

was routinely over 95%. For morphological studies, cytospin preparations from purified DC subsets were stained with May-Grunwald-Giemsa solution. For analysis of leukocytes, cells were subjected to density-gradient centrifugation in 40% to 75% (vol/vol) Percoll (approximately density, 1.058 g/ml and 1.093 g/ml, respectively) after enzyme treatment. Cells collected from the interface were washed and were used as lamina propria leukocytes in assays. Naive CD4<sup>+</sup> T cells from the spleens of OT-II transgenic mice and B220<sup>+</sup> cells from the peritoneal cavities of C57BL/6 mice were purified by magnetic sorting with mouse anti-CD4 beads and mouse anti-B220 beads, respectively. Peritoneal cells from C57BL/6 mice were incubated with fluorescein isothiocyanate-conjugated anti-IgD (11-26c.2a; 553439; BD Pharmingen) and phycoerythrin-indotricarbocyanine-conjugated anti-IgM (R6-60.2; 553409; BD Pharmingen) after blockade of Fc receptors. Naive B cells were sorted on the basis of their expression of IgD and IgM with a FACSVantage SEM or FACSAria (BD Biosciences). The purity of the sorted cells was routinely over 95%.

**In vitro T cell differentiation.** OT-II transgenic CD4<sup>+</sup> T cells ( $1 \times 10^6$ ) were cultured with CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs or SPDCs ( $1 \times 10^5$ ) in the presence of OVA protein (100 µg/ml), unsupplemented or supplemented with flagellin (1 µg/ml) or LPS (1 µg/ml), respectively. After 4 d, cells were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Calbiochem) in the presence of GolgiStop (BD Pharmingen), then cells producing IL-17 and IFN-γ were analyzed by flow cytometry.

**Immunization.** CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs or SPDCs were cultured for 12 h with OVA protein (100 µg/ml) in the presence or absence of flagellin (1 µg/ml) or LPS (1 µg/ml). Antigen-loading cells ( $5 \times 10^4$  per mouse) were injected on days 0 and 14 into the peritoneal cavities of naive *Thr5*<sup>-/-</sup> mice (CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs) or *Thr4*<sup>-/-</sup> mice (SPDCs); control mice were treated with PBS. At 1 week after the final immunization, splenocytes were collected and were cultured for 4 d with OVA protein (10 µg/ml). The concentration of IFN-γ, IL-17 and IL-4 in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA).

**In vitro IgA<sup>+</sup> plasma cell differentiation.** Peritoneal IgM<sup>+</sup>IgD<sup>+</sup> cells or B220<sup>+</sup> cells ( $1 \times 10^6$ ) were cultured in medium supplemented with B cell-activating factor (50 ng/ml) together with CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs or SPDCs ( $1 \times 10^5$  to  $5 \times 10^5$ ) in the presence or absence of flagellin (1 µg/ml) or LPS (1 µg/ml), respectively. After 5 d, cells were analyzed by flow cytometry and the concentration of IgA in culture supernatants was measured by ELISA.

**Flow cytometry.** Before staining, Fc receptors were blocked for 15 min at 4 °C. Low-density LPCs were stained with the following biotinylated monoclonal antibodies: anti-CD11b (MI170; 557395), anti-CD11c (HL3; 553800), anti-CD40 (3/23; 553789), anti-CD80 (16-10A1; 553767), anti-CD86 (GL1; 553690), anti-I-A/I-E (2G9; 553622) and anti-CD103 (M290; 557493; all from BD Pharmingen); anti-F4/80 (A3-1; MF48015; Caltag Laboratories); and anti-DEC-205 (NLDC-145; CL89145PE; Cedarlane Laboratories). The surfaces of cocultured T cells were stained with fluorescein isothiocyanate-labeled anti-CD4 (L3T4; 553055; BD Pharmingen). Then, cells were fixed and made permeable with Cytotfix/Cytoperm (BD Pharmingen) and were stained intracellularly with phycoerythrin-labeled anti-IL-17 (TCC11-18H10.1; 559502) and allophycocyanin-labeled anti-IFN-γ (XMG1.2; 554413; both from BD Pharmingen). The surfaces of cocultured B cells or lamina propria leukocytes were stained with phycoerythrin-labeled anti-B220 (RA3-6B2; 553090; BD Pharmingen). Then, cells were fixed and made permeable with Cytotfix/Cytoperm and were stained intracellularly with fluorescein isothiocyanate-labeled anti-IgA (CI0-3; 559354) or were incubated with biotin-conjugated IgA (CI0-1; 556978) and then stained intracellularly with allophycocyanin-labeled streptavidin (all from BD Pharmingen). CCR9 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti-mouse CCR9 (242503; FAB2160A; R&D Systems). CCR10 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti-mouse CCR10 (248918; FAB2815A; R&D Systems). Samples were acquired on a FACSCalibur with CELLQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

**RT-PCR and quantitative real-time PCR.** RNA (1 µg) was reverse-transcribed with Superscript2 (Invitrogen) according to the manufacturer's instructions with random hexamers as primers. Primer pairs specific for *Thr2*, *Thr4*, *Thr5*, *Thr7*, *Thr9*, *Aicda*<sup>30</sup> or *Actb* (Supplementary Table 1 online) and Taq polymerase<sup>18</sup> (Takara Shuzo) were used for PCR of 25 cycles at 97 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; products were separated by agarose gel electrophoresis. A 7700 Sequence Detector (Applied Biosystems) was used for quantitative real-time PCR of cDNA amplified as described above with 2× PCR Master Mix (Applied Biosystems) and primers for 18S rRNA (as an internal control; Applied Biosystems) or primers specific for *Thr5*, *Rorc*, *Aldh1a1*, *Aldh1a2* or *Aldh1a3* (Applied Biosystems), in a final volume of 25 µl. After incubation at 95 °C for 10 min, products were amplified by 35 cycles of 95 °C for 15 s, 60 °C for 60 s and 50 °C for 120 s.

**Measurement of cytokines in supernatants.** The concentrations of IFN-γ, IL-17, IL-4, IL-6, IL-10 and IL-12p40 were measured with the Bio-plex system (Bio-Rad) according to the manufacturer's instructions. The concentrations of IL-21, IL-23 and IgA were determined by ELISA (R&D Systems, eBioscience and Southern Biotech, respectively).

**Immunohistochemical analysis.** For analysis of the number of IgA<sup>+</sup> cells in the small intestinal lamina propria, fluorescein isothiocyanate-conjugated anti-mouse IgA (CI0-3; 559354; BD Pharmingen) was applied overnight at 4 °C to sections cut from frozen tissue. Immunohistochemical staining was analyzed with a Radiance 2100 Bio-Rad confocal laser microscope (Bio-Rad).

**Bacterial infection.** *S. enterica* serovar *typhimurium* has been described<sup>18</sup>. *S. typhimurium* was grown in Luria-Bertani medium without shaking at 37 °C. The concentration of bacteria was determined on the basis of the absorbance at 600 nm. Bacteria were injected orally into mice.

**Statistical analysis.** Statistical significance was evaluated with an unpaired two-tailed Student's *t*-test in all experiments except Supplementary Figure 8d. A *P* value of less than 0.05 was considered significant. Kaplan-Meier plots and log-rank tests were used to assess the survival differences of control and mutant mice after bacterial infection (Supplementary Fig. 8d).

