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RANK-RANKL Signaling Pathway Is Critically Involved in the Function of CD4⁺CD25⁺ Regulatory T Cells in Chronic Colitis¹

Teruji Totsuka,2* Takanori Kanai,2,3* Yasuhiro Nemoto,* Takayuki Tomita,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Tetsuya Nakamura,* Naoya Sakamoto,* Hisaya Akiba,† Ko Okumura,† Hideo Yagita,† and Mamoru Watanabe*

It is now clear that functional CD4⁺CD25⁺ regulatory T (T₉) cells exist as part of the normal immune population and prevent the development of intestinal inflammation. We have recently shown that CD4⁺CD25⁺ T₉ 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⁺ T₉ cells in the intestine. We first found that RANKL was preferentially expressed on both CD4⁺CD25⁺ T₉ cells and colitogenic CD4⁺ T cells, whereas RANK was expressed on dendritic cells. Although neutralizing anti-RANKL mAb did not affect T₉ activity of CD4⁺CD25⁺ T₉ cells to suppress the proliferation of CD4⁺ responder cells in vitro, in vivo administration of anti-RANKL mAb abrogated CD4⁺CD25⁺ T₉ cell-mediated suppression of colitis induced by adoptive transfer of CD4⁺CD45RBbright T cells into SCID mice. Interestingly, an adoptive transfer experiment using Ly5.1⁺CD4⁺CD45RBhigh cells and Ly5.2⁺CD4⁺CD25⁺ T₉ cells revealed that the ratio of CD4⁺CD25⁺ T₉ 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⁺ T₉ 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⁺ T₉ 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 intestinal and systemic immune responses (2, 3). In this regard, CD4⁺CD25⁺ regulatory T (T₉) cells play a central role in the maintenance of immunological homeostasis (6, 7). CD4⁺CD25⁺ T₉ cells have been detected mainly in lymphoid sites, including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated the capacity of CD4⁺CD25⁺ T₉ 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⁺ T₉ 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 naive, 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 where CD4⁺CD25⁺ T₉ 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 in the inductive sites by CD4⁺CD25⁺ T₉ 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 T₉ activity in vitro and therefore questioned whether these inductive sites alone were involved in the induction and suppression of intestinal inflammation (11). We also reported that peripheral CD4⁺CD25⁺ T₉ cells actually migrated to the intestine and suppressed the development of colitis in the CD4⁺CD45RBhigh cell transfer model of colitis without the involvement of lymph nodes in lymph node-null LTA⁻⁻/- × RAG-2⁻⁻/- recipient mice (12). Consistent with our previous reports, it has recently been reported that CD4⁺CD25⁺ T₉ cells were detected in peripheral tissues and at sites of ongoing immune responses, such as synovial fluid from rheumatoid arthritis patients (13), tumors (14), transplants (15), skin lesions in mice infected

*Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
†Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan
‡Address correspondence to Dr. Takanori Kanai at the current address: Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan. E-mail address: takagast@sc.itc.keio.ac.jp
§Abbreviations used in this paper: IBD, inflammatory bowel disease; LP, lamina propria; MLN, mesenteric lymph node; RANK, receptor activator of NF-κB; SP, spleen; RANKL, receptor activator of NF-κB ligand; T₉, regulatory T; IEL, intraepithelial lymphocyte; HFF, high-power field; Fwd, forward; Rev, reverse; MMC, mitomycin C; DC, dendritic cell; RA, retinoic acid.

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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<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> cells in the intestine to suppress intestinal inflammation. In the present study, we show that both CD4<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> cells and colitogenic CD4<sup>+</sup> T cells preferentially express a TNF family member, receptor activator of NF-κB ligand (RANKL), and that blockade of the signaling pathway via RANKL and its receptor activator of NF-κB (RANK) (20) by administering neutralizing anti-RANKL mAb abrogates the CD4<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> cell-mediated suppression of colitis induced by adoptive transfer of CD4<sup>+</sup> CD45RB<sup>high</sup> T cells into SCID mice, indicating a critical role for the RANKL/RANK signaling pathway in the function of intestinal CD4<sup>+</sup> CD25<sup>+</sup> 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 Central Laboratories for Experimental Animals. Mice were maintained under specific pathogen-free conditions in the animal care facility of Tokyo Medical and Dental University. 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-mouse CD4 (rat IgG2a); 7D4, FITC-conjugated anti-mouse CD25 (rat IgM); PC06, PE-conjugated anti-mouse CD25 (rat IgG1); H1.3F3, FITC-conjugated anti-CD69 (Ham IgG1); FJK-16s, allophycocyanin-conjugated anti-mouse Foxp3 (rat IgG2a); DAATK2, PE-conjugated anti-integrin α<sub>β</sub>2 (rat IgG2a); M290, PE-conjugated anti-integrin α<sub>β</sub>5 (rat IgG2a); 24G203, PE-conjugated anti-CCR9 (rat IgG2b); isotype control Abs, biotin-conjugated rat IgG2, FITC-conjugated rat IgM, PE-conjugated rat IgG2a, and PE-conjugated mouse IgG2α; PE-conjugated streptavidin; and CyChrome-conjugated streptavidin. The neutralizing anti-mouse RANKL mAb (IK22-5, rat IgG2a), and anti-mouse RANK mAb (12-31, rat IgG2a) were prepared as described previously (21).

