Intestinal Lamina Propria Retaining CD4+ CD25+ Regulatory T Cells Is A Suppressive Site of Intestinal Inflammation

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Intestinal Lamina Propria Retaining CD4⁺CD25⁺ Regulatory T Cells Is A Suppressive Site of Intestinal Inflammation

Shin Makita,* Takanori Kanai,†‡ Yasuhiro Nemoto,* Teruji Totsuka,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Masafumi Yamamoto,‡ Hiroshi Kiyono,‡ and Mamoru Watanabe*

It is well known that immune responses in the intestine remain in a state of controlled inflammation, suggesting that not only does active suppression by regulatory T (TREG) cells play an important role in the normal intestinal homeostasis, but also that its dysregulation of immune response leads to the development of inflammatory bowel disease. In this study, we demonstrate that murine CD4⁺CD25⁺ T cells residing in the intestinal lamina propria (LP) constitutively express CTLA-4, glucocorticoid-induced TNFR, and Foxp3 and suppress proliferation of responder CD4⁺ T cells in vitro. Furthermore, cotransfer of intestinal LP CD4⁺CD25⁺ T cells prevents the development of chronic colitis induced by adoptive transfer of CD4⁺ CD45RBhigh T cells into SCID mice. When lymphoxin (LT)α-deficient intercrossed Rag2 double knockout mice (LTα⁻/⁻ × Rag2⁻/⁻), which lack mesenteric lymph nodes and Peyer’s patches, are transferred with CD4⁺ CD45RBhigh T cells, they develop severe wasting disease and chronic colitis despite the delayed kinetics as compared with the control LTα⁺/⁺ × Rag2⁻/⁻ mice transferred with CD4⁺ CD45RBhigh T cells. Of note, when a mixture of splenic CD4⁺ CD25⁺ TREG cells and CD4⁺ CD45RBhigh T cells are transferred into LTα⁻/⁻ × Rag2⁻/⁻ recipients, CD4⁺ CD25⁺ TREG cells migrate into the colon and prevent the development of colitis in LTα⁻/⁻ × Rag2⁻/⁻ recipients as well as in the control LTα⁺/⁺ × Rag2⁻/⁻ recipients. These results suggest that the intestinal LP harboring CD4⁺ CD25⁺ TREG cells contributes to the intestinal immune suppression. *The Journal of Immunology, 2007, 178: 4937–4946.

Intestinal mucosal surfaces are exposed to alimentary and bacterial Ags of the intestinal flora (1). The gut-associated immune system finely tunes potentially harmful intestinal Ags from systemic circulation and induces systemic tolerance against luminal Ags. In contrast, inflammatory bowel disease is associated with activation of the local intestinal and systemic immune responses (2, 3). CD4⁺ CD25⁺ regulatory T (TREG) cells fulfill a central role in the maintenance of immunological homeostasis and self-tolerance (4, 5). CD4⁺ CD25⁺ TREG cells have been detected mainly in lymphoid sites including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated a capacity of TREG cells to prevent the induction of immune responses and because suppression requires direct cell-cell contact with responder T cells or APCs, it is conceivable that TREG cells act as central regulators within lymphoid tissues (6–8).

The gut-associated lymphoid tissue can be divided into effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria (LP) of the mucosa and organized lymphoid tissues (inductive sites) that are responsible for the induction phase of the immune response (1, 9). These include Peyer’s patches (PPs), mesenteric lymph nodes (MLNs), and isolated lymphoid follicles (ILFs). It is thought that presentation of Ags to immune naive and effector cells is concentrated at these inductive sites of organized mucosal lymphoid follicles, and thus APCs tune the delicate balance between intestinal immune tolerance and inflammation.

In addition to the inductive sites for the development of colitis, however, it also remains unclear where CD4⁺ CD25⁺ TREG cells suppress the development of colitis. Although it is reasonable to hypothesize that mechanisms for the induction, maintenance, and suppression of colitis would be centrally controlled by CD4⁺ CD25⁺ TREG cells in the inductive sites, we question in this study whether these inductive sites are solely involved in the induction and suppression of intestinal inflammation because we recently demonstrated that human intestinal LP CD4⁺ CD25bright T cells as well as peripheral CD4⁺ CD25bright T cells obtained from normal individuals possess TREG activity in vitro (10). Consistent with our previous report, it has been recently reported that CD4⁺ CD25⁺ TREG cells were detected in peripheral tissues and at sites of ongoing immune responses such as synovial fluid from rheumatoid arthritis patients (11), tumors (12), transplants (13), skin lesions in mice infected with Leishmania major (14), lungs from mice infected with Pneumocystis carinii (15), islets of Langerhans in diabetes models (16), and lesions in delayed-type hypersensitivity models (17) as well as in inflamed mucosa in colitic mice (8, 18). In the present study, we conducted a series of the adoptive transfer experiments focusing on intestinal...
Specific T cells to understand where and how CD4+CD25+ TREG cells control the mucosal immune system in vivo.

