This information is current as of April 19, 2017.

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*J Immunol* 2009; 182:6079-6087; doi: 10.4049/jimmunol.0711823
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RANK-RANKL Signaling Pathway Is Critically Involved in the Function of CD4⁺CD25⁺ Regulatory T Cells in Chronic Colitis

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It is now clear that functional CD4⁺CD25⁺ regulatory T (Tₐ) cells exist as part of the normal immune population and prevent the development of intestinal inflammation. We have recently shown that CD4⁺CD25⁺ Tₐ cells reside in the intestine and control intestinal homeostasis in humans and mice. In this study, we demonstrate that the TNF family molecule RANKL and its receptor RANK are critically involved in controlling the function of CD4⁺CD25⁺ Tₐ cells in the intestine. We first found that RANKL was preferentially expressed on both CD4⁺CD25⁺ Tₐ cells and colitogenic CD4⁺ T cells, whereas RANK was expressed on dendritic cells. Although neutralizing anti-RANKL mAb did not affect Tₐ activity of CD4⁺CD25⁺ Tₐ cells to suppress the proliferation of CD4⁺ responder cells in vitro, in vivo administration of anti-RANKL mAb abrogated CD4⁺CD25⁺ Tₐ cell-mediated suppression of colitis induced by adoptive transfer of CD4⁺CD45RB⁺ T cells into SCID mice. Interestingly, an adoptive transfer experiment using Ly5.1⁺CD4⁺CD45RB⁺High cells and Ly5.2⁺CD4⁺CD25⁺ Tₐ cells revealed that the ratio of CD4⁺CD25⁺ Tₐ cells in total CD4⁺ T cells in inflamed mucosa was significantly decreased by anti-RANKL mAb treatment. Consistent with this, the expression of RANK on lamina propria CD11c⁺ cells from colitic mice was significantly increased as compared with that from normal mice, and in vitro treatment with anti-RANKL mAb suppressed the expansion of CD4⁺Foxp3⁺ Tₐ cells in culture with colitic lamina propria CD11c⁺ cells. Together, these results suggest that the RANK-RANKL signaling pathway is critically involved in regulating the function of CD4⁺CD25⁺ Tₐ cells in colitis. The Journal of Immunology, 2009, 182: 6079–6087.

Intestinal mucosal surfaces are exposed to a large number of dietary and bacterial Ags (1–5). However, the gut-associated immune system fences off harmful Ags from systemic circulation and induces systemic tolerance against luminal Ags. In contrast, inflammatory bowel diseases (IBD)⁶ and animal models of T cell-mediated chronic colitis are associated with the activation of intestinal and systemic immune responses (2, 3). In this regard, CD4⁺CD25⁺ regulatory T (Tₐ) cells play a central role in the maintenance of immunological homeostasis (6, 7). CD4⁺CD25⁺ Tₐ cells have been detected mainly in lymphoid sites, including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated the capacity of CD4⁺CD25⁺ Tₐ cells to prevent the induction of immune responses and this suppression requires direct cell-cell contact with responder T cells or APCs, it is conceivable that CD4⁺CD25⁺ Tₐ cells act as a central regulator within lymphoid tissues (6–8).

The GALT can be divided into effector sites, which consist of lymphocytes scattered throughout the lamina propria (LP) of the intestinal mucosa and organized lymphoid tissues (inductive sites) such as mesenteric lymph nodes (MLNs) and Peyer’s patches, which are responsible for the induction phase of immune responses (2, 9). It is thought that presentation of Ags to naïve, effector, and memory T cells is concentrated at these inductive sites of organized mucosal lymphoid follicles, and thus APCs finely tune the balance between intestinal immune tolerance and inflammation. In addition to the inductive sites, however, it remains unclear whether these inductive sites alone were involved in the induction and suppression of intestinal inflammation (11). We also reported that peripheral CD4⁺CD25⁺ Tₐ cells actually migrated to the intestine and suppressed the development of colitis. Although it is reasonable to hypothesize that mechanisms for the induction, maintenance, and suppression of colitis would be centrally controlled in the inductive sites by CD4⁺CD25⁺ Tₐ cells, two-thirds of which constitutively express the lymph node-homing receptor CD62L (10), we previously demonstrated that human intestinal LP CD4⁺CD25bright T cells obtained from normal individuals possess Tₐ activity in vitro and therefore questioned whether these inductive sites alone were involved in the induction and suppression of intestinal inflammation (11).

Received for publication July 5, 2007. Accepted for publication March 13, 2009.

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This study was supported in part by Grants-in-Aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare, the Japan Medical Association, and the Foundation for Advancement of International Science.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0711823

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with *Leishmania major* (16), lungs from mice infected with *Pneumocystis carinii* (17), and diseased lesions in delayed-type hypersensitivity models (18), as well as in inflamed mucosa of colitic mice (8, 19).