**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 cells (96–97% pure, as estimated by FACSsCalibur; BD Biosciences) were then labeled with PE-conjugated anti-CCR9 (rat IgG2a); 7D4, FITC-conjugated anti-CD25 (rat IgM), 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) and, further purified by Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4<sup>+</sup> T cells from normal mice, and we isolated CD4<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> cells in inflamed mucosa of colitic mice, we conducted another in vivo setting. First, RAG-2<sup>−/−</sup> mice were transferred with CD4<sup>+</sup> CD45RB<sup>high</sup> T cells and, 4 wk after transfer, these colitic mice were treated with 250 μg of anti-RANKL mAb or control IgG two times within 1 day. Then they were retransferred with splenic Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells from normal mice, and we evaluated the cell number of Ly5.1<sup>+</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> and Foxp3<sup>+</sup> recovered from LP, spleen (SP), and MLNs at 24 h after the retransfer.

**Disease monitoring and clinical scoring**

The recipient SCID mice after T cell transfer were weighed initially, 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 and assessed for a clinical score as the sum of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 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 6% 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 colonic mucosa 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**

Colonial 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 two times 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 desorbed, 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> LP T cells were obtained by positive selection using an anti-CD4 (L3T4) MACS magnetic separation system. The resultant cells when analyzed by FACSsCalibur contained >96% CD4<sup>+</sup> cells.

**RT-PCR**

Total cellular RNA was extracted from 7 × 10<sup>6</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 QuantiText SYBER green PCR kit using Applied Biosytems 7500 real-time PCR system and 7500 system SDS software with the following primers: RANK forward 5′-CGA TGC CAT GAG TAT CAG CTC TTC TTC CAT GAC-3′ and reverse 5′-CTA CTG GCG CTG CCA AGG CAG T-3′; and G3PDH forward 5′-GCC ATG AAC TTC GCA GCC TCA GCC 3′- and reverse 5′-TGA GAC TGG GCA GGT AGG ACC CC-3′. The 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.
with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-integrin experiments, a CFSE dilution assay was performed. To this end, splenic proliferating cells was measured during the last 9 h of culture. In some experiments, 2 μg/ml anti-CD3 mAb (BD Biosciences), human 10 ng/ml rTGF-β, and 50 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME. Incorporation of [3H]thymidine (1 μCi/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 C57BL/6 mice using a CD4 T Cell Isolation Kit (Miltenyi Biotec). CD11c+ responder T and CD4+CD25+ Tc25 cells were separately 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+CD45RB+ T cells. All populations were >92% pure on reanalysis. Then, CD4+CD25+ responder T cells were labeled with 1 μM CFSE (Molecular Probes). CFSE-labeled CD4+CD25− responder T cells (1 × 105) were cocultured with unlabelled Ly5.2+CD4+CD25− Tq cells (0.33 × 105) with CD11c+ DC (2 × 105) and anti-CD3 (1 μg/ml) in the presence of the 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. Prodigium iodide was added to exclude dead cells. Proliferation analysis was based on division times of responder CFSE+CD4+ T cells at the condition that can discriminate 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 × 10^6 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 rIL-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 α9β1, 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 purified 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.

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% CO2 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 × 10^6) and mitomycin C (MMC)-treated CD4− cells (5 × 10^6) as APCs, with or without CD4+CD25+ cells (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 [3H]thymidine (1 μCi/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 C57BL/6 mice using a CD4 T Cell Isolation Kit (Miltenyi Biotec). CD11c+ responder T and CD4+CD25+ Tc25 cells were separately 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+CD45RB+ T cells. All populations were >92% pure on reanalysis. Then, CD4+CD25+ responder T cells were labeled with 1 μM CFSE (Molecular Probes). CFSE-labeled CD4+CD25− responder T cells (1 × 10^6) were cocultured with unlabelled 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 the 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. Prodigium iodide was added to exclude dead cells. Proliferation analysis was based on division times of responder CFSE+CD4+ T cells at the condition that can discriminate the unlabeled CD4+CD25− Tq cells and CD11c+ DC cells.