Materials and Methods

Animals

Female BALB/c, C.B-17 SCID, and C57BL/6-Ly5.2 mice were purchased from JapanCLEA, C57BL/6-Ly5.1 and C57BL/6-Ly5.2 Rag2-deficient (Rag2−/−) mice were obtained from Taconic Farms. Ly5.2 background lymphocytotoxin (LT)α−/− (LTα−/−) mice were purchased from The Jackson Laboratory. LTα−/− mice were intercrossed into Rag2−/− mice to generate LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Abs and reagents

The following mAbs except DTA-1, biotinylated anti-mouse glucocorticoid-induced TNFR (GITR; eBioscience) and FJK-16b, PE-conjugated anti-mouse CD3 (145-2C11). Biotinylated Abs were detected with PE- or CyChrome-streptavidin (BD Pharmingen). FITC-conjugated anti-mouse CD45RB (16A), FITC-conjugated anti-mouse Ly5.1 (CD45.1, A20), PE-conjugated anti-mouse CD25 (PC61), PE-conjugated anti-mouse CD103 (αβ7 integrin) (M290), PE-conjugated anti-mouse αβ integrin (DATK32), PE-conjugated anti-CTLA-4 (UC10-4F10-11), FITC-conjugated anti-mouse CD5RB (16A), FITC-conjugated anti-mouse Ly5.1 (CD45.1, A20), and FITC- and PerCP-conjugated anti-mouse CD3 (145-2C11). Biotinylated Abs were detected with PE- or CyChrome-streptavidin (BD Pharmingen).

Purification of T cell subsets

CD4+ T cells were isolated from normal spleen and colon using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer’s instructions. To isolate normal LP CD4+ T cells, the entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca2+−/−, Mg2+−/−-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase and 0.01% DNase (both Worthington, New Jersey) for 2 h. The cells were pelleted two times through 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4+ T cells from the spleen and the colon (spleen, 94–97% pure; colon, 80–90%, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), PE-Cy5- and allophyocyanin-conjugated anti-mouse CD4 (L3T4), FITC-conjugated anti-mouse CD25 (7D4), PE-conjugated anti-mouse CD25 (PC61), PE-conjugated anti-mouse CD103 (αβ7 integrin) (M290), PE-conjugated anti-mouse αβ integrin (DATK32), PE-conjugated anti-CTLA-4 (UC10-4F10-11), FITC-conjugated anti-mouse CD5RB (16A), FITC-conjugated anti-mouse Ly5.1 (CD45.1, A20), and FITC-conjugated anti-mouse CD3 (145-2C11). Biotinylated Abs were detected with PE- or CyChrome-streptavidin (BD Pharmingen).

In vivo experimental design

A series of in vivo experiments was conducted to investigate the role of intestinal LP CD4+CD25+ T cells in the suppression of murine chronic colitis. In Experiment 1, to assess the role of intestinal LP CD4+CD25+ T cells obtained from normal mice in the protection of colitis, we transferred 3 × 105 splenic CD4+CD45RBbright T cells from normal BALB/c mice with or without 1 × 106 intestinal LP CD4+CD25+ T cells into syngeneic C.B-17 SCID mice. All recipient SCID mice were sacrificed at 7 wk after transfer. In Experiment 2, to assess the necessity of gut-associated lymphoid tissues including MLNs in the development and the protection of colitis, we transferred 3 × 105 splenic CD4+CD45RBbright T cells from normal C57BL/6-Ly5.2 mice with or without 1 × 105 splenic CD4+CD25+ TREG cells from C57BL/6-Ly5.1 mice into LTα−/− × Rag2−/− mice and the control LTα−/− × Rag2−/− mice. In Experiment 3, to exclude a possible role of spleen in the suppression of colitis in addition to MLNs, we transferred 3 × 105 colitogenic LP CD4+ T cells (Ly5.2+) from established CD4+CD45RBbright T cell-transferred mice (19) with or without 3 × 105 splenic CD4+CD25+ TREG cells from C57BL/6-Ly5.1 mice into splenectomized LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice.

FIGURE 1. Identification and characterization of murine intestinal LP CD4+CD25+ T cells in terms of TREG cells in vitro. A. Freshly isolated murine spleen (SP) and LP mononuclear cells were assessed by a FACSCalibur. Representative sorting gates of the two cell populations, CD4+CD25+ and CD4+CD25−, are shown. Percentages in the upper right quadrant represent CD25+ cells at indicated site. B. Murine intestinal CD4+CD25+ constitutively express CTLA-4, GITR, and Fasp3 and partially express αβ integrins on or in LP CD4+CD25+ T cells. Thick line histogram represents staining with mAbs against the indicated markers. Thin line histogram represents staining with isotype-matched control IgG. C. Murine LP CD4+CD25+ subsets suppress the proliferation of CD4+ responder T cells in vitro. Splenic CD4+CD25−/CD4+CD25+ and LP CD4+CD25+ populations were isolated from MACS-purified CD4+ T cells by FACS sorting. The suppressive activity of the indicated subpopulations was determined by coculturing with splenic CD4+CD25− responder T cells at a 1:1 ratio of responder to TREG cells in the presence of anti-CD3 mAb and mitomycin C-treated APCs for 72 h. [3H]Thymidine uptake was determined for the last 9 h. Data are represented as the mean ± SD of triplicate samples. *, p < 0.05 compared with culture in splenic CD4+CD25+ responder cells alone.
Disease monitoring and clinical scoring

The recipient mice, after T cell transfer, were weighed initially and then three times per week thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed at the indicated time point and assayed for a clinical score that is the sum (0–8 points) of four parameters assessed for a clinical score that is the sum (0–8 points) of four parameters in the stool: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed at the indicated time point and assayed for a clinical score that is the sum (0–8 points) of four parameters assessed for a clinical score that is the sum (0–8 points) of four parameters