However, it remains largely unknown which molecular mechanisms actually control the function of CD4^+^ CD25^+^ T<sub>R</sub> cells in the intestine to suppress intestinal inflammation. In the present study, we show that both CD4^+^ CD25^+^ T<sub>R</sub> cells and colitogenic CD4^+^ T cells preferentially express a TNF family member, receptor activator of NF-κB (RANK) (20) by administering neutralizing anti-RANKL mAb against the CD4^+^ CD25^+^ T<sub>R</sub> cell-mediated suppression of colitis induced by adoptive transfer of CD4^+^ CD45RB<sup>hi</sup> T cells into SCID mice, indicating a critical role for the RANKL/RANK signaling pathway in the function of intestinal CD4^+^ CD25^+^ T<sub>R</sub> cells in attenuating colitis.

**Materials and Methods**

**Animals**
Female BALB/c, CB-17 SCID, and C57BL/6-Ly5.2 mice were purchased from Japan Clea. C57BL/6-Ly5.1 mice and C57BL/6-Ly5.2 Rag-2-deficient (RAG-2<sup>-/-</sup>) mice were obtained from Taconic Farms and the National Institutes of Health, respectively. Mice were used at 7–12 wk of age. All experiments were approved by the regional animal study committees.

**Antibodies**

The following mAbs except anti-CCR9 mAb (R&D Systems) and reagents were purchased from BD PharMingen: RM4-5, PE, or PerCP-conjugated anti-CD4 (rat IgG2a); 7D4, FITC-conjugated anti-mouse CD25 (rat IgM); PC61, PE-conjugated anti-mouse CD25 (rat IgG1); H1.3F3, FITC-conjugated anti-CD69 (Ham IgG1); FJK-16s, allophycocyanin-conjugated anti-mouse Foxp3 (rat IgG2a); DATK32, PE-conjugated anti-integrin α<sub>β</sub>2 (rat IgG2a); M290, PE-conjugated anti-integrin α<sub>β</sub>2 (rat IgG2a); 242503, PE-conjugated anti-CR3 (rat IgG2b); isotype control Abs, biotin-conjugated streptavidin. The neutralizing anti-mouse RANKL mAb (H9260), anti-mouse Foxp3 (rat IgG2a); 12-31, rat IgG2a) were conjugated with streptavidin. The neutralizing anti-mouse RANKL mAb (H9260), anti-mouse Foxp3 (rat IgG2a); 12-31, rat IgG2a) were conjugated with streptavidin. The neutralizing anti-mouse RANKL mAb (H9260), anti-mouse Foxp3 (rat IgG2a), anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer's instructions. Enriched CD4^+^ T<sub>R</sub> cells (96–97% pure, as estimated by FACS-Calibur; BD Biosciences) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-CD45RB (16A), FITC-conjugated anti-CD25 (7D4), and streptavidin-PE. Subpopulations of CD4<sup>+</sup> cells were isolated by two-color sorting on a FACSVantage (BD Biosciences). All populations were >98.0% pure on reanalysis.

**Purification of T cell subsets**

CD4<sup>+</sup> T cells were isolated from spleen cells of BALB/c mice using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer's instructions. Enriched CD4<sup>+</sup> T<sub>R</sub> cells (96–97% pure, as estimated by FACS-Calibur) were further purified by Ficoll-Hypaque (Pharmacia) density gradient centrifugation; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and an additional point was added if gross blood was noted) (12).

**Histological examination**

Tissue samples were fixed in PBS containing 2% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colons was calculated using a modification of a previously described scoring system (12), as follows: mucosal damage, 0; normal, 1; 3–10 intraepithelial lymphocytes (IEL)/high-power field (HPF) and focal damage, 2>10 IEL/HPF and rare crypt abscesses, 3; and >10 IEL/HPF, multiple crypt abscesses and erosion ulceration, submucosal damage, 0; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, and 3; extensive leukocyte infiltration with transmural effacement of the muscularis.

**Preparation of mucosal LP mononuclear cells**

Colonic LP mononuclear cells were isolated using a method described previously (22). In brief, the entire length of intestine was opened longitudinally, washed with PBS, and cut into small (~5–mm) pieces. To remove epithelium including IEL, the dissected mucosa was incubated twice with Ca<sup>2+</sup>-Mg<sup>2+</sup>-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 30 min and then serially incubated two times in medium containing 0.75 mM EDTA (Sigma-Aldrich) for 60 min at 37°C under gentle shaking. The supernatants from these incubations, which included the epithelium and IEL, were depleted, and the residual fragments were pooled and treated with 2 mg/ml collagenase A (Worthington Biomedical) and 0.01% DNase (Worthington Biochemical) in 5% CO<sub>2</sub>-humidified air at 37°C for 2 h. The cells were then pelleted two times through a 40% isotonic Percoll solution and further purified by Ficoll-Hypaque (Pharmacia) density gradient centrifugation (40%/75%). Enriched CD4<sup>+</sup> T<sub>R</sub> cells were obtained by positive selection using an anti-CD4 (L3T4) MACS magnetic separation system. The resultant cells when analyzed by FACSCalibur contained >96% CD4<sup>+</sup> cells.