FIGURE 1.

A. 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/in 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+CD45RBhigh or CD45RBlow 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+CD45RBhigh T
cells and age-matched normal BALB/c mice at a protein level by flow cytometry. As shown in Fig. 1B, splenic CD4⁺ CD25⁺ and CD4⁺ CD25⁻ cells in normal mice slightly but substantially expressed RANKL, while CD4⁺ CD25⁻ cells did not. We confirmed that almost all CD4⁺ CD25⁻ T cells expressed Foxp3, while CD4⁺ CD25⁺ T cells did not, indicating that CD4⁺ CD25⁻ and CD4⁺ CD25⁻ T cells are TR and previously activated effector T cells, respectively (Fig. 1B). Interestingly, although colitic SCID mice were reconstituted with a splenic CD4⁺ CD45RB⁻ T cell population that lacks CD4⁺ CD25⁺ TR cells, inducible CD4⁺ CD25⁺Foxp3⁺ T cells as well as activated CD4⁺ CD25⁻Foxp3⁻ 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⁺ CD45RB⁻ T cells were contaminated with CD4⁺ Foxp3⁻ TR cells, we examined the expression of Foxp3 in those cells by flow cytometry. Although almost one-third of the control CD4⁺ CD45RB⁻ T cells expressed Foxp3, we confirmed that a small part of CD4⁺ CD45RB⁻Foxp3⁻ T cells had differentiated into CD4⁺ Foxp3⁻ TR 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 TR activity of CD4⁺ CD25⁻ T cells

Having evidence that activated CD4⁺ T cells or CD4⁺ CD25⁻ T cells express RANKL, we next assessed whether TR 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 TR to 1 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 TR activity of CD4⁺ CD25⁻ TR cells (Fig. 2A).

To further precisely assess the role of the RANK/RANKL pathway in the suppressive activity of CD4⁺ CD25⁻ TR 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 SP or LP CD11c⁺ 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⁻ TR 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⁻ TR cells with any CD11c⁻ DC regardless of SP or LP and normal or colitic DC. In contrast, the addition of CD4⁺ CD25⁻ TR 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⁻ TR cells at least in vitro.

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

Although blockade of the RANK/RANKL signaling pathway did not affect the TR 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⁺ CD45RB⁻ cells alone or a...
Anti-RANKL mAb abrogates CD4 \(^+\)CD25 \(^+\) T\(_R\) cell-mediated suppression of Th1 and Th17 cells in vivo. A, SP and LP CD4 \(^+\) T (B) cells were isolated from the colon at 6 wk after adoptive T cell transfer and treated as described in Fig. 3, and the number of CD4 \(^+\) cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, p < 0.01. B, Cytokine production by CD4 \(^+\) lamina propria lymphocyte. LP CD4 \(^+\) cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SEM of six mice in each group, *, p < 0.01.

mixture of CD4 \(^+\)CD45RB\(^{hi}\) cells and CD4 \(^+\)CD25 \(^+\) T cells (Fig. 3A). As a positive control, mice transfere\nred with CD4 \(^+\)CD45RB\(^{hi}\) 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 transfere\nd with both CD4 \(^+\)CD45RB\(^{hi}\) and CD4 \(^+\)CD25 \(^+\) T\(_R\) 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\(^{hi}\)Foxp3\(^+\) effector T cells express RANKL (Fig. 1), the administration of anti-RANKL mAb to mice transfere\ned with CD4 \(^+\)CD45RB\(^{hi}\) cells alone did not affect the severity of colitis as compared with the control IgG-treated mice transfere\ned with CD4 \(^+\)CD45RB\(^{hi}\) cells alone (Fig. 3, A–E), suggesting that the RANK/RANKL pathway between RANKL-expressing activated effector CD4 \(^+\) T cells and RANK-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 transfere\ned with CD4 \(^+\)CD45RB\(^{hi}\) cells and CD4 \(^+\)CD25 \(^+\) T\(_R\) cells, indicating that the anti-RANKL mAb treatment abolished the CD4 \(^+\)CD25 \(^+\) T\(_R\) cell-mediated in vivo suppression of colitis induced by adoptive transfer of CD4 \(^+\)CD45RB\(^{hi}\) T cells.