**Accession codes.** UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org/>): A002297 and A001174.

*Note: Supplementary information is available on the Nature Immunology website.*

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#### AUTHOR CONTRIBUTIONS

K.F. and S.U. did most of the experiments; S.U., K.J.I. and M.H.J. designed all the experiments; B.-G.Y. helped with the immunohistochemical analysis; Y.-J.J. and M.N. helped to isolate cells; S.S., T.T. and M.Y. provided advice for the experiments; Y.Y. provided *Id2*<sup>-/-</sup> mice; H.K. and M.M. provided advice for the experiments and manuscript; S.U. and S.A. prepared the manuscript; and S.A. directed the research.

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# Intestinal Lamina Propria Retaining CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells Is A Suppressive Site of Intestinal Inflammation<sup>1</sup>

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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 (T<sub>REG</sub>) 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<sup>+</sup>CD25<sup>+</sup> T cells residing in the intestinal lamina propria (LP) constitutively express CTLA-4, glucocorticoid-induced TNFR, and Foxp3 and suppress proliferation of responder CD4<sup>+</sup> T cells in vitro. Furthermore, cotransfer of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells prevents the development of chronic colitis induced by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into SCID mice. When lymphotoxin (LT) $\alpha$ -deficient intercrossed Rag2 double knockout mice (LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup>), which lack mesenteric lymph nodes and Peyer's patches, are transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, they develop severe wasting disease and chronic colitis despite the delayed kinetics as compared with the control LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Of note, when a mixture of splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells and CD4<sup>+</sup>CD45RB<sup>high</sup> T cells are transferred into LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> recipients, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells migrate into the colon and prevent the development of colitis in LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> recipients as well as in the control LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> recipients. These results suggest that the intestinal LP harboring CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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 fences off 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<sup>+</sup>CD25<sup>+</sup> regulatory T (T<sub>REG</sub>)<sup>3</sup> cells fulfill a central role in the maintenance of immunological homeostasis and self-tolerance (4, 5). CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells have been detected mainly in lymphoid sites including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated a capacity of T<sub>REG</sub> 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 T<sub>REG</sub> 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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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<sup>+</sup>CD25<sup>bright</sup> T cells as well as peripheral CD4<sup>+</sup>CD25<sup>bright</sup> T cells obtained from normal individuals possess T<sub>REG</sub> activity in vitro (10). Consistent with our previous report, it has been recently reported that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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

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<sup>3</sup> Abbreviations used in this paper: T<sub>REG</sub>, regulatory T; LP, lamina propria; PP, Peyer's patch; MLN, mesenteric lymph node; ILF, isolated lymphoid follicle; GITR, glucocorticoid-induced TNFR; LT, lymphotoxin.

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LP CD4<sup>+</sup>CD25<sup>+</sup> T cells to understand where and how CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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 Japan CLEA. C57BL/6-Ly5.1 and C57BL/6-Ly5.2 Rag2-deficient (Rag2<sup>-/-</sup>) mice were obtained from Taconic Farms. Ly5.2 background lymphotoxin (LT) $\alpha$ -deficient (LT $\alpha$ <sup>-/-</sup>) mice were purchased from The Jackson Laboratory. LT $\alpha$ <sup>-/-</sup> mice were intercrossed into Rag2<sup>-/-</sup> mice to generate LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> and LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> 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

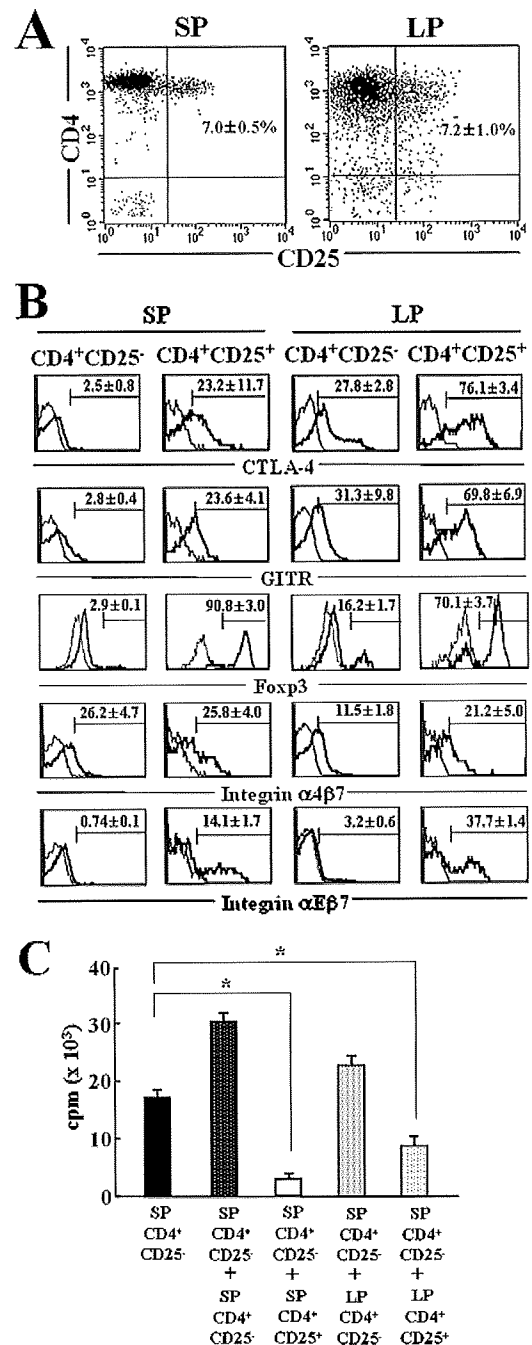
The following mAbs except DTA-1, biotinylated anti-mouse glucocorticoid-induced TNFR (GITR; eBioscience) and FJK-16s, PE-conjugated anti-mouse Foxp3 (eBioscience) were obtained from BD Pharmingen for purification of cell populations and flow cytometry analysis: PE-conjugated anti-mouse CD4 (RM4-5), PE-Cy5- and allophycocyanin-conjugated anti-mouse CD4 (L3T4), FITC-conjugated anti-mouse CD25 (7D4), PE-conjugated anti-mouse CD25 (PC61), PE-conjugated anti-mouse CD103 ( $\alpha_E$  integrin) (M290), PE-conjugated anti-mouse  $\alpha_4\beta_7$  integrin (DATK32), PE-conjugated anti-CTLA-4 (UC10-4F10-11), FITC-conjugated anti-mouse CD45RB (16A), FITC-conjugated anti-mouse Ly5.1 (CD45.1, A20), FITC-conjugated anti-mouse Ly5.2 (CD45.2, 104), 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<sup>+</sup> 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<sup>+</sup> T cells, the entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca<sup>2+</sup>, Mg<sup>2+</sup>-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 Biomedical) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4<sup>+</sup> 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), FITC-conjugated anti-CD45RB (16A), and FITC-conjugated anti-CD25 (7D4). Subpopulations of CD4<sup>+</sup> cells were generated by two-color sorting on FACSVantage (BD Biosciences). All populations were >98.0% pure on reanalysis.