**RT-PCR**

Total cellular RNA was extracted from 4 × 10<sup>5</sup> cells using a RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was reverse-transcribed using the Superscript II Reverse Transcriptase (Invitrogen). RANK and RANKL levels were measured with a QuantiTect SYBER green PCR kit using Applied Biosystems 7500 real-time PCR system and 7500 system software with the following primers: RANK forward: 5′-TTG CAG GTC TTC ATG GCA-3′ and reverse (Rev): 5′-TGA GAC TGG GCC GCA GGT AAG CCA-3′; RANKL: Fwd: 5′-TTG CAC ACC TCA CCA TCA ATG-3′ and Rev, 5′-TTA GAC ATC TGG GCC CAG CAC GTG-3′; and G3PDH: Fwd: 5′-CTA CGG CGG CTA AGG CAG T-3′ and Rev: 5′-GCC ATG AGG TCC ACC ACC CTG-3′. PCR cycling conditions consisted of 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s. Data are expressed as the relative amount of indicated mRNA as normalized against G3PDH.

**Flow cytometry**

To detect the surface expression of various molecules, isolated splenocytes or LP mononuclear cells were preincubated with a FcγR-blocking mAb
(CD16/32, 2.4G2; BD Pharmingen) for 15 min, then incubated with specific FITC-, PE-, or biotin-labeled Abs for 20 min on ice. Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Intracellular Foxp3 staining was performed with the allophycocyanin-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 with CellQuest software. Background fluorescence was assessed by staining with control isotype-matched mAbs.

**Cytokine ELISA**

To measure cytokine production, 1 × 10^6 LP CD4^+ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (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 regulatory function of CD4^+ CD25^+ T cells**

Spleen cells from BALB/c mice were separated into unfractionated whole CD4^+ T cells and CD4^+ CD25^+ T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACS Vantage as described above. Responder CD4^+ cells (7 × 10^5) and mitomycin C (MMC)-treated CD4^- cells (5 × 10^5) as APCs, with or without CD4^+ CD25^+ (1 × 10^5), were cultured in the presence or absence of neutralizing anti-RANKL mAb or anti-RANK mAb (1, 3, or 10 μg/ml) for 72 h in round-bottom 96-well plates in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME. Incorporation of [H]thymidine ([H]Ci/well) by proliferating cells was measured during the last 9 h of culture. In some experiments, a CFSE dilution assay was performed. To this end, splenic CD4^+ T cells were negatively obtained from whole spleen cells of normal BALB/c mice using a CD4 T Cell Isolation Kit (Miltenyi Biotec). CD11c^- dendritic cells (DC) were isolated using anti-CD25 MACS beads (Miltenyi Biotec) from the CD4^+ T cells. CD11c^- dendritic cells (DC) were isolated from SP and LP of normal C57BL/6 mice and colitic C57BL/6 background RAG-2^−/− mice previously transferred with CD4^+ T cells. All populations were >92% pure on reanalysis. Then, CD4^+ CD25^+ T cells were labeled with 1 μM CFSE (Molecular Probes). CFSE-labeled CD4^+ CD25^- responder T cells (1 × 10^5) were cocultured with unlabeled Ly5.2^− CD4^+ CD25^− Tq cells (0.33 × 10^5) with CD11c^+ DC (2 × 10^5) and anti-CD3 (1 μg/ml) in the presence of anti-RANKL mAb (1 μg/ml) or control IgG (1 μg/ml) in 96-well round-bottom plates for 120 h in triplicates. After incubation, cells were collected and analyzed by FACS. Propidium iodide was added to exclude dead cells. Proliferation analysis was based on division times of responder CFSE-labeled CD4^+ T cells at the condition that eliminated the unlabeled CD4^+ CD25^- Tq cells and CD11c^+ DC cells.

**In vitro induction of gut-homing receptors and CD4^+ Foxp3^+ T_R cells**

For the isolation of DC from LP, MLN, and SP of colitic mice, total cells obtained by digestion with collagenase were passed through a 40-μm cell strainer. CD11c^- DC were further purified using an anti-CD11c MACS magnetic separation system, resulting in >80% purity. In vitro assay for the induction of gut-homing receptors was performed by the modified protocol established by others (23). In short, 2 × 10^5 CD4^+ T cells purified from normal BALB/c mice were cultured with 2 × 10^5 purified LP, MLN, or splenic CD11c^- DC from colitic mice in addition to a soluble 1 μg/ml anti-CD3 mAb (BD Biosciences), human 10 ng/ml RTGF-β (PeproTech), all-trans retinoic acid (RA), and 5 ng/ml human hIL-2 (Shionogi Pharm) with or without 1 μg/ml anti-RANKL mAb. On day 4, cells were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-integrin α4β1, FITC-conjugated anti-integrin α4β7, or FITC-conjugated anti-CCR9 mAb. In some experiments, 2 × 10^5 CD4^+ T cells purified from normal BALB/c mice were cultured with 2 × 10^5 LP, MLN, or SP CD11c^- DC from colitic mice in addition to soluble 1 μg/ml anti-CD3 mAb (BD Biosciences) with or without 1 μg/ml anti-RANKL mAb. On day 4, intracellular Foxp3 staining was performed with the allophycocyanin-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer’s instructions.

