; 2.4G2; BD PharMingen) for 15 minutes then incubated with specific FITC-, PE-, PerCP-, allophycocyanin- or biotin-labeled antibodies for 20 minutes on ice. Biotinylated antibodies were detected with PE-streptavidin. Standard 3- or 4-color flow cytometric analyses were obtained using the FACS Calibur with CellQuest software. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAbs.

Statistical Analysis

First, we examined the normality of each group. If either of 2 groups was not normally distributed, we assessed the difference between the 2 groups, with the Mann-Whitney *U* test. If both groups were normally distributed, we assessed the variance of population to which each group belonged with the *F* test. With homoscedasticity of both populations, we assessed the difference between 2 groups, using the Student *t* test. Without homoscedasticity, we assessed the difference with the Welch *t* test. We used the program Statcell (OMS Ltd Tokorozawa, Saitama, Japan) for all statistical analysis. The results were expressed as the mean \pm standard error of the mean. Differences were considered to be statistically significant when $P < .05$.

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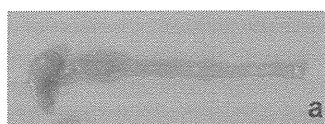
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Supplementary Table 1. Patients Enrolled in the Study

No.	Sex	Age, y	Pathologic diagnosis
UC1	Male	69	Ulcerative colitis, active phase of the colon and rectum
UC2	Male	68	Ulcerative colitis with toxic megacolon
UC3	Male	29	Adenocarcinoma of the descending colon. Ulcerative colitis
UC4	Male	53	Advanced rectal cancer. Ulcerative colitis

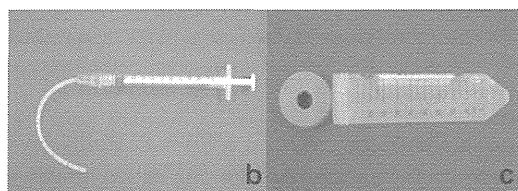
-Pre-medication

- 1, Give recipient mice 1ml of Niflec[®] (Ajinomoto Pharma Co., Tokyo), which is an oral bowel cleaner for human, at the concentration of 69g/L (standard concentration for human) three times at intervals of 1 hour, using oral catheter.
- 2, Stop any feed except water for recipient mice.



a. Gross appearance of the colon at day 2, after the pre-medication with Niflec[®].

- 3, Six hours after the administration of Niflec[®], insert the anal catheter into the colon of recipient mice to the depth of 3cm, and give 100 μ l 50% Ethanol through the catheter.
- 4, One hour after administration of Ethanol, 100 μ l 5% pronase (kaken Pharmaceutical Co., Ltd., Tokyo) was given to mice intrarectally as mentioned above.