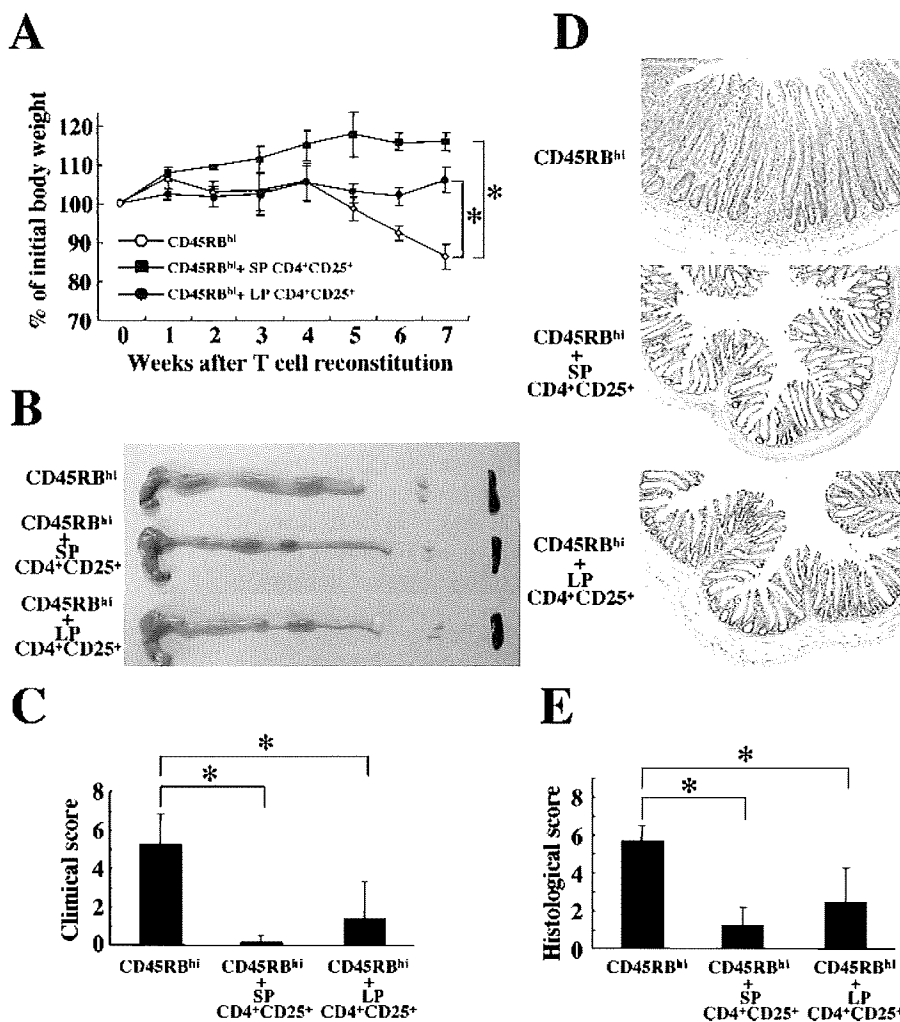
### In vivo experimental design

A series of in vivo experiments was conducted to investigate the role of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells in the suppression of murine chronic colitis. In Experiment 1, to assess the role of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells obtained from normal mice in the protection of colitis, we transferred 3  $\times$  10<sup>5</sup> splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from normal BALB/c mice with or without 1  $\times$  10<sup>5</sup> intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> 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  $\times$  10<sup>5</sup> splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from normal C57BL/6-Ly5.2 mice with or without 1  $\times$  10<sup>5</sup> splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells from C57BL/6-Ly5.1 mice into LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice and the control LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice. In Experiment 3, to exclude a possible role of spleen in the suppression of colitis in addition to MLNs, we transferred 3  $\times$  10<sup>5</sup> colitogenic LP CD4<sup>+</sup> T cells (Ly5.2<sup>+</sup>) obtained from established CD4<sup>+</sup>CD45RB<sup>high</sup> T cell-transferred mice (19) with or without 3  $\times$  10<sup>5</sup> splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells from C57BL/6-Ly5.1 mice into splenectomized LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> and LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice.



**FIGURE 1.** Identification and characterization of murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells in terms of T<sub>REG</sub> 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<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup>, are shown. Percentages in the upper right quadrant represent CD25<sup>+</sup> cells at indicated site. *B*, Murine intestinal CD4<sup>+</sup>CD25<sup>+</sup> constitutively express CTLA-4, GITR, and Foxp3 and partially express  $\alpha_4\beta_7$  and  $\alpha_E\beta_7$  integrins on or in LP CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> subsets suppress the proliferation of CD4<sup>+</sup> responder T cells in vitro. Splenic CD4<sup>+</sup>CD25<sup>-</sup>/CD4<sup>+</sup>CD25<sup>+</sup> and LP CD4<sup>+</sup>CD25<sup>-</sup>/CD4<sup>+</sup>CD25<sup>+</sup> populations were isolated from MACS-purified CD4<sup>+</sup> T cells by FACS sorting. The suppressive activity of the indicated subpopulations was determined by coculturing with splenic CD4<sup>+</sup>CD25<sup>-</sup> responder T cells at a 1:1 ratio of responder to T<sub>REG</sub> cells in the presence of anti-CD3 mAb and mitomycin C-treated APCs for 72 h. [<sup>3</sup>H]Thymidine uptake was determined for the last 9 h. Data are represented as the mean  $\pm$  SD of triplicate samples. \*,  $p < 0.05$  compared with culture in splenic CD4<sup>+</sup>CD25<sup>-</sup> responder cells alone.

**FIGURE 2.** Murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells as well as splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells inhibit the development of colitis induced by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into SCID mice. Seven SCID mice in each group were injected i.p. with the following T cell subpopulations: 1) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone (3 × 10<sup>5</sup> cells); 2) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (3 × 10<sup>5</sup> cells) + splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 × 10<sup>5</sup> cells); or 3) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (3 × 10<sup>5</sup> cells) + LP CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 × 10<sup>5</sup> cells). **A**, Body weight during 7 wk after transfer. \*, *p* < 0.05 compared with mice transferred with CD45RB<sup>high</sup> T cells alone at 7 wk after transfer. **B**, Gross appearance of the colon, MLNs, and spleen from SCID mice transferred with splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone (*upper*), splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells + splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (*middle*), or splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells + LP CD4<sup>+</sup>CD25<sup>+</sup> T cells (*lower*) at 7 wk after transfer. **C**, Clinical score at 7 wk after transfer. \*, *p* < 0.05 compared with mice transferred with CD45RB<sup>high</sup> T cells alone. **D**, Histopathology of distal colon at 7 wk after transfer. Original magnification, ×40. **E**, Histological score at 7 wk after transfer. \*, *p* < 0.05 compared with mice transferred with CD45RB<sup>high</sup> T 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 assessed for a clinical score that is the sum (0–8 points) of four parameters as follows: 0 or 1, hunching and wasting; 0–3, colon thickening (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); 0–3, stool consistency (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool); and an additional point was added if gross blood was noted (19). To monitor the clinical sign during the observed period over time, the disease activity index is defined as the sum (0–5 points) of the described parameters except colon thickening.

**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<sup>+</sup> dendritic cells and CD4<sup>+</sup> 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 FACSCalibur method and CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

**Cytokine ELISA**

To measure cytokine production, 1 × 10<sup>5</sup> LP CD4<sup>+</sup> T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in 96-well plates (Costar) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; 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 T<sub>REG</sub> cell activity**

LP mononuclear cells and splenocytes from normal BALB/c mice were separated into unfractionated CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACS Vantage as described. Cells (5 × 10<sup>4</sup>) and mitomycin C-treated BALB/c CD4<sup>-</sup> cells (2 × 10<sup>5</sup>) 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 CD3ε mAb. In coculture experiments, the same number of splenic CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> cells, or LP CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> cells (5 × 10<sup>4</sup>), were added into wells with the fixed dose of splenic CD4<sup>+</sup>CD25<sup>+</sup> responder cells (5 × 10<sup>4</sup>) and mitomycin C-treated

CD4<sup>-</sup> cells ( $2 \times 10^5$ ), as APCs. Incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) by proliferating cells was measured during the last 9 h of culture.