Histological examination and immunohistology

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (19). To detect CD11c+ dendritic cells and CD4+ T cells in the LP, consecutive cryostat sections (6 μm) were fixed and stained with the following rat Abs: purified CD4 (L3T4) and biotinylated anti-CD11c (HL3) (BD Pharmingen). Alexa Fluor 594 goat anti-rat IgG and streptavidin-Alexa Fluor 488 (Molecular Probes) were used as second Abs. All confocal microscopy was conducted on a BioZero BZ8000 (Keyence).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes or LP mononuclear cells were preincubated with an FcγR-blocking mAb (CD16/32, 2.4G2; BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PE-Cy5-, or biotin-labeled Abs for 30 min on ice. Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Intracellular Foxp3 staining was performed with the PE anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer’s instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACS caliber software and CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1 × 105 LP CD4+ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO2 in 96-well plates (Costar) precoated with 5 μg/ml hamster anti-mouse CD3e mAb (14-5-2C11; BD Pharmingen) and 2 μg/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer’s recommendation (R&D Systems).

In vitro TREG cell activity

LP mononuclear cells and splenocytes from normal BALB/c mice were separated into unfractioned CD4+ T cells, CD4+CD25+ and CD4+CD25- T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACSVantage as described. Cells (5 × 105) as APCs were cultured for 72 h in round-bottom 96-well plates in RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME. Cells were stimulated with 1 μg/ml anti-mouse CD3e mAb. In coculture experiments, the same number of splenic CD4+CD25+ or CD4+CD25- cells, or LP CD4+CD25+ or CD4+CD25- cells (5 × 105), were added into wells with the fixed dose of splenic CD4+CD25+ responder cells (5 × 105) and mitomycin C-treated
CD4+ cells (2 × 10^5), as APCs. Incorporation of [3H]thymidine (1 μCi/well) by proliferating cells was measured during the last 9 h of culture.

Statistical analysis

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

Results

Characterization of intestinal LP CD4+CD25+ in terms of TREG cell in vitro

Paired samples of spleen and colon obtained from normal BALB/c mice were analyzed by flow cytometry for the presence of the CD4+CD25+ T cells. Consistent with previous reports described, in naturally occurring CD4+CD25+ TREG cells (4–6), 7.0 ± 0.5% of the splenic CD4+ T cells were CD25+ (Fig. 1A). Similarly, 7.2 ± 1.0% of the colonic LP CD4+ T cells were also CD25+ (Fig. 1A). Because we previously demonstrated that human intestinal LP CD4+CD25bright T cells obtained from healthy individuals function as TREG cells in vitro (10), we postulated that intestinal LP as well as MLNs is another important site of regulation of immune responses for intestinal homeostasis in vivo. To prove it, we first assessed whether murine intestinal LP CD4+CD25+ T cells also express well-known TREG markers, such as CTLA-4, GITR, and Foxp3. Like the control splenic CD4+CD25+ T cells, the expression of CTLA-4, GITR, and Foxp3 was markedly up-regulated in or on the intestinal LP CD4+CD25+ T cells (Fig. 1B) compared with the paired CD4+CD25+ T cells. Unexpectedly, but consistent with our human study (10), colonic LP CD4+CD25+ T cells expressed CTLA-4, albeit to lesser extent compared with the paired colonic CD4+CD25+ T cells (Fig. 1B). To further investigate the migration property of these CD4+CD25+ T cells, we assessed the expression of αβ2/αβ3 integrins, which are gut-homing receptors essential to migrate into the colon. As shown in Fig. 1B, ~10–30% of cells in each subpopulation expressed αβ2/3 integrin. In contrast, αβ2 integrin was predominantly expressed on the splenic and LP CD4+CD25+ T cells, but not on the paired CD4+CD25− T cells, indicating that a part of splenic and LP CD4+CD25+ T cells can directly migrate into the gut.

We next investigated the TREG activity of the murine intestinal LP CD4+CD25+ T cells by testing their ability to suppress the proliferative responses of the splenic CD4+CD25− responder T cells. As shown in Fig. 1C, both the splenic and LP CD4+CD25+ T cells were able to suppress the proliferation of the splenic CD4+CD25− responder cells when cocultured at a ratio of 1:1 TREG to responder in the presence of mitomycin C-treated CD4+CD25− APCs and soluble anti-CD3 mAb (Fig. 1C), indicating that the LP CD4+CD25+ T cells were TREG cells as well as the splenic CD4+CD25+ T cells at least in vitro. As a control, it was shown that titration of the same dose of the splenic and LP CD4+CD25− cells with the splenic CD4+CD25− responder cells into the cultures did not affect the degree of proliferation, thereby excluding the possibility that an increase in total responder cell number was responsible for the suppressive effect (Fig. 1C).