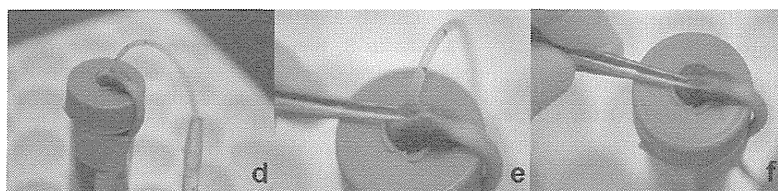


b. Rectal catheter with 1ml syringe

c. Mice fixation case

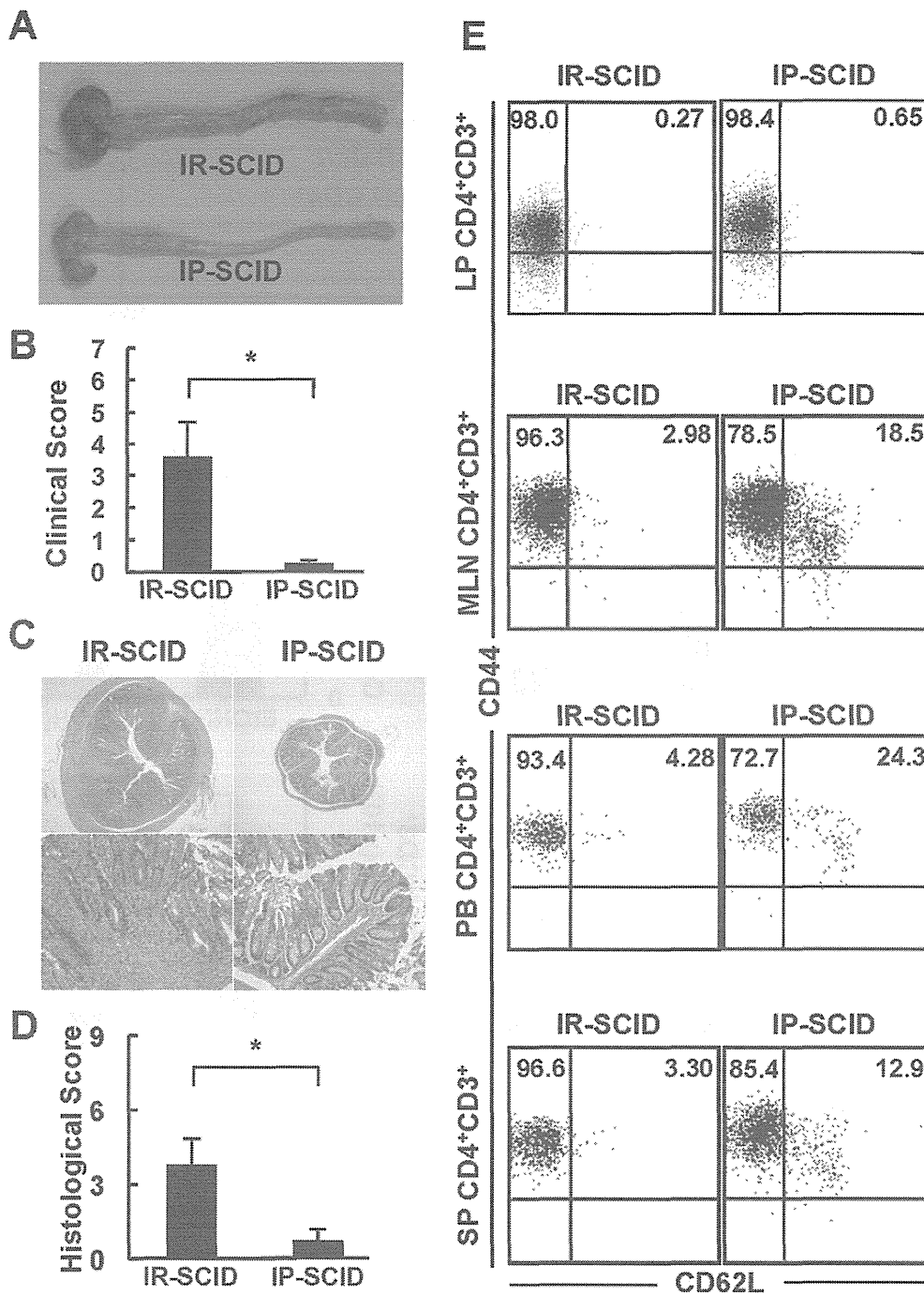
-Cell transfer

- 1, One hour after the pre-medication, insert the anal catheter into colon of recipient mice to the depth of 3cm (d), and give cells suspended to 200 μ l PBS. Reduce the defluvium using tweezers (e).
- 2, Shut the anal of recipient mice with the adhesive (f).