#### Statistical analysis

The results were expressed as the mean  $\pm$  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

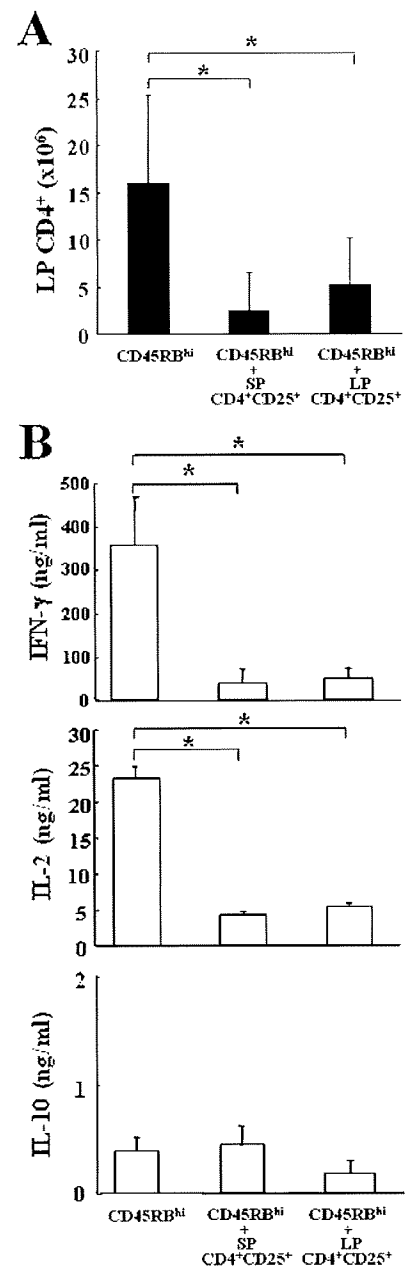
#### Characterization of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> in terms of T<sub>REG</sub> cell in vitro

Paired samples of spleen and colon obtained from normal BALB/c mice were analyzed by flow cytometry for the presence of the CD4<sup>+</sup>CD25<sup>+</sup> T cells. Consistent with previous reports described, in naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells (4–6),  $7.0 \pm 0.5\%$  of the splenic CD4<sup>+</sup> T cells were CD25<sup>+</sup> (Fig. 1A). Similarly,  $7.2 \pm 1.0\%$  of the colonic LP CD4<sup>+</sup> T cells were also CD25<sup>+</sup> (Fig. 1A). Because we previously demonstrated that human intestinal LP CD4<sup>+</sup>CD25<sup>bright</sup> T cells obtained from healthy individuals function as T<sub>REG</sub> 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<sup>+</sup>CD25<sup>+</sup> T cells also express well-known T<sub>REG</sub> markers, such as CTLA-4, GITR, and Foxp3. Like the control splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells, the expression of CTLA-4, GITR, and Foxp3 was markedly up-regulated in or on the intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 1B) compared with the paired CD4<sup>+</sup>CD25<sup>-</sup> T cells. Unexpectedly, but consistent with our human study (10), colonic LP CD4<sup>+</sup>CD25<sup>-</sup> T cells expressed CTLA-4, albeit to lesser extent compared with the paired colonic CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 1B). To further investigate the migration property of these CD4<sup>+</sup>CD25<sup>+</sup> T cells, we assessed the expression of  $\alpha_4\beta_7/\alpha_E\beta_7$  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  $\alpha_4\beta_7$  integrin. In contrast,  $\alpha_E\beta_7$  integrin was predominantly expressed on the splenic and LP CD4<sup>+</sup>CD25<sup>+</sup> T cells, but not on the paired CD4<sup>+</sup>CD25<sup>-</sup> T cells, indicating that a part of splenic and LP CD4<sup>+</sup>CD25<sup>+</sup> T cells can directly migrate into the gut.

We next investigated the T<sub>REG</sub> activity of the murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells by testing their ability to suppress the proliferative responses of the splenic CD4<sup>+</sup>CD25<sup>-</sup> responder T cells. As shown in Fig. 1C, both the splenic and LP CD4<sup>+</sup>CD25<sup>+</sup> T cells were able to suppress the proliferation of the splenic CD4<sup>+</sup>CD25<sup>-</sup> responder cells when cocultured at a ratio of 1:1 T<sub>REG</sub> to responder in the presence of mitomycin C-treated CD4<sup>-</sup> APCs and soluble anti-CD3 mAb (Fig. 1C), indicating that the LP CD4<sup>+</sup>CD25<sup>+</sup> T cells were T<sub>REG</sub> cells as well as the splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells at least in vitro. As a control, it was shown that titration of the same dose of the splenic or LP CD4<sup>+</sup>CD25<sup>-</sup> cells with the splenic CD4<sup>+</sup>CD25<sup>-</sup> 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).

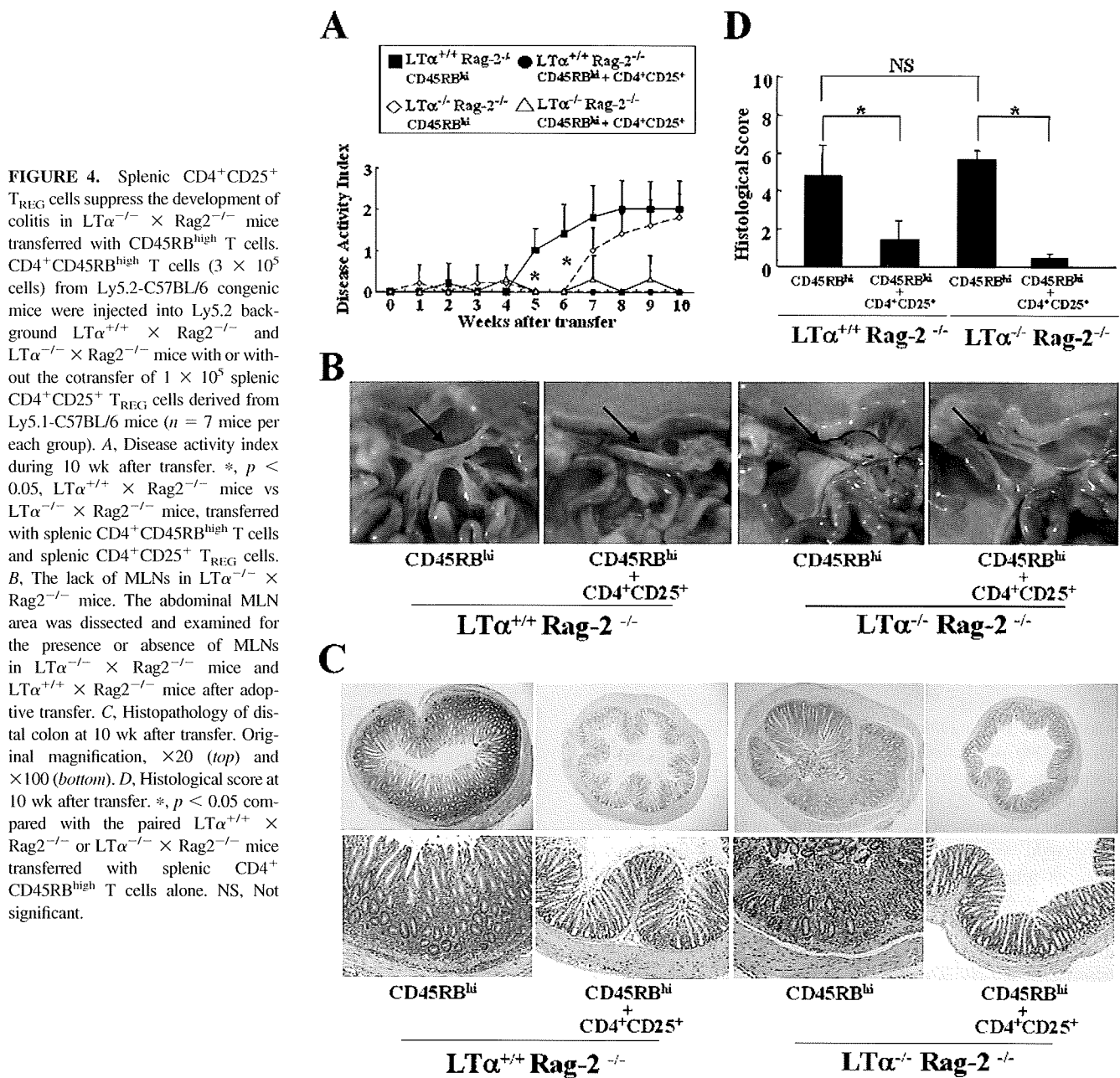
#### Murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the development of the CD4<sup>+</sup>CD45RB<sup>high</sup> T cell-transferred colitis