**FIGURE 1.** Expression of RANK/RANKL in spleen cells from normal and colitic mice. A, Expression of RANK, RANKL, and G3PDH mRNA was determined by RT-PCR using five SP samples each from normal and colitic mice and is shown as relative amount of the indicated mRNA normalized to G3PDH. Data are represented as the mean ± SEM of five samples. *p < 0.005, B, Phenotypic characterization of splenic CD4^+ T cells expressing CD25 and Foxp3 by FACS. Correlation of CD25/Foxp3 and RANKL expression on splenic CD4^+ T cells obtained from normal or colitic mice. Representatives of four separate samples in each group. C, Expression of Foxp3 in a population of CD4^+ CD45RB^high or CD45RB^low T cells purified from spleen of normal BALB/c mice.

**Statistical analysis**

The results are expressed as the mean ± SEM. Groups of data were compared using the Mann-Whitney U test. Differences were considered to be statistically significant when p < 0.05.

**Results**

**Murine splenic CD4^+ CD25^+ cells constitutively express RANKL.**

To assess the role of the RANK/RANKL axis in the pathogenesis of chronic colitis in terms of stimulatory and inhibitory effects, we first assessed the expression of RANK and RANKL molecules in normal splenic CD4^+ T cells and CD11c^- DC by RT-PCR. As shown in Fig. 1A, CD4^+ T cells preferentially expressed RANKL, but not RANK mRNA, while CD11c^- cells preferentially expressed RANK, but not RANKL mRNA. We further assessed the expression of RANKL on CD4^+ T cells obtained from colitic SCID mice induced by adoptive transfer of CD4^+ CD45RB^high T cells purified from spleen of normal BALB/c mice.
cells and age-matched normal BALB/c mice at a protein level by flow cytometry. As shown in Fig. 1B, splenic CD4+CD25high and CD4+CD25low T cells in normal mice slightly but substantially expressed RANKL, while CD4+CD25+ cells did not. We confirmed that almost all CD4+CD25high T cells expressed Foxp3, while CD4+CD25low T cells did not, indicating that CD4+CD25high and CD4+CD25low T cells are Treg and previously activated effector T cells, respectively (Fig. 1B). Interestingly, although colitic SCID mice were reconstituted with a splenic CD4+CD45RBhigh T cell population that lacks CD4+CD25+ Treg cells, inducible CD4+CD25highFoxp3+ T cells as well as activated CD4+CD25lowFoxp3+ T cells were found in the spleen and these cells expressed the RANKL molecule, in contrast to CD4+CD25− T cells (Fig. 1B). To exclude the possibility that isolated CD4+CD45RBhigh T cells were contaminated with CD4+Foxp3+ Treg cells, we examined the expression of Foxp3 in those cells by flow cytometry. As shown in Fig. 1C, Foxp3+ T cells (Fig. 1C), indicating that a small part of CD4+CD45RBhighFoxp3+ T cells had differentiated into CD4+Foxp3+ Treg cells in LP or MLNs in the periphery and migrated to the spleen in colitic mice.

Blockade of the RANK/RANKL signaling pathway did not affect in vitro Treg activity of CD4+CD25+ cells

Having evidence that activated CD4+ T cells or CD4+CD25− Treg cells express RANKL, we next assessed whether Treg activity of splenic CD4+CD25+ T cells is modulated by blocking the RANK/RANKL pathway using neutralizing anti-RANKL and anti-RANK mAbs in in vitro coculture assay. To this end, we used two approaches, [3H]thymidine uptake and CFSE dilution assay, to assess the proliferation of responder CD4+ T cells. As shown in Fig. 2A, splenic CD4+CD25+ T cells were able to suppress the proliferation of splenic CD4+ responder T cells when cocultured at a ratio of 1:1 Treg to responder in the presence of MMC-treated CD4− APCs, soluble anti-CD3 mAb, and control rat IgG. Similarly, neither anti-RANKL nor anti-RANK mAb at concentrations of 1–10 μg/ml affected the Treg activity of CD4+CD25+ Treg cells (Fig. 2A). To further precisely assess the role of the RANK/RANKL pathway in the suppressive activity of CD4+CD25+ Treg cells to block the proliferation of CD4+CD25− responder T cells at the same ratio of the following in vivo adoptive transfer experiment, we next used CD4+CD25− T cells as responder T cells and sorted CD4+CD25− DC from normal or colitic mice and adopted the CFSE dilution assay to assess the proliferation of responder CD4+CD25− T cells without the impact of the proliferation of cocultured CD4+CD25+ Treg cells. As shown in Fig. 2B, the proliferation of CD4+CD25− responder T cells is seen in the right part of the histogram, and they extensively proliferated in response to anti-CD3 mAb in the absence of CD4+CD25+ Treg cells with any CD11c+ DC regardless of SP or LP and normal or colitic DC. In contrast, the addition of CD4+CD25+ Treg cells prevented the proliferation of CD4+CD25− responder T cells in the presence of anti-RANKL mAb to a similar extent in the presence of control IgG, indicating that the blockade of the RANK/RANKL pathway does not affect the suppressive activity of CD4+CD25+ Treg cells at least in vitro.