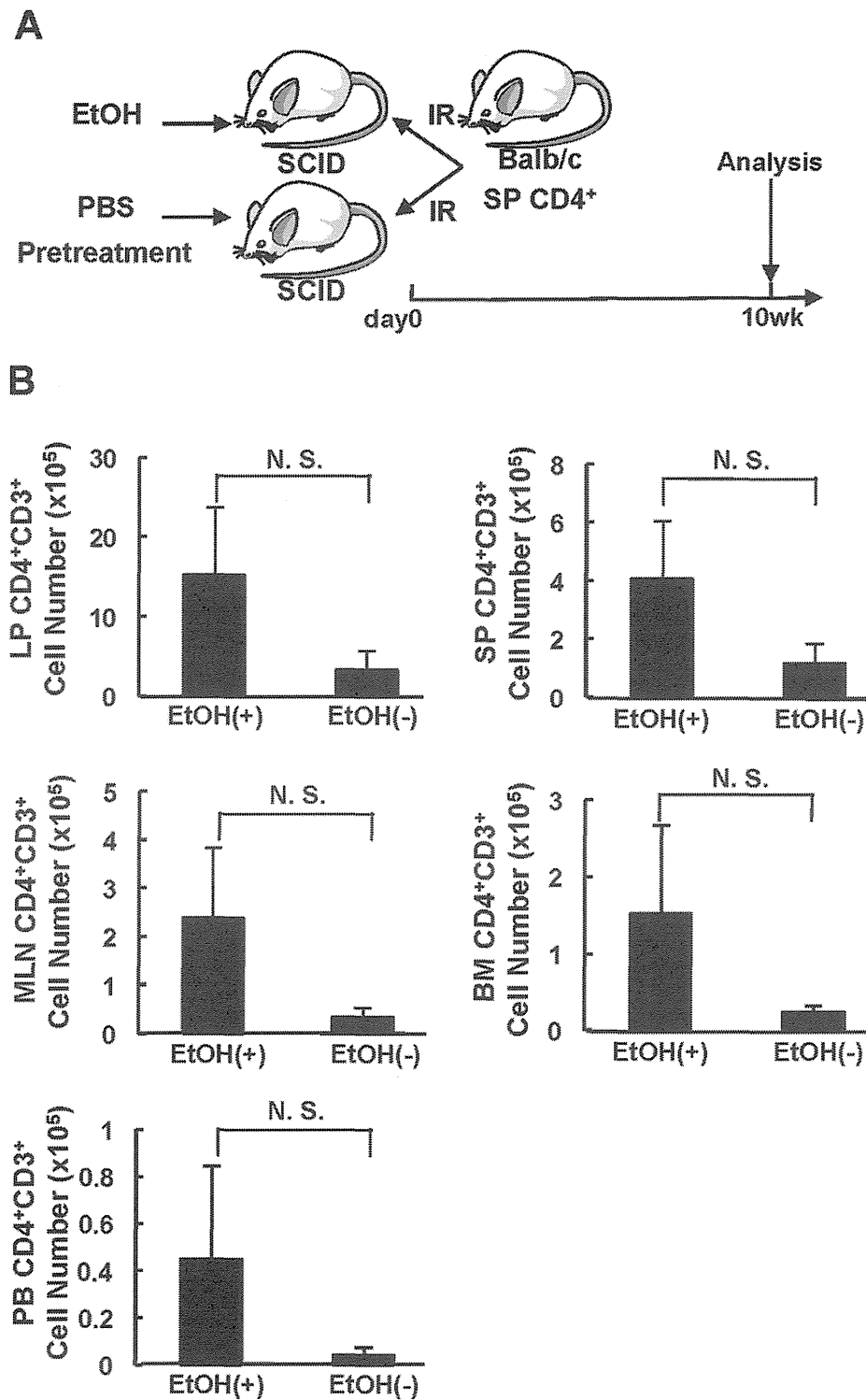


- 3, Six hours after the cell transfer, remove the adhesive from every mice.
- 4, Start feeding.

Supplementary Figure 1. Procedure of intrarectal administration of CD4⁺ T cells into mice (a) Gross appearance of colon 6 after Niflec treatment. (b) Catheter with 1-mL syringe for pronase and ethanol treatment and intrarectal administration. (c) Mice fixation case. This device is made from a 50-mL Falcon tube. (d-f) Procedures for intrarectal cell administration.



Supplementary Figure 2. Intrarectal administration of splenic CD4⁺ T cells into SCID mice induces chronic colitis. C.B-17 SCID recipient mice were administered with splenic CD4⁺ T cells from normal BALB/c mice intrarectally (5×10^6 , IR-SCID mice, n = 9) or intraperitoneally (5×10^5 , IP-SCID mice, n = 9). (A) Gross appearance of the colon from IR- and IP-SCID mice at 10 weeks after cell administration. (B) Clinical scores were determined at 10 weeks after administration as described in the Materials and Methods section. Data are indicated as the mean \pm standard error of mean (SEM) of 9 mice in each group. * $P < .01$. (C) Histologic examination of the colon at 10 weeks after administration. Original magnification, $\times 40$ (upper panel) and $\times 100$ (lower panel). (D) Histologic scores were determined at 10 weeks after transfer as described in the Materials and Methods section. Data are indicated as mean \pm SEM of 9 mice in each group. * $P < .05$. (E) Phenotypic characterization of CD3⁺CD4⁺-gated T cells expressing CD44/CD62L in lamina propria (LP), mesenteric lymph node (MLN), peripheral blood (PB), and spleen (SP) of each group. Representatives of 9 separate samples in each group.



Supplementary Figure 3. Intrarectal administration of splenic CD4⁺ T cells into SCID mice induces chronic colitis. (A) C.B-17 SCID recipient mice with or without pretreatment with 50% ethanol were intrarectally administered 5×10^6 CD4⁺ T cells from normal BALB/c mice ($n = 5$ in each group). (B) LP, MLN, and spleen CD3⁺CD4⁺ T cells were isolated from the colon at 10 weeks after T-cell administration, and the number of CD3⁺CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean \pm standard error of mean of 9 mice in each group. *N. S.*, not significant.