To next analyze the functional role of murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cell subset in vivo, we tested the T<sub>REG</sub> activity of the intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells using the classical SCID-transferred colitis model induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (19). C.B-17 SCID mice were injected i.p. with one or two subpopulations of sorted CD4<sup>+</sup> T cell in PBS: 1) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone ( $3 \times 10^5$  per mouse) as a positive control, 2) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> ( $3 \times 10^5$  per



**FIGURE 3.** Cotransfer of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells inhibits the expansion of LP CD4<sup>+</sup> T cells and Th1 cytokine production in SCID mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Transfer protocol is described in Fig. 2. *A*, Recovered LP CD4<sup>+</sup> T cells at 7 wk after transfer. Data are indicated as the mean  $\pm$  SD of seven mice in each group. \*,  $p < 0.05$  compared with mice transferred with CD45RB<sup>high</sup> T cells alone. *B*, Cytokine production by LP CD4<sup>+</sup> T cells. LP CD4<sup>+</sup> T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 72 h. Cytokines in the supernatants were measured by ELISA. Data are indicated as the mean  $\pm$  SD of seven mice in each group. \*,  $p < 0.05$  compared with mice transferred with CD45RB<sup>high</sup> T cells alone.

mouse) with splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $1 \times 10^5$ ) as a negative control, and 3) splenic CD4<sup>+</sup>CD45RB<sup>high</sup> ( $3 \times 10^5$ ) with LP CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $1 \times 10^5$ ). The results clearly demonstrated that control of intestinal inflammation resided predominantly within the intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> subpopulation as well as the splenic CD4<sup>+</sup>CD25<sup>+</sup> 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



**FIGURE 4.** Splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells suppress the development of colitis in LTα<sup>-/-</sup> × Rag2<sup>-/-</sup> mice transferred with CD45RB<sup>high</sup> T cells. CD4<sup>+</sup>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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</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<sup>+</sup>CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</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<sup>+</sup>CD45RB<sup>high</sup> T cells alone. NS, Not significant.

CD4<sup>+</sup>CD45RB<sup>high</sup> and LP CD4<sup>+</sup>CD25<sup>+</sup> T cells exhibited no detectable pathological changes and were indistinguishable from colons from mice reconstituted with a mixture of CD4<sup>+</sup>CD45RB<sup>high</sup> plus splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2*B*). In contrast, mice reconstituted with CD4<sup>+</sup>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 (Fig. 2*C*). Clinical score for mice transferred with a mixture of CD4<sup>+</sup>CD45RB<sup>high</sup> and LP CD4<sup>+</sup>CD25<sup>+</sup> T cells was significantly decreased as compared with that for mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone. Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the LP of the colon from the control mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone (Fig. 2*D*). In contrast, the glandular elongation was mostly abrogated and only a few mononuclear cells were observed in the colonic LP from mice reconstituted with a mixture of CD4<sup>+</sup>CD45RB<sup>high</sup> plus splenic or LP CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig.

2*D*). This difference was also confirmed by histological scores of multiple colon sections, which were 5.63 ± 0.89 in control mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, 1.21 ± 0.97 in mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells plus splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells, and 2.40 ± 1.83 in mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells plus LP CD4<sup>+</sup>CD25<sup>+</sup> T cells (*p* < 0.05, mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone vs mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells plus splenic or LP CD4<sup>+</sup>CD25<sup>+</sup> T cells) (Fig. 2*E*).

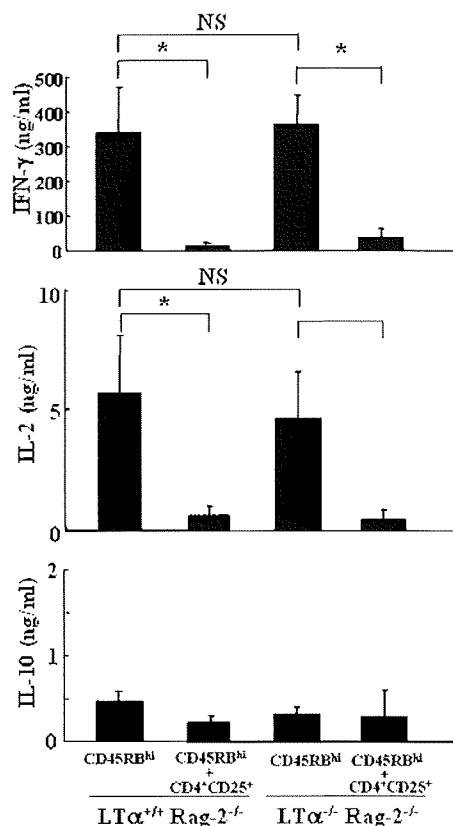
A further quantitative evaluation of CD4<sup>+</sup> T cell infiltration was made by isolating LP mononuclear cells from the resected colons. A significantly less number of CD4<sup>+</sup> T cells was recovered from the colonic tissue of mice reconstituted with CD4<sup>+</sup>CD45RB<sup>high</sup> and with LP or splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells as compared with mice reconstituted with CD4<sup>+</sup>CD45RB<sup>high</sup> alone (Fig. 3*A*). To next determine the effect of cotransfer of LP CD4<sup>+</sup>CD25<sup>+</sup> T cells on Th1/Th2 development, we measured IFN-γ, IL-2, and IL-10 production by anti-CD3/CD28 mAb-stimulated LP CD4<sup>+</sup> T cells.

As shown in Fig. 3B, production of Th1 cytokines (IFN- $\gamma$ , IL-2) was significantly reduced in LP CD4<sup>+</sup> T cells from the mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> plus LP or splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells as compared with those transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone ( $p < 0.05$ ). In contrast, production of IL-10 was not significantly affected among the groups (Fig. 3B).

*Splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the development of colitis in LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells*

To further investigate the origin of LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells and their role in suppressing the development of colitis, we generated LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice, which lack conventional lymphoid tissues (inductive sites) including MLNs, PPs, and ILFs, 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<sup>+</sup>CD25<sup>+</sup> 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 splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from normal C57BL/6 mice into LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice and the littermate control LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice to assess the role of MLNs as inductive sites in inducing colitis. When CD4<sup>+</sup>CD45RB<sup>high</sup> T cells were transferred into the control LT $\alpha$ <sup>+/+</sup>  $\times$  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<sup>+</sup>CD45RB<sup>high</sup> T cells were transferred into the LT $\alpha$ <sup>-/-</sup>  $\times$  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 LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells 10 wk after transfer (Fig. 4A). The rapid onset of colitis in the recipient LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice could easily be explained by the existence of MLNs in these mice and migration of effector CD4<sup>+</sup> cells primed in these sites into the colon, but the evidence that the recipient LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice, albeit delayed, developed colitis indicates that there must be other sites where CD4<sup>+</sup> T cells could be primed besides the MLNs. These LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> and LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</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 LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice macroscopically lacked MLNs (Fig. 4B) and other peripheral LNs (data not shown) in contrast to control LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice (Fig. 4B). Tissue sections from LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> and LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</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<sup>+</sup>CD45RB<sup>high</sup> T cells, we next asked whether splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells can migrate into the LP, and suppress the development of colitis in the absence of MLNs. Expectedly, LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells did not show weight loss and clinical signs of colitis throughout the entire observation period (Fig. 4A). Of note, LT $\alpha$ <sup>-/-</sup>  $\times$  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 also did not manifest clinical signs of colitis (Fig. 4A). Consistent with the lack of clinical signs of colitis, LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> or LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> recipients cotransferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells displayed no histological evidence of intes-



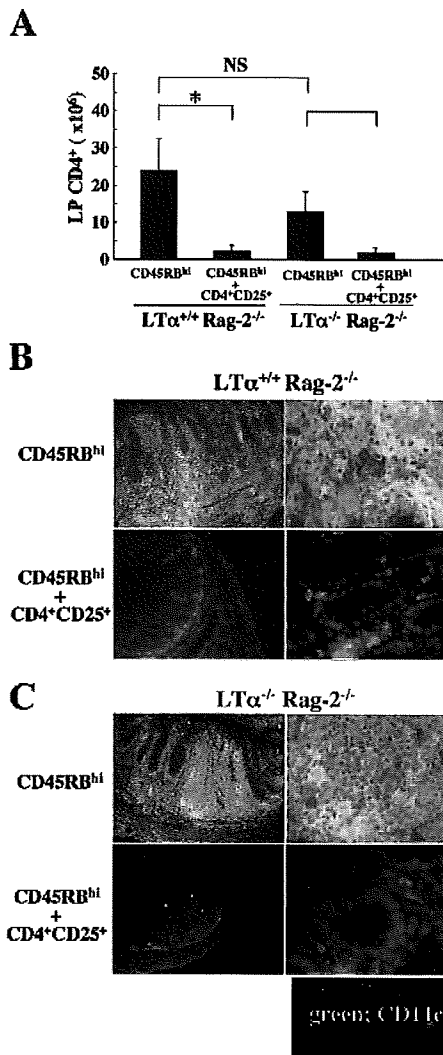
**FIGURE 5.** Splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells suppress the production of Th1 cytokines in LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD45RB<sup>high</sup> T cells. CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $3 \times 10^5$  cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> and LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice with or without the cotransfer of  $1 \times 10^5$  splenic CD4<sup>+</sup>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. Cytokine production by LP CD4<sup>+</sup> T cells was measured by specific ELISA. LP CD4<sup>+</sup> T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 72 h. Cytokines in the supernatants were measured by ELISA. Data are indicated as the mean  $\pm$  SD of seven mice in each group. \*,  $p < 0.05$  compared with the paired LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> or LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with splenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone. NS, Not significant.

tinal inflammation (Fig. 4C). The difference among each group was also confirmed by histological scoring of multiple colon sections, which was  $4.85 \pm 1.58$  in LT $\alpha$ <sup>+/+</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, and  $1.40 \pm 0.96$  in LT $\alpha$ <sup>+/+</sup>  $\times$  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 ( $p < 0.05$ ), and  $5.60 \pm 0.40$  in LT $\alpha$ <sup>-/-</sup>  $\times$  Rag2<sup>-/-</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, and  $0.43 \pm 0.23$  in LT $\alpha$ <sup>-/-</sup>  $\times$  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 ( $p < 0.05$ ) (Fig. 4D).

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

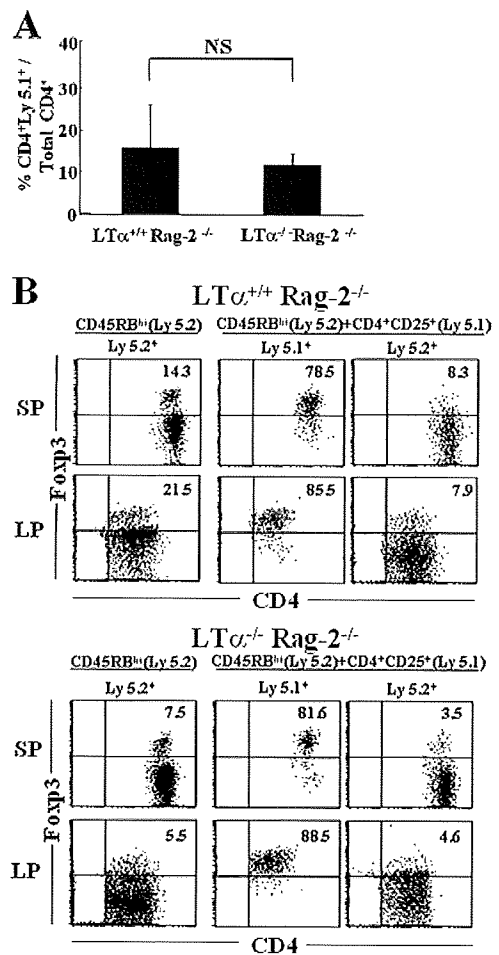
Consistent with the reduction in the histological scores by the cotransfer of splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells, there was also a striking reduction in the recovered number of LP CD4<sup>+</sup> T cells both in





**FIGURE 6.** Splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells suppress the expansion of pathogenic LP CD4<sup>+</sup> T cells in LTα<sup>-/-</sup> × Rag2<sup>-/-</sup> mice transferred with CD45RB<sup>high</sup> T cells. CD4<sup>+</sup>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<sup>+</sup>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<sup>+</sup> 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<sup>+</sup>CD45RB<sup>high</sup> T cells alone. **B**, Distribution of CD11c<sup>+</sup> dendritic cells (green) and CD4<sup>+</sup> T cells (red) in the colon after adoptive transfer. Original magnification, ×100 (left) and ×400 (right).

LTα<sup>+/+</sup> × Rag2<sup>-/-</sup> and LTα<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 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 LTα<sup>+/+</sup> × Rag2<sup>-/-</sup> and LTα<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 LTα<sup>+/+</sup> × Rag2<sup>-/-</sup> and LTα<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 pos-