Blockade of the RANK/RANKL signaling pathway abolished CD4+CD25+ Treg cell-mediated suppression of colitis in vivo

Although blockade of the RANK/RANKL signaling pathway did not affect the Treg function of CD4+CD25+ cells in the coculture assay (Fig. 2), in vitro assays do not always reflect function in vivo. Thus, we next administered neutralizing anti-RANKL mAb to SCID mice transferred with CD4+CD45RBhigh cells alone or a

FIGURE 2. Blockade of the RANK/RANKL signaling pathway does not affect Treg activity in in vitro coculture assay. A, Splenic CD4+CD25+ T cells suppress the proliferation of responder (Res) CD4+ T cells in vitro in the presence of neutralizing anti-RANKL or anti-RANK mAb. Responder CD4+ T cells and sorted CD4+CD25+ T cells were cocultured for 72 h with anti-CD3 mAb at a 1:1 ratio in the presence of MMC-treated APCs and in the presence of various concentrations (1, 3, or 10 μg/ml) of control IgG, anti-RANKL, or anti-RANK mAb. [3H]Thymidine ([3H]TdR) uptake was determined for the last 9 h. Data are represented from four independent experiments.

B, Colitic mice LP CD11c+ T cells and normal mice LP CD11c+ T cells. As shown in Fig. 2B, the proliferation of CD4+CD25− responder T cells is seen in the right part of the histogram, and they extensively proliferated in response to anti-CD3 mAb in the absence of CD4+CD25+ Treg cells with any CD11c+ DC regardless of SP or LP and normal or colitic DC. In contrast, the addition of CD4+CD25+ Treg cells prevented the proliferation of CD4+CD25− responder T cells in the presence of anti-RANKL mAb to a similar extent in the presence of control IgG, indicating that the blockade of the RANK/RANKL pathway does not affect the suppressive activity of CD4+CD25+ Treg cells at least in vitro.

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starting at the time of T cell transfer. mAb by i.p. injection at a dose of 250 μg H9262 CD45RBhigh (3 x 10⁶/mouse) alone or CD4⁺ CD45RBhigh (3 x 10⁶) + CD4⁺ CD25⁺ T cells (1 x 10⁶) and treated with control IgG or anti-RANKL mAb by i.p. injection at a dose of 250 μg three times per week over 6 wk starting at the time of T cell transfer. B. Change in body weight over time is expressed as percentage of the original weight. Data are represented as the mean ± SEM of seven mice in each group. *, p < 0.05. C. Clinical scores were determined at 6 wk after transfer. Original magnification, ×100. E. Histological scores were determined at 6 wk after transfer as described in Materials and Methods. Data are indicated as the mean ± SEM of seven mice in each group. *, p < 0.05.

mixture of CD4⁺ CD45RBhigh cells and CD4⁺ CD25⁺ T cells (Fig. 3A). As a positive control, mice transferred with CD4⁺ CD45RBhigh T cells alone and treated with control rat IgG developed wasting disease, which became evident 3–5 wk after transfer (Fig. 3B), and clinical (Fig. 3C) and histological evidence of severe chronic colitis as estimated at 6 wk after transfer (Fig. 3, D and E). As a negative control, mice transferred with both CD4⁺ CD45RBhigh and CD4⁺ CD25⁺ TR cell populations and administered with the control rat IgG did not develop wasting disease or colitis (Fig. 3, A–E). Although we showed that activated CD4⁺ CD25⁺Foxp3⁺ effector T cells express RANKL (Fig. 1), the administration of anti-RANKL mAb to mice transferred with CD4⁺ CD45RBhigh cells alone did not affect the severity of colitis as compared with the control IgG-treated mice transferred with CD4⁺ CD45RBhigh cells alone (Fig. 3, A–E), suggesting that the RANK/RANKL pathway between RANKL-expressing APCs is not critically involved in the development of colitis. Surprisingly, however, the administration of anti-RANKL mAb did induce severe colitis in mice transferred with CD4⁺ CD45RBhigh cells and CD4⁺ CD25⁺ TR cell populations, indicating that the anti-RANKL mAb treatment abolished the CD4⁺ CD25⁺ TR cell-mediated in vivo suppression of colitis induced by adoptive transfer of CD4⁺ CD45RBhigh T cells.

A further quantitative evaluation of CD4⁺ T cell infiltration was made by isolating spleen cells and LP cells from the resectedbowels. Significantly higher numbers of CD4⁺ T cells were recovered from the spleen (Fig. 4A) and LP (Fig. 4B) of the mice transferred with both CD4⁺ CD45RBhigh and CD4⁺ CD25⁺ TR cell populations and administered with anti-RANKL mAb as compared with control IgG, being comparable to those from mice transferred with CD4⁺ CD45RBhigh T cells alone and treated with anti-RANKL mAb or the control rat IgG. We also examined the cytokine production by CD4⁺...
lamina propria lymphocyte from the four groups of mice. As shown in Fig. 4C, IFN-γ and IL-17 production by LP CD4+ T cells was markedly suppressed by cotransfer of CD4+CD25+ T R cells with CD4+CD45RB<sup>high</sup> T cells, but this suppression was partially but significa-
antly abrogated by the treatment with anti-RANKL mAb to the level of LP CD4+ T cells from the mice transferred with CD4+CD45RB<sup>high</sup> T cells alone and treated with anti-RANKL mAb or control IgG.