Murine intestinal LP CD4+CD25+ T cells suppress the development of the CD4+CD45RBhigh T cell-transferred colitis

To next analyze the functional role of murine intestinal LP CD4+CD25+ T cell subset in vivo, we tested the TREG activity of the intestinal LP CD4+CD25+ T cells using the classical SCID-transferred colitis model induced by the adoptive transfer of CD4+CD45RBhigh T cells (19). C.B-17 SCID mice were injected i.p. with one or two subpopulations of sorted CD4+ T cell in PBS: 1) splenic CD4+CD45RBhigh T cells alone (3 × 10^5 per mouse) as a positive control, 2) splenic CD4+CD45RBhigh (3 × 10^5 per mouse) with splenic CD4+CD25+ T cells (1 × 10^5) as a negative control, and 3) splenic CD4+CD45RBhigh (3 × 10^5) with LP CD4+CD25+ T cells (1 × 10^5). The results clearly demonstrated that control of intestinal inflammation resides predominantly within the intestinal LP CD4+CD25+ subpopulation as well as the splenic CD4+CD25+ T cells, as these cells significantly inhibited the development of wasting disease (Fig. 2A) and colitis (Fig. 2, B–E). Colons from mice reconstituted with a mixture of
CD4⁺ CD45RB<sup>high</sup> and LP CD4⁺ CD25⁺ T cells exhibited no detectable pathological changes and were indistinguishable from colons from mice reconstituted with a mixture of CD4⁺ CD45RB<sup>high</sup> plus splenic CD4⁺ CD25⁺ T cells (Fig. 2B). In contrast, mice reconstituted with CD4⁺ CD45RB<sup>high</sup> cells alone developed wasting disease and severe colitis (Fig. 2). Totally, the assessment of colitis by clinical scores showed a clear difference among three groups (0.05, mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus splenic CD4⁺ CD25⁺ T cells, and 2.40 ± 1.83 in mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus LP CD4⁺ CD25⁺ T cells (p < 0.05; mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells alone vs mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus splenic or LP CD4⁺ CD25⁺ T cells) (Fig. 2E).

A further quantitative evaluation of CD4⁺ T cell infiltration was made by isolating LP mononuclear cells from the resected colons. A significantly less number of CD4⁺ T cells was recovered from mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells alone, 1.21 ± 0.97 in mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus splenic CD4⁺ CD25⁺ T cells and 2.40 ± 1.83 in mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus LP CD4⁺ CD25⁺ T cells (p < 0.05; mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells alone vs mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells plus splenic or LP CD4⁺ CD25⁺ T cells) (Fig. 2F).

FIGURE 4. Splenic CD4⁺ CD25⁺ T<sub>Rag2</sub> cells suppress the development of colitis in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4⁺ CD45RB<sup>high</sup> T cells, CD4⁺ CD45RB<sup>high</sup> T cells (3 × 10⁶ cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice with or without the cotransfer of 1 × 10⁶ splenic CD4⁺ CD25⁺ T<sub>Rag2</sub> cells derived from Ly5.1-C57BL/6 mice (n = 7 mice per each group). A, Disease activity index during 10 wk after transfer. *, p < 0.05, LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice vs LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice, transferred with splenic CD4⁺ CD45RB<sup>high</sup> T cells and splenic CD4⁺ CD25⁺ T<sub>Rag2</sub> cells. B, The lack of MLNs in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice. The abdominal MLN area was dissected and examined for the presence or absence of MLNs in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice after adoptive transfer. C, Histopathology of distal colon at 10 wk after transfer. Original magnification, ×20 (top) and ×100 (bottom). D, Histological score at 10 wk after transfer. *, p < 0.05 compared with the paired LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> or LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with splenic CD4⁺ CD45RB<sup>high</sup> T cells alone. NS, Not significant.
As shown in Fig. 3B, production of Th1 cytokines (IFN-γ, IL-2) was significantly reduced in LP CD4+ T cells from the mice transferred with CD4+ CD45RB<sup>b</sup> plus LP or splenic CD4+ CD25+ T cells as compared with those transferred with CD4+ CD45RB<sup>b</sup> T cells alone (p < 0.05). In contrast, production of IL-10 was not significantly affected among the groups (Fig. 3B).

**Spleenic CD4+ CD25+ T cells suppress the development of colitis in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells**

To further investigate the origin of LP CD4+ CD25+ T<sub>REG</sub> cells and their role in suppressing the development of colitis, we generated LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice, which lack conventional lymphoid tissues (inductive sites) including MLNs, PPs, and ILEs, as recipients for the adoptive transfer experiments. We excluded the impact of these inductive sites because it was possible that it is essential for LP CD4+ CD25+ T<sub>REG</sub> cells to be instructed to differentiate to gut-homing LP T<sub>REG</sub> cells in these inductive sites. Before addressing this issue, we first transferred splenetic CD4+ CD45RB<sup>b</sup> T cells from normal C57BL/6 mice into LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice and the littermate control LTα<sup>+/+</sup> × Rag2<sup>−/−</sup> mice to assess the role of MLNs as inductive sites in inducing colitis. When CD4+ CD45RB<sup>b</sup> T cells were transferred into the control LTα<sup>+/+</sup> × Rag2<sup>−/−</sup> mice, expectedly, the recipients rapidly developed severe wasting disease associated with clinical signs of severe colitis, in particular, weight loss, persistent diarrhea and occasionally also bloody stool and anal prolapses (Fig. 4A). When CD4+ CD45RB<sup>b</sup> T cells were transferred into the LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice, however, the recipients also developed severe wasting chronic colitis despite the delayed onset and kinetics (Fig. 4A). Clinical scores in these mice eventually reached almost the same with those in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells 10 wk after transfer (Fig. 4A). The rapid onset of colitis in the recipient LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice could easily be explained by the existence of MLNs in these mice and migration of effector CD4+ T cells primed in the these sites into the colon, but the evidence that the recipient LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice, albeit delayed, developed colitis indicates that there must be other sites where CD4+ T cells could be primed besides the MLNs. These LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells had an enlarged colon with a significantly thickened wall 10 wk after the transfer (data not shown). At the autopsy of mice, we confirmed that our established LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice macroscopically lacked MLNs (Fig. 4B) and other peripheral LNs (data not shown) in contrast to control LTα<sup>+/+</sup> × Rag2<sup>−/−</sup> mice (Fig. 4B). Tissue sections from LTα<sup>+/+</sup> × Rag2<sup>−/−</sup> and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells were characterized by inflammatory infiltrate, epithelial hyperplasia, crypt cell damage, and goblet cell depletion (Fig. 4C).