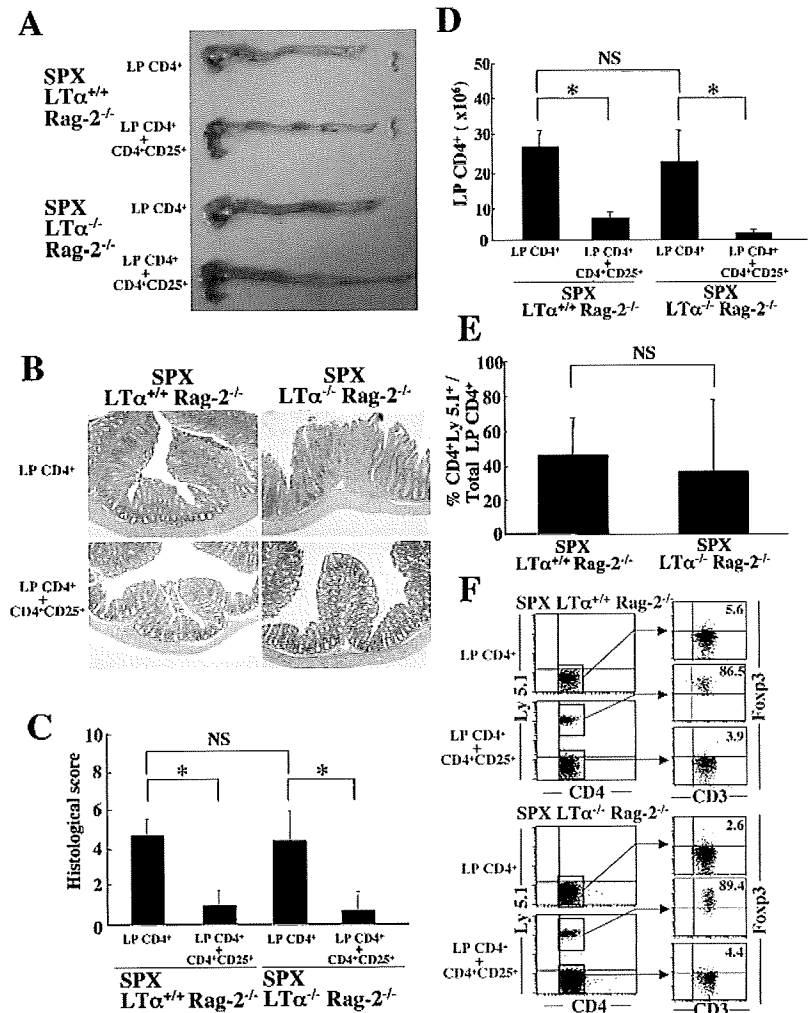
**FIGURE 7.** Splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells migrate into the colonic LP and are sustained in the LP in LTα<sup>-/-</sup> × Rag2<sup>-/-</sup> mice transferred with CD45RB<sup>high</sup> T cells. **A**, The ratio of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> (Ly5.1<sup>+</sup>) cells to total CD4<sup>+</sup> 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<sup>+</sup> 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> cells were gated and analyzed for the presence of converted Ly5.2<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells and Ly5.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells, respectively. Number in upper right quadrant represents the percentage of inducible CD4<sup>+</sup>Foxp3<sup>+</sup> cells per CD4<sup>+</sup> cells.

sible role of LP as a site for actively interacting between CD11c<sup>+</sup> dendritic cells and CD4<sup>+</sup> T cells.

*Splenic 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- to Ly5.1-derived cells. As shown in Fig. 7A, ~10–15% of total LP CD4<sup>+</sup> T cells both in LTα<sup>+/+</sup> × Rag2<sup>-/-</sup> and LTα<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 and secondarily by inhibiting the development of pathogenic Th1 cells producing IFN-γ and IL-2.

**FIGURE 8.** Splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells migrate into the gut and inhibit the development of colitis induced by adoptive transfer of colitogenic LP CD4<sup>+</sup> T cells into splenectomized (SPX) LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice. Seven Rag2 $^{-/-}$  mice in each group were injected i.p. with the following T cell subpopulations: 1) colitogenic LP Ly5.2<sup>+</sup>CD4<sup>+</sup> T cells ( $3 \times 10^5$  cells) into splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  mice; 2) colitogenic LPLy5.2<sup>+</sup>CD4<sup>+</sup> T cells ( $3 \times 10^5$  cells) + splenic Ly5.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $3 \times 10^5$  cells) into splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  mice; 3) colitogenic LP Ly5.2<sup>+</sup>CD4<sup>+</sup> T cells ( $3 \times 10^5$  cells) into splenectomized LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice; or 4) colitogenic LP Ly5.2<sup>+</sup>CD4<sup>+</sup> T cells ( $3 \times 10^5$  cells) + splenic Ly5.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $3 \times 10^5$  cells) into splenectomized LT $\alpha^{-/-}$   $\times$  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,  $\times 40$ . **C**, Histological score at 7 wk after transfer. **D**, Number of recovered LP CD4<sup>+</sup> T cells at 7 wk after transfer. Data are indicated as the mean  $\pm$  SD of seven mice in each group. **E**, Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> (Ly5.1<sup>+</sup>) cells to total CD4<sup>+</sup> T cells (Ly5.1<sup>+</sup> + Ly5.2<sup>+</sup>) at 7 wk after transfer was analyzed by gating Ly5.1 or Ly5.2 on CD4<sup>+</sup> 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<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup> cells were gated and analyzed for the presence of Foxp3<sup>+</sup> cells. The percentage of induced Foxp3<sup>+</sup> cells per CD3<sup>+</sup> cells is indicated in upper right quadrant of enlarged gate.



However, it was also possible that a part of Ly5.2-derived CD4<sup>+</sup>CD45RB<sup>high</sup> T cells was converted into inducible CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells rather than pathogenic CD4<sup>+</sup> T cells in the gut. Thus, to evaluate this possibility that the LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells are composed of naturally arising CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.1<sup>+</sup>), inducible CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.2<sup>+</sup>) and pathogenic CD4<sup>+</sup> T cells (Ly5.2<sup>+</sup>), 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<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells by staining CD25 molecule. Indeed, only ~3–16% of splenic and LP Ly5.2<sup>+</sup> cells were converted into inducible CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>REG</sub> cells in both LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone or with a mixture of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells, but most Ly5.1-derived CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells (78–88%) retained Foxp3 in both LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  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. 7B).

#### Splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells suppressed the expansion of colitogenic LP CD4<sup>+</sup> T cells in the gut

With respect to the site for suppression of effector and memory CD4<sup>+</sup> T cells, it was also possible that naturally arising CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the activation of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and the expansion of the differentiated effector CD4<sup>+</sup> T

cells in the spleen rather than in the gut. To clarify that CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the expansion of colitogenic effector and memory CD4<sup>+</sup> T cells in the gut, we finally transferred colitogenic LP CD4<sup>+</sup> T cells obtained from colitic mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (Ly5.2<sup>+</sup>) (19) with or without splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.1<sup>+</sup>) into splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice to exclude the impact of spleen. Both splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice transferred with colitic LP CD4<sup>+</sup> T cells (Ly5.2<sup>+</sup>) developed wasting disease (data not shown) and colitis by assessing histological findings (Fig. 8, A–C) and the recovered CD4<sup>+</sup> cell numbers from the LP (Fig. 8D). In contrast, splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice transferred with a mixture of colitic LP CD4<sup>+</sup> T cells (Ly5.2<sup>+</sup>) and splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.1<sup>+</sup>) 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<sup>+</sup> T cells in splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice cotransferred with a mixture were derived from Ly5.1<sup>+</sup> cells (Fig. 8E). Furthermore, we confirmed that CD4<sup>+</sup>Foxp3<sup>+</sup> T cells residing in the LP were mostly derived from Ly5.1<sup>+</sup> population in both splenectomized LT $\alpha^{+/+}$   $\times$  Rag2 $^{-/-}$  and LT $\alpha^{-/-}$   $\times$  Rag2 $^{-/-}$  mice transferred with a mixture of colitic LP CD4<sup>+</sup> T cells (Ly5.2<sup>+</sup>) and CD4<sup>+</sup>CD25<sup>+</sup> T cells (Ly5.1<sup>+</sup>) (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<sup>+</sup> T cells.