**Balance between effector CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells in the inflamed mucosa was dysregulated by treatment with anti-RANKL mAb**

To further assess the balance in cell numbers between effector CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells in recipient SCID mice, we transferred Ly5.1<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> cells and Ly5.2<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells into C57BL/6 RAG-2<sup>−/−</sup> mice to distinguish between effector CD4<sup>+</sup> T cells and T<sub>R</sub> cells (Fig. 5A). We first confirmed that C57BL/6 RAG-2<sup>−/−</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells did develop colitis with the marked expansion of CD4<sup>+</sup> T cells when treated with anti-RANKL mAb, but not with control IgG (data not shown). As expected, the absolute number of total CD4<sup>+</sup> T cells (Ly5.1<sup>+</sup> plus Ly5.2<sup>+</sup> cells) and Ly5.1<sup>+</sup> effector CD4<sup>+</sup> T cells in LP of anti-RANKL mAb-treated mice was signifi-
cantly increased due to the presence of colitis as compared with that in control IgG-treated mice with no colitis (Fig. 5B). Also, the abso-
lute number of Ly5.2<sup>+</sup> T cells and Ly5.2<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>R</sub> cells in LP of colitic anti-RANKL mAb-treated mice was signifi-
cantly higher than that in non-colitic control IgG-treated mice (Fig. 5B), suggesting that both effector CD4<sup>+</sup> T cells and T<sub>R</sub> cells extensively proliferated in LP of anti-RANKL mAb-treated colitic mice. Interestingly, how-
ever, the ratio of Foxp3<sup>+</sup> cells to total Ly5.2<sup>+</sup> CD4<sup>+</sup> T cells at 6 wk after transfer was analyzed by flow cytometry. Data are indicated as the mean ± SEM of six mice per group. *<em>p</em> < 0.01.

**Blockade of the RANK/RANKL pathway does not affect migration of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells to the inflamed mucosa, but suppresses their expansion in the inflamed mucosa**

To assess why blockade of the RANK/RANKL signaling pathway affected the expansion and/or migration of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells, we conducted several in vitro and in vivo experiments. First, to assess the possibility that blockade of the RANK/RANKL pathway skews the expression of gut-homing receptors on T<sub>R</sub> cells, splenic CD4<sup>+</sup> T cells were cultured for 72 h in the presence or absence of anti-RANKL mAb with a stimulating

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**FIGURE 5.** Blockade of the RANK/RANKL pathway induces the dysregu-
lated cell balance between effector CD4<sup>+</sup> T cell and T<sub>R</sub> cells in the inflamed mucosa of colitic mice. A, C57BL/6 RAG-2<sup>−/−</sup> mice were injected i.p. with Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> (3 × 10<sup>5</sup>/mouse) alone or Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> (3 × 10<sup>5</sup>) + Ly5.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 × 10<sup>5</sup>) and treated with control IgG or anti-RANKL mAb by i.p. injection at a dose of 250 µg three times per week over 6 wk starting at the time of transfer. B, Absolute number of total CD4<sup>+</sup> T cells (Ly5.1<sup>+</sup> + Ly5.2<sup>+</sup> cells), Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells, Ly5.2<sup>+</sup>CD4<sup>+</sup> T cells, Ly5.2<sup>+</sup>CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>R</sub> cells in LP, SP, MLN, and liver of control IgG- or anti-RANKL mAb-treated mice. Data are indicated as the mean ± SEM of 12 mice per group for LP, SP, and MLN and six mice per group for liver. *<em>p</em> < 0.01. C, Ratio of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> (Ly5.2<sup>+</sup>) cells per total CD4<sup>+</sup> T cells (Ly5.1<sup>+</sup> + Ly5.2<sup>+</sup>) at 6 wk after transfer was analyzed by flow cytometry. Data are indicated as the mean ± SEM of six mice per group. *<em>p</em> < 0.01. D, Ratio of Foxp3<sup>+</sup> cells to total Ly5.2<sup>+</sup> CD4<sup>+</sup> T cells at 6 wk after transfer was analyzed by flow cytometry. Data are indicated as the mean ± SEM of six mice per group.