Having evidence that LN-null mice developed chronic colitis induced by the adoptive transfer of CD4+ CD45RB<sup>b</sup> T cells, we next asked whether splenetic CD4+ CD25+ T<sub>REG</sub> cells can migrate into the LP, and suppress the development of colitis in the absence of MLNs. Expectedly, LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells and splenic CD4+ CD25+ T<sub>REG</sub> cells did not show weight loss and clinical signs of colitis throughout the entire observation period (Fig. 4A). Of note, LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with a mixture of CD4+ CD45RB<sup>b</sup> T cells and splenic CD4+ CD25+ T cells also did not manifest clinical signs of colitis (Fig. 4A). Consistent with the lack of clinical signs of colitis, LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> recipients cotransferred with CD4+ CD45RB<sup>b</sup> T cells and splenic CD4+ CD25+ T cells displayed no histological evidence of intestinal inflammation (Fig. 4C). The difference among each group was also confirmed by histological scoring of multiple colon sections, which was 4.85 ± 1.58 in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells, 1.40 ± 0.96 in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells plus splenic CD4+ CD25+ T cells (p < 0.05), and 5.60 ± 0.40 in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells alone, 0.43 ± 0.23 in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4+ CD45RB<sup>b</sup> T cells plus splenic CD4+ CD25+ T cells (p < 0.05) (Fig. 4D).

We also examined the cytokine production by LP CD4+ T cells from each group of mice. As shown in Fig. 5, LP CD4+ T cells from the LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> recipients transferred with CD4+ CD45RB<sup>b</sup> T cells alone produced significantly higher amount of IFN-γ and IL-2 as compared with those transferred with CD4+ CD45RB<sup>b</sup> T cells alone produced significantly higher amount of IFN-γ and IL-2 as compared with those transferred with CD4+ CD45RB<sup>b</sup> T cells and splenic CD4+ CD25+ T cells upon in vitro anti-CD3/CD28 mAbs stimulation. In contrast, the production of IL-10 was not significantly affected.

Consistent with the reduction in the histological scores by the cotransfer of splenetic CD4+ CD25+ T cells, there was also a striking reduction in the recovered number of LP CD4+ T cells both in
FIGURE 6. Splenic CD4+CD25+ T<sub>REG</sub> cells suppress the expansion of pathogenic LP CD4+ T cells in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD45RB<sup>high</sup> T cells. CD4+CD45RB<sup>high</sup> T cells (3 × 10<sup>5</sup> cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> and LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice with or without the cotransfer of 1 × 10<sup>5</sup> splenic CD4+CD25<sup>+</sup> T<sub>REG</sub> cells derived from Ly5.1-C57BL/6 mice (n = 7 mice per each group) as described in Fig. 4. A, Recovered LP CD4+ T cells at 10 wk after transfer. Data are indicated as the mean ± SD of seven mice in each group. *, p < 0.05 compared with the paired LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> or LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with splenic CD4+CD45RB<sup>high</sup> T cells alone. B, Distribution of CD11c<sup>+</sup> dendritic cells (green) and CD4+ T cells (red) in the colon after adoptive transfer. Original magnification, ×100 (left) and ×400 (right).

FIGURE 7. Splenic CD4+CD25+ T<sub>REG</sub> cells migrate into the colonic LP and are sustained in the LP in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD45RB<sup>high</sup> T cells. A. The ratio of CD4+CD25<sup>+</sup> T<sub>REG</sub> (Ly5.1<sup>+</sup>) cells to total CD4+ T cells (Ly5.1<sup>+</sup> + Ly5.2<sup>+</sup>) at 10 wk after transfer was analyzed by gating Ly5.1 or Ly5.2 on CD4+ cells. Results shown are from seven mice per group. NS. Not significant. B, Spleen (SP) and LP cells were collected and labeled for Ly5.1, Ly5.2, CD4, and intracellular Foxp3. Ly5.2<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells were gated and analyzed for the presence of converted Ly5.2<sup>+</sup>CD4+Foxp3<sup>+</sup> cells and Ly5.1<sup>+</sup>CD4+Foxp3<sup>+</sup> cells, respectively. Number in upper right quadrant represents the percentage of inducible CD4+Foxp3<sup>+</sup> cells per CD4+ cells.

L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> and L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with a mixture of CD4+CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells 10 wk after transfer (Fig. 6A) compared with those in the paired recipients transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone. To further assess the role of LP as inductive and/or suppressive site, the expression of CD11c in the colon was investigated by immunohistochemistry (Fig. 6B). Immunohistochemical analysis of the colons revealed that significant numbers of CD11c<sup>+</sup> dendritic cells were surrounded by many CD4<sup>+</sup> T cells in both in L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> and L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, but with few CD4<sup>+</sup> T cells and CD11c<sup>+</sup> cells in L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> and L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with a mixture of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 6B), suggesting a possible role of LP as a site for actively interacting between CD11c<sup>+</sup> dendritic cells and CD4<sup>+</sup> T cells.