## Discussion

In this study, we demonstrate that intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells residing in normal mice constitutively express CTLA-4, GITR, and Foxp3 and suppress the proliferation of responder CD4<sup>+</sup> T cells *in vitro*. Furthermore, cotransfer of intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells prevents the development of CD4<sup>+</sup>CD45RB<sup>high</sup> T cell-transferred colitis. Surprisingly, when LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice, which lack MLNs, ILFs, and PPs, were transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, they did develop severe wasting disease and colitis despite the delayed onset and kinetics as compared with the control LT $\alpha^{+/+}$  × Rag2 $^{-/-}$  mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Of note, splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells can migrate into the LP, and prevent the development of CD4<sup>+</sup>CD45RB<sup>high</sup> T cell-transferred colitis in MLN-null LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  recipient mice. These results suggest that at least in part intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells without the instruction by an MLN environment directly migrate into the gut and act as T<sub>REG</sub> cells, and therefore may contribute to the intestinal immune homeostasis *in vivo*.

We have recently demonstrated that human CD4<sup>+</sup>CD25<sup>bright</sup> T cells resided in the intestinal LP, expressed CTLA-4, GITR, and Foxp3, and possessed T<sub>REG</sub> activity *in vitro* (10). Although the results indicate that these cells might serve as mucosal (nonlymphoid) T<sub>REG</sub> 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<sup>+</sup>CD25<sup>+</sup> T cells isolated from normal mice suppress the development of colitis induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into SCID mice. As shown in Fig. 2, we found the clinical score in SCID mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells at a ratio of 3:1 was significantly decreased as compared with that in SCID mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, indicating that the murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells maintain intestinal homeostasis to suppress the development of colitis *in vivo*. Consistent with this, murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed constitutively CTLA-4, GITR, and Foxp3 and suppressed the proliferation of responder cells *in vitro*, such as human LP CD4<sup>+</sup>CD25<sup>bright</sup> T cells (10). Furthermore, because we also found that LP CD4<sup>+</sup>CD25<sup>+</sup> T cells did partially express  $\alpha_4\beta_7$  and  $\alpha_E\beta_7$  integrins, it is conceivable that these gut-homing receptor-expressing LP CD4<sup>+</sup>CD25<sup>+</sup> T cells might migrate into the colon from outside of the gut. Although it has been reported that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells reside in nonlymphoid tissues (10–18), our current data now provide the first experimental evidence that intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells prevent the development of colitis *in vivo*.

Having the evidence that the murine intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T cells suppressed the development of colitis induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, we next asked whether MLNs are not fully essential for the suppression of colitis by splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells because it was still possible that 1) a part of the LP CD4<sup>+</sup>CD25<sup>+</sup> T cells was needed to be instructed in MLNs to differentiate to gut-homing receptor-expressing T<sub>REG</sub> cells (17) to migrate to the gut, and also possible that 2) the transferred LP CD4<sup>+</sup>CD25<sup>+</sup> T cells acted as T<sub>REG</sub> 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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells without the impact of MLNs, which are

thought to be representative inductive and suppressive sites for classical splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells because high expression levels of CD62 ligand enable both naive CD4<sup>+</sup> T cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells to efficiently enter the Ag-draining lymph nodes from the bloodstream. As the second approach to address this issue, thus, we generated LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice as recipients for the following adoptive transfer experiment. Before starting the experiment, it was unclear whether the LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> 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 LT $\alpha^{+/+}$  × Rag2 $^{-/-}$  mice 10 wk after transfer, although it took a longer period to establish colitis as compared with the LT $\alpha^{+/+}$  × 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 LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells obtained from normal mice prevent the development of colitis in LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells as well as in LT $\alpha^{+/+}$  × Rag2 $^{-/-}$  recipients, indicating that splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells can suppress the development of colitis in the absence of MLNs. Moreover, we demonstrated that Ly5.1-CD4<sup>+</sup>CD25<sup>+</sup> T cells resided in the colon in MLN-null LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  mice cotransferred with Ly5.2-derived CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and Ly5.1-derived splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells, suggesting that the LP might be a regulatory site between colitogenic effector/memory cells and T<sub>REG</sub> cells to suppress intestinal inflammation probably as a second line of suppression (17). It was also possible, however, that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells prevented the expansion of pathogenic effector CD4<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells prevented the development of colitis induced by adoptive transfer of colitogenic LP CD4<sup>+</sup> T cells in splenectomized LT $\alpha^{-/-}$  × Rag2 $^{-/-}$  recipients (Fig. 8). Because colitogenic LP CD4<sup>+</sup> T cells that have a phenotype of effector/memory CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>-</sup> cells (21) should have migrated to the gut and expanded in the gut, it is very likely that splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells can directly migrate to the gut and suppress the expansion of these colitogenic CD4<sup>+</sup> T cells in the gut. With respect to the equilibrium of pathogenic CD4<sup>+</sup> T cells and T<sub>REG</sub> cells, however, further studies will be needed because we found that effector to T<sub>REG</sub> cell ratio varied by different experimental settings (Figs. 6 and 8).

Finally, it should be discussed the protective mechanism by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells of this SCID/Rag2 $^{-/-}$  colitis model induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from the standpoint of the sites of active suppression. Indeed, Mottet et al. (18) previously demonstrated that not only effector CD4<sup>+</sup> T cells but also CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells accumulate in the intestinal LP in addition to the MLNs in the cured SCID mice by retransferring splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells 3–4 wk after the first transfer

of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, it remains to be determined whether intestinal inflammation can be suppressed solely by LP or MLN CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells in this setting. In contrast, Denning et al. (8) recently demonstrated that  $\beta_7$  integrin-deficient ( $\beta_7^{-/-}$ ) CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> 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  $\alpha_4\beta_7/\alpha_E\beta_7$  integrin molecules, are capable of preventing intestinal inflammation, suggesting T<sub>REG</sub> accumulation in the intestine is dispensable for the protection of this colitis model. In this protection protocol, indeed, it is possible that  $\beta_7^{+/+}$  CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells are not needed to suppress the development of colitis because  $\beta_7^{-/-}$  CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells directly migrate to MLNs and can inhibit naive CD4<sup>+</sup>CD45RB<sup>high</sup> T cell activation and proliferation within Ag-draining MLNs, resulting in suppressing the development of the gut-seeking activated effector CD4<sup>+</sup> T cells instructed to express the gut-homing receptors such as  $\alpha_4\beta_7/\alpha_E\beta_7$  integrin. However, it still remains unknown whether mucosal CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells are necessary for the suppression of mucosal pathogenic effector CD4<sup>+</sup> T cell *ex vivo* especially in the therapeutic protocol that can be assessed and whether LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells as effector T<sub>REG</sub> cells can suppress the surrounding LP effector CD4<sup>+</sup> T cells *ex vivo*. In our adoptive transfer experiment using splenectomized MLN-null LT $\alpha^{-/-}$   $\times$  Rag2<sup>-/-</sup> mice, however, we clearly demonstrated that cotransfer of splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells suppressed the development of colitis despite the lack of spleen and MLNs and found that these T<sub>REG</sub> cells migrated to the effector sites, in this case, the intestine, suggesting that intestinal LP CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells play an important role at least in part for the suppression of intestinal inflammation in the gut.

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<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells directly control the pathogenic effector CD4<sup>+</sup> cells as a second line of suppression (effector T<sub>REG</sub>) site together with the MLNs as a first line of suppression (naïve T<sub>REG</sub>) site.

## Disclosures

The authors have no financial conflict of interest.

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