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To further assess the balance in cell numbers between effector CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells in recipient SCID mice, we trans-
ferred Ly5.1<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> cells and Ly5.2<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells into C57BL/6 RAG-2<sup>−/−</sup> mice to distinguish between effector CD4<sup>+</sup> T cells and T<sub>R</sub> cells (Fig. 5A). We first confirmed that C57BL/6 RAG-2<sup>−/−</sup> mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells did develop colitis with the marked expansion of CD4<sup>+</sup> T cells when treated with anti-RANKL mAb, but not with control IgG (data not shown). As expected, the absolute number of total CD4<sup>+</sup> T cells (Ly5.1<sup>+</sup> plus Ly5.2<sup>+</sup> cells) and Ly5.1<sup>+</sup> effector CD4<sup>+</sup> T cells in LP of anti-RANKL mAb-treated mice was signifi-
cantly increased due to the presence of colitis as compared with that in control IgG-treated mice with no colitis (Fig. 5B). Also, the abso-
To further assess the possibility that blockade of the RANK/RANKL pathway does not affect the migration of CD4<sup>+</sup> T<sub>reg</sub> cells to the inflamed mucosa of colitic mice, we performed an additional in vivo adoptive transfer experiment. To this end, RAG-2<sup>−/−</sup> mice, since it has been reported that RANK is expressed on RAG-2<sup>−/−</sup> bone marrow, since it has been reported that RANK is expressed on RAG-2<sup>−/−</sup> bone marrow. To assess the effect of anti-RANKL mAb on the trafficking of TR cells to inflamed mucosa of colitic mice, RAG-2<sup>−/−</sup> mice were transferred with splenic CD4<sup>+</sup> T cells to inflamed mucosa of colitic mice. To finally assess the possibility that colitic LP DC cells modulate the expansion of T<sub>reg</sub> cells in the inflamed mucosa in a RANK/RANKL-dependent manner, we evaluated the expression of RANK on CD11c<sup>+</sup> DC cells obtained from the spleen, MLN, and LP of colitic and normal mice. As shown in Fig. 7A, the expression of RANK on colitic LP DC was significantly increased as compared with that on normal LP DC. In contrast, the expression of RANK in SP and MLN was similar in normal and colitic mice. Given the up-regulated expression of RANK on colitic LP DC cells, we next assessed the possibility that the RANK/RANKL pathway is involved in the expansion of T<sub>reg</sub> cells. To this end, splenic CD4<sup>+</sup> T cells recovered from normal mice were cultured with colitic LP, MLN, or SP CD11c<sup>+</sup> DC in the presence of anti-CD3 mAb with control IgG or anti-RANKL mAb for 72 h, and the ratio of CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells per total CD4<sup>+</sup> T cells (B) and the number of CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells (C) recovered from culture with colitic LP, MLN, or SP CD11c<sup>+</sup> DC in the presence or absence of anti-RANKL mAb were evaluated by flow cytometry. Data are indicated as the mean ± SEM of seven samples per group, *p < 0.05.

Ly5.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>−</sup> T cells in SP, MLN, and LP was found not to be modified by anti-RANKL-treatment at all (Fig. 6B).

To finally assess the possibility that colitic LP DC cells modulate the expansion of T<sub>reg</sub> cells in the inflamed mucosa in a RANK/RANKL-dependent manner, we evaluated the expression of RANK on CD11c<sup>+</sup> DC cells obtained from the spleen, MLN, and LP of colitic and normal mice. As shown in Fig. 7A, the expression of RANK on colitic LP DC was significantly increased as compared with that on normal LP DC. In contrast, the expression of RANK in SP and MLN was similar in normal and colitic mice. Given the up-regulated expression of RANK on colitic LP DC cells, we next assessed the possibility that the RANK/RANKL pathway is involved in the expansion of T<sub>reg</sub> cells. To this end, splenic CD4<sup>+</sup> T cells recovered from normal mice were cultured with colitic LP, MLN, or SP CD11c<sup>+</sup> DC in the presence of anti-CD3 mAb with or without anti-RANKL mAb for 72 h. The ratio of CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells per total of CD4<sup>+</sup> T cells (Fig. 7B) and the number of CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells (Fig. 7C) recovered from culture with colitic LP, but not MLN

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Blockade of the RANK/RANKL pathway does not affect the migration of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells to the inflamed mucosa of colitic mice. A, CD4<sup>+</sup> T cells (2 × 10<sup>5</sup>) purified from normal BALB/c mice were cultured with purified SP CD11c<sup>+</sup> DC (2 × 10<sup>3</sup>) from colitic mice in addition to soluble 1 μg/ml anti-CD3 mAb, 10 ng/ml human rTGF-β, all-trans RA, and 5 ng/ml rIL-2 with or without 1 μg/ml anti-RANKL mAb. On day 4, cells were stained with PerCP-conjugated anti-CD4 mAb and PE-conjugated anti-integrin α<sub>β</sub>7, PE-conjugated anti-integrin α<sub>d</sub>β<sub>7</sub>, or PE-conjugated anti-CCR9 mAb, followed by intracellular staining by allophycocyanin-conjugated Foxp3 mAb. Data are indicated as the mean ± SEM of six samples per group, *p < 0.01. B, Anti-RANKL mAb treatment does not affect the migration of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>−</sup> T cells in vivo. To assess the effect of anti-RANKL mAb on the trafficking of T<sub>reg</sub> cells to inflamed mucosa of colitic mice, RAG-2<sup>−/−</sup> mice were transferred with CD4<sup>+</sup>CD45RB<sup>−</sup> T cells and, 4 wk after transfer, they were treated with 250 μg of anti-RANKL mAb or control IgG two times in 1 day (or on 2 consecutive days). They were then transferred with splenic Ly5.1<sup>+</sup>CD4<sup>+</sup> T cells from normal mice and, 24 h after the second transfer, the cell number of Ly5.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>−</sup> (lower) or Foxp3<sup>+</sup> (upper) T cells recovered from LP, SP, and MLN was evaluated by flow cytometry.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Blockade of the RANK/RANKL pathway suppresses the expansion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in the inflamed mucosa. A, Expression of RANK on LP, SP, or MLN DC cells obtained from colitic (■) or normal (□) mice. B and C, Splenic CD4<sup>+</sup> T cells from normal mice were cultured with colitic LP, MLN, or SP CD11c<sup>+</sup> DC in the presence of anti-CD3 mAb with control IgG (□) or anti-RANKL mAb (■) for 72 h, and the ratio of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells per total CD4<sup>+</sup> T cells (B) and the number of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells (C) recovered from culture with colitic LP, MLN, or SP CD11c<sup>+</sup> DC in the presence or absence of anti-RANKL mAb were evaluated by flow cytometry. Data are indicated as the mean ± SEM of six samples per group, *p < 0.01.
or SP, CD11c+ DC in the presence of anti-RANKL mAb was significantly decreased as compared with that in the presence of control IgG, suggesting that the RANK/RANKL pathway is critically involved in the expansion of LP TR cells through the direct interaction with colitic RANK-expressing LP DC and TR cells.