Spleenic CD4<sup>+</sup>CD25<sup>+</sup> T cells migrate into the intestinal LP in LTα<sup>−/−</sup> × Rag2<sup>−/−</sup> mice

To next assess the in vivo expansion of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells after adoptive transfer at a 3:1 ratio (CD4<sup>+</sup>CD45RB<sup>high</sup> (Ly5.2<sup>+</sup>) to CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.1<sup>+</sup>)), colonic LP CD4<sup>+</sup> cells were analyzed for the ratio of Ly5.2<sup>+</sup> to Ly5.1<sup>+</sup>-derived cells. As shown in Fig. 7A, ~10–15% of total LP CD4<sup>+</sup> T cells both in L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> and L<sub>α</sub>α<sup>−/−</sup> × Rag2<sup>−/−</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells plus splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells were derived from Ly5.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in the colon. These results suggested that splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells migrated to the colon, and prevented the development of colitis primarily by inhibiting the expansion and/or infiltration of pathogenic CD4<sup>+</sup> T cells in the colon and secondarily by inhibiting the development of pathogenic Th1 cells producing IFN-γ and IL-2.
FIGURE 8. Splenic CD4+CD25+ TREG cells migrate into the gut and inhibit the development of colitis induced by adoptive transfer of colitic LP CD4+ T cells into splenectomized (SPX) LTα−/− × Rag2−/− mice. Seven Rag2−/− mice in each group were injected i.p. with the following T cell subpopulations: 1) colitogenic LP Ly5.2+CD4+ T cells (3 x 10^6 cells) into splenectomized LTα−/− × Rag2−/− mice; 2) colitogenic LP Ly5.2+CD4+ T cells (3 x 10^6 cells) + splenic Ly5.1+CD4+CD25+ T cells (3 x 10^6 cells) into splenectomized LTα−/− × Rag2−/− mice; 3) colitogenic LP Ly5.2+CD4+ T cells (3 x 10^6 cells) + splenic Ly5.1+CD4+CD25+ HD9251 T cells (3 x 10^6 cells) into splenectomized LTα−/− × Rag2−/− mice. A, Gross appearance of the colon and MLN at 7 wk after transfer. B, Histopathology of distal colon at 7 wk after transfer. Original magnification, ×40. C, Histological score at 7 wk after transfer. +, p < 0.05. D, Number of recovered LP CD4+ T cells at 7 wk after transfer. Data are indicated as the mean ± SD of seven mice in each group. +, p < 0.05. E, Percentage of CD4+CD25+ TREG (Ly5.1+) cells to total CD4+ T cells (Ly5.1+ + Ly5.2+) at 7 wk after transfer was analyzed by gating Ly5.1 or Ly5.2 on CD4+ T cells. Results shown are from seven mice per group. NS, Not significant. F, LP cells were collected and labeled for Ly5.1, Ly5.2, CD4, and Foxp3. Ly5.2+ and Ly5.1+ CD4+ T cells were gated and analyzed for the presence of Foxp3+ cells. The percentage of induced Foxp3 cells per CD3+ cells is indicated in upper right quadrant of enlarged gate.

However, it was also possible that a part of Ly5.2-derived CD4+CD45RBhigh CD4+ cells was converted into inducible CD4+CD25+ TREG cells rather than pathogenic CD4+ T cells in the gut. Thus, to evaluate this possibility that the LP CD4+CD25+ TREG cells are composed of naturally arising CD4+CD25+ T cells (Ly5.1+), inducible CD4+CD25+ T cells (Ly5.2+), and pathogenic CD4+ T cells (Ly5.2+), we performed three-color flow cytometry analysis (Fig. 7B). In this setting, we stained intracellular Foxp3 because it was difficult to distinguish between activated/ pathogenic CD4+CD25+ T cells and CD4+CD25+ TREG cells by staining CD25 molecule. Indeed, only ~3–16% of splenic and LP Ly5.2+ cells were converted into inducible CD4+Foxp3+ TREG cells in both LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice transferred with CD4+CD45RBhigh T cells alone or with a mixture of CD4+CD45RBhigh T cells and splenic CD4+CD25+ T cells, but most Ly5.1-derived CD4+CD25+ TREG cells (78–88%) retained Foxp3 in both LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice transferred with a mixture of CD4+CD45RBhigh T cells and splenic CD4+CD25+ T cells (Fig. 7B).

Splenic CD4+CD25+ T cells suppressed the expansion of colitogenic LP CD4+ T cells in the gut

With respect to the site for suppression of effector and memory CD4+ T cells, it was also possible that naturally arising CD4+CD25+ T cells suppress the activation of CD4+CD45RBhigh T cells and the expansion of the differentiated effector CD4+ T cells in the spleen rather than in the gut. To clarify that CD4+CD25+ T cells suppress the expansion of colitogenic effector and memory CD4+ T cells in the gut, we finally transferred colitogenic LP CD4+ T cells obtained from colitic mice transferred with CD4+CD45RBhigh HD9251 T cells (Ly5.2+) (19) with or without splenic CD4+CD25+ T cells (Ly5.1+) into splenectomized LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice to exclude the impact of spleen. Both splenectomized LTα−/− × Rag2−/− mice transferred with colitic LP CD4+ T cells (Ly5.2+) developed wasting disease (data not shown) and colitis by assessing histological findings (Fig. 8, A–C) and the recovered CD4+ T cell numbers from the LP (Fig. 8D). In contrast, splenectomized LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice transferred with a mixture of colitic LP CD4+ T cells (Ly5.2+) and splenic CD4+CD25+ T cells (Ly5.1+) at a 1:1 ratio did not develop wasting disease (data not shown) and colitis (Fig. 8, A–D). Of note, we found that ~30–40% of LP CD4+ T cells in splenectomized LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice cotransferred with a mixture were derived from Ly5.1+ cells (Fig. 8E). Furthermore, we confirmed that CD4+Foxp3+ T cells residing in the LP were mostly derived from Ly5.1+ population in both splenectomized LTα−/− × Rag2−/− and LTα−/− × Rag2−/− mice transferred with a mixture of colitic LP CD4+ T cells (Ly5.2+) and CD4+CD25+ T cells (Ly5.1+) (Fig. 8F), indicating that LP acts as a suppressive site, and spleen is not solely essential to act as a suppressive site to inhibit the expansion of effector CD4+ T cells.
Discussion
In this study, we demonstrate that intestinal LP CD4+CD25+ T cells residing in normal mice constitutively express CTLA-4, GITR, and Foxp3 and suppress the proliferation of responder CD4+ T cells in vitro. Furthermore, cotransfer of intestinal LP CD4+CD25+ T cells prevents the development of CD4+CD45RBhigh T-cell transferred colitis. Surprisingly, when LTα−/−×Rag2−/− mice, which lack MLNs, ILFs, and PPs, were transferred with CD4+CD45RBhigh T cells, they did develop severe wasting disease and colitis despite the delayed onset and kinetics as compared with the control LTα+/+×Rag2−/− mice transferred with CD4+CD45RBhigh T cells. Of note, splenic CD4+CD25+ T cells can migrate into the LP, and prevent the development of CD4+CD45RBhigh T cell-transferred colitis in MLN-null LTα−/−×Rag2−/− recipient mice. These results suggest that at least in part intestinal LP CD4+CD25+ T cells without the instruction by an MLN environment directly migrate into the gut and act as TREG cells, and therefore may contribute to the intestinal immune homeostasis in vivo.

We have recently demonstrated that human CD4+CD25bright T cells resided in the intestinal LP, expressed CTLA-4, GITR, and Foxp3, and possessed TREG activity in vitro (10). Although the results indicate that these cells might serve as mucosal (non-lymphoid) TREG cells to maintain intestinal homeostasis against many luminal Ags, it was impossible to determine whether they actually suppress the development of colitis in vivo using any human studies. To answer the question, it was necessary to translate into the mouse experimental system. To address this issue, we proceeded with two approaches using the different adoptive transfer experiments in this study. We first directly assessed whether the cotransfer of murine intestinal LP CD4+CD25+ T cells isolated from normal mice suppress the development of colitis induced by the adoptive transfer of CD4+CD45RBhigh T cells into SCID mice. As shown in Fig. 2, we found the clinical score in SCID mice transferred with CD4+CD45RBhigh T cells and intestinal LP CD4+CD25+ T cells at a ratio of 3:1 was significantly decreased as compared with that in SCID mice transferred with CD4+CD45RBhigh T cells alone, indicating that the murine intestinal LP CD4+CD25+ T cells maintain intestinal homeostasis to suppress the development of colitis in vivo. Consistent with this, murine intestinal LP CD4+CD25+ T cells expressed constitutively CTLA-4, GITR, and Foxp3 and suppressed the proliferation of responder cells in vitro, such as human LP CD4+CD25bright T cells (10). Furthermore, because we also found that LP CD4+CD25+ T cells did partially express αβ2 and αβ2 integrins, it is conceivable that these gut-homing receptor-expressing LP CD4+CD25+ T cells might migrate into the colon from outside of the gut. Although it has been reported that CD4+CD25+ T REG cells reside in nonlymphoid tissues (10–18), our current data now provide the first experimental evidence that intestinal LP CD4+CD25+ T cells prevent the development of colitis in vivo.

Having the evidence that the murine intestinal LP CD4+CD25+ T cells suppressed the development of colitis induced by the adoptive transfer of CD4+CD45RBhigh T cells, we next asked whether MLNs are not fully essential for the suppression of colitis by splenic CD4+CD25+ T cells because it was still possible that 1) a part of the LP CD4+CD25+ T cells was needed to be instructed in MLNs to differentiate to gut-homing receptor-expressing T REG cells (17) to migrate to the gut, and also possible that 2) the transferred LP CD4+CD25+ T cells acted as T REG cells in MLNs rather than in the intestine in the first adoptive transfer experiment (Figs. 2 and 3). To address these issues, it was important to assess the CD4+CD25+ T REG cells without the impact of MLNs, which are thought to be representative inductive and suppressive sites for classical splenic CD4+CD25+ T REG cells because high expression levels of CD62 ligand enable both naive CD4+ T cells and splenic CD4+CD25+ T REG cells to efficiently enter the Ag-draining lymph nodes from the bloodstream. As the second approach to address this issue, thus, we generated LTα−/−×Rag2−/− mice as recipients for the following adoptive transfer experiment. Before starting the experiment, it was unclear whether the LTα−/−×Rag2−/− mice transferred with CD4+CD45RBhigh T cells alone develop colitis, or rather it was likely to envisage that these mice did not develop colitis because MLNs are thought to be very important as inductive sites for the development of colitis. However, it was noteworthy that these mice did develop wasting disease and colitis to a similar extent of the transferred LTα−/−×Rag2−/− mice 10 wk after transfer, although it took a longer period to establish colitis as compared with the LTα−/−×Rag2−/− recipients (Fig. 4A). Although this fact is actually not a main focus in this study, it is possible that spleen and/or LP are complimentary inductive sites to develop colitis under the absence of MLNs. Consistent with this hypothesis, it has been reported that naive T cells can recruit to the inflamed intestinal mucosa, although these cells are usually excluded from uninfamed nonlymphoid tissues (20). However, the delayed kinetics of the development of colitis in the LTα−/−×Rag2−/− mice transferred with CD4+CD45RBhigh T cells indicates that MLNs are involved in the induction of colitis by their functioning as a professional inductive site. Further study will be needed to address this initial immune response for the development of colitis.