Discussion

In the present study, we demonstrated that 1) CD4+CD25+ T cells including CD4+CD25high TR cells and activated CD4+CD25low effector cells rather than CD4+CD25− T cells preferentially express the RANKL molecule and 2) blockade of the RANK/RANKL signaling pathway suppresses the expansion of CD4+CD25+ TR cells and subsequently abolishes the TR cell-mediated suppression of colitis due to dysregulation of the cell balance between effector CD4+ T cells and TR cells in the inflamed intestine. Interestingly, although activated effector CD4+ T cells and inducible CD4+CD25− TR cells also express RANKL molecules in SCID mice transferred with CD4+CD45RBhigh T cells alone, the administration of this mAb did not affect the course of colitis. Collectively, these findings indicate that the RANK-RANKL signaling pathway is critically involved in intestinal mucosal tolerance by controlling the expansion and function of CD4+CD25+ TR cells in the inflamed mucosa.

Although many previous reports have established the role of the RANK/RANKL signaling pathway in osteoclastogenesis and bone loss in various chronic T cell-mediated inflammatory diseases including IBDs (20, 25–29), the role of this pathway in the local mucosa of the colon. In our colitis model induced by adoptive transfer of CD45RBhigh T cells and previously activated CD4+TR cells at a ratio of 3:1, TR cells preferentially expanded in draining lymph nodes by CD4+TR cells, but also

Discussion

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that $\beta_7^{-/-}$CD4$^+$CD25$^+$ Treg cells are not needed to suppress the development of colitis, because $\beta_7^{-/-}$ CD4$^+$CD25$^+$ Treg cells directly migrate to MLNs and can inhibit naive CD4$^+$CD45RB$^{hi}$ T cell activation and proliferation within Ag-draining MLNs, resulting in suppression of the development of the gut-seeking activated effector CD4$^+$ T cells instructed to express the gut-homing receptors such as integrins $\alpha_4\beta_7$ and $\alpha_4\beta_7$. However, it remains unknown whether mucosal CD4$^+$CD25$^+$ Treg cells are necessary for the suppression of mucosal pathogenic effector CD4$^+$ T cells ex vivo, especially in an ongoing colitis system in which it can be assessed whether LP CD4$^+$CD25$^+$ Treg cells as effector Treg cells can suppress the surrounding LP effector CD4$^+$ T cells ex vivo.

In this regard, we have previously demonstrated that human CD4$^+$CD25$^{hi}$ and mouse CD4$^+$CD25$^+$ T cells reside in the intestinal LP, express CTLA-4, GITR, and Foxp3 and possess T reg suppress the development of colitis. Having evidence that the murine intestinal LP CD4$^+$CD25$^+$ T cells express GITR and Foxp3, we further asked whether MLNs are fully essential for the suppression of colitis by splenic CD4$^+$CD25$^+$ T cells. As a second approach to this issue, we also found that the cotransfer of splenic CD4$^+$CD25$^+$ Treg cells prevented the development of colitis in the lymph node-null Lta$^{-/-}$ × Rag-2$^{-/-}$ mice transfected with CD4$^+$CD45RB$^{hi}$ T cells, indicating that splenic CD4$^+$CD25$^+$ T cells can suppress the development of colitis in the absence of MLNs (12). Moreover, we demonstrated that CD4$^+$CD25$^+$ Treg cells actually migrated and resided in the colon in Lta$^{-/-}$ × Rag-2$^{-/-}$ mice cotransfected with Ly5.2-derived CD4$^+$CD45RB$^{hi}$ 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 Treg cells to suppress intestinal inflammation, probably as a second line of suppression (yy). Along with the present findings that the RANK/RANKL interaction is critically involved in the function of CD4$^+$CD25$^+$ Treg cells in the intestine, our research suggests that therapeutic approaches enhancing the migration of CD4$^+$CD25$^+$ Treg cells, such as the specific induction of RANKL on CD4$^+$CD25$^+$ Treg cells, may be feasible in the treatment of IBD.

Disclosures

The authors have no financial conflict of interest.

References


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