As our focus in this study, we also found that the cotransfer of splenic CD4+CD25+ T cells obtained from normal mice prevent the development of colitis in LTα−/−×Rag2−/− mice transferred with CD4+CD45RBhigh T cells as well as in LTα−/−×Rag2−/− recipients, indicating that splenic CD4+CD25+ T cells can suppress the development of colitis in the absence of MLNs. Moreover, we demonstrated that Ly5.1-CD4+CD25+ T cells resided in the colon in MLN-null LTα−/−×Rag2−/− mice cotransferred with Ly5.2-derived CD4+CD45RBhigh T cells and Ly5.1-derived splenic CD4+CD25+ T cells, suggesting that the LP might be a regulatory site between colitogenic effector/memory cells and T REG cells to suppress intestinal inflammation probably as a second line of suppression (17). It was also possible, however, that CD4+CD25+ T REG cells prevented the expansion of pathogenic effector CD4+ T cells and the migration to the gut in the recipient’s spleen rather in the gut. With respect to this issue, we also demonstrated that cotransfer of splenic CD4+CD25+ T cells prevented the development of colitis induced by adoptive transfer of colitogenic LP CD4+ T cells in splenicotomized LTα−/−×Rag2−/− recipients (Fig. 8). Because colitogenic LP CD4+ T cells that have a phenotype of effector/memory CD4+CD44high CD62L− cells (21) should have migrated to the gut and expanded in the gut, it is very likely that splenic CD4+CD25+ T cells can directly migrate to the gut and suppress the expansion of these colitogenic CD4+ T cells in the gut. With respect to the equilibrium of pathogenic CD4+ T cells and T REG cells, however, further studies will be needed because we found that effector to T REG cell ratio varied by different experimental settings (Figs. 6 and 8). Finally, it should be discussed the protective mechanism by CD4+CD25+ T REG cells of this SCID/Rag2−/− colitis model induced by the adoptive transfer of CD4+CD45RBhigh T cells from the standpoint of the sites of active suppression. Indeed, Mottet et al. (18) previously demonstrated that not only effector CD4+ T cells but also CD4+CD25+ T REG cells accumulate in the intestinal LP in addition to the MLNs in the cured SCID mice by retransferring splenic CD4+CD25+ T cells 3–4 wk after the first transfer.
of CD4+CD45RBhigh T cells, it remains to be determined whether intestinal inflammation can be suppressed solely by LP or MLN CD4+CD25+ TREG cells in this setting. In contrast, Deneg et al. (8) recently demonstrated that β7 integrin-deficient (β7−/−) CD4+CD25+ TREG cells that preferentially migrate to MLNs, but are impaired in their ability to migrate to the intestine because of the lack of the gut-homing α4β7/α6β7 integrin molecules, are capable of preventing intestinal inflammation, suggesting TREG accumulation in the intestine is dispensable for the protection of this colitis model. In this protection protocol, indeed, it is possible that β7−/− CD4+CD25+ TREG cells are not needed to suppress the development of colitis because β7−/− CD4+CD25+ TREG cells directly migrate to MLNs and can inhibit naive CD4+CD45RBhigh T cell activation and proliferation within Ag-draining MLNs, resulting in suppressing the development of the gut-seeking activated effector CD4+ T cells instructed to express the gut-homing receptors such as α4β7/α6β7 integrin. However, it still remains unknown whether mucosal CD4+CD25+ TREG cells are necessary for the suppression of mucosal pathogenic effector CD4+ T cell ex vivo especially in the therapeutic protocol that can be assessed and whether LP CD4+CD25+ TREG cells as effector TREG cells can suppress the surrounding LP effector CD4+ T cells ex vivo. In our adoptive transfer experiment using splenectomized MLN-null LTα−/−×Rag2−/− mice, however, we clearly demonstrated that cotransfer of splenic CD4+CD25+ TREG cells suppressed the development of colitis despite the lack of spleen and MLNs and found that these TREG cells migrated to the effector sites, in this case, the intestine, suggesting that intestinal LP CD4+CD25+ TREG cells play an important role at least in part for the suppression of intestinal inflammation in vivo.

In conclusion, our findings showed that intestinal LP functions not only as a critical effector site for inflammatory responses but also as a regulatory (suppressive) site that CD4+CD25+ TREG cells directly control the pathogenic effector CD4+ cells as a second line of suppression (effector TREG) site together with the MLNs as a first line of suppression (naive TREG) site.

Disclosures
The authors have no financial conflict of interest.

References