A further quantitative evaluation of CD4 \(^+\) T cell infiltration was made by isolating spleen cells and LP cells from the resected bowels. Significantly higher numbers of CD4 \(^+\) T cells were recovered from the spleen (Fig. 4A) and LP (Fig. 4B) of the mice transfere\ned with both CD4 \(^+\)CD45RB\(^{hi}\) and CD4 \(^+\)CD25 \(^+\) T\(_R\) cell populations and administered with anti-RANKL mAb as compared with control IgG, being comparable to those from mice transfere\ned with CD4 \(^+\)CD45RB\(^{hi}\) T cells alone and treated with anti-RANKL mAb or the control rat IgG. We also examined the cytokine production by CD4 \(^+\)
To further assess the balance in cell numbers between effector CD4⁺ T cells and CD4⁺CD25⁺ TR cells in the inflamed mucosa was dysregulated by treatment with anti-RANKL mAb.

Balance between effector CD4⁺ T cells and CD4⁺CD25⁺ TR cells in the inflamed mucosa was dysregulated by treatment with anti-RANKL mAb.

To further assess the balance in cell numbers between effector CD4⁺ T cells and CD4⁺CD25⁺ TR cells in recipient SCID mice, we transferred Ly5.1⁺ CD4⁺CD45RB⁺ T cells and Ly5.2⁺ CD4⁺CD25⁺ TR cells into C57BL/6 RAG-2⁻/⁻ mice to distinguish between effector CD4⁺ T cells and TR cells (Fig. 5A). We first confirmed that C57BL/6 RAG-2⁻/⁻ mice transferred with CD4⁺CD45RB⁺ T cells and CD4⁺CD25⁺ TR cells did develop colitis with the marked expansion of CD4⁺ T cells when treated with anti-RANKL mAb, but not with control IgG (data not shown). As expected, the absolute number of total CD4⁺ T cells (Ly5.1⁺ plus Ly5.2⁺ cells) and Ly5.1⁺ effector CD4⁺ T cells in LP of anti-RANKL mAb-treated mice was significantly increased due to the presence of colitis as compared with that in control IgG-treated mice with no colitis (Fig. 5B). Also, the absolute number of Ly5.2⁺ T cells and Ly5.2⁺CD45RB⁺Foxp3⁺ TR cells in LP of colitic anti-RANKL mAb-treated mice was significantly higher than that in non-colitic control IgG-treated mice (Fig. 5B), suggesting that both effector CD4⁺ T cells and TR cells extensively proliferated in LP of anti-RANKL mAb-treated colitic mice. Interestingly, however, the ratio of Ly5.2⁺CD4⁺CD25⁺ TR cells to total CD4⁺ T cells in the recipient mice treated with anti-RANKL mAb was markedly decreased in the intestine as compared with that in the recipient mice treated with control IgG, although there were no significant differences in the spleen, MLN, or liver between the two groups (Fig. 5C). These results suggested that the blockade of the RANK/RANKL signaling pathway affected the expansion and/or migration of CD4⁺CD25⁺ TR cells, resulting in dysregulated cell balance between effectors and TR cells in the inflamed mucosa.

Blockade of the RANK/RANKL pathway does not affect migration of CD4⁺CD25⁺ TR cells to the inflamed mucosa, but suppresses their expansion in the inflamed mucosa.

To assess why blockade of the RANK/RANKL pathway abolished TR function in mice transferred with CD4⁺CD45RB⁺ TR cells and CD4⁺CD25⁺ TR 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 TR cells, splenic CD4⁺ T cells were cultured for 72 h in the presence or absence of anti-RANKL mAb with a stimulating...
mixture including anti-CD3 mAb, soluble IL-2, TGF-β, and RA, which is known to induce the gut-homing receptors (integrin α4β7, α6β1, and CCR9) (23). As expected, the mixture strongly induced integrin α4β7, α6β1, and CCR9, but the addition of anti-RANKL mAb did not affect the expression of these molecules (Fig. 6A).

To further assess the possibility that blockade of the RANK/RANKL pathway affected the TR cell migration to the inflamed mucosa, we performed an additional in vivo adoptive transfer experiment, since it has been reported that RANK is expressed on endothelial cells (24). To this end, RAG-2−/− mice were transferred with Ly5.2+CD4+CD45Rohigh T cells and, 4 wk after transfer, they were treated with control IgG or anti-RANKL mAb twice in 1 day. On the following day, they were transferred with splenic CD4+CD25− T cells to inflamed mucosa of colitic mice. A, CD4+ T cells (2 × 10^5) purified from normal BALB/c mice were cultured with purified SP CD11c+ DC (2 × 10^5) 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 Rl-2 or with or without 1 μg/ml anti-RANKL mAb. On day 4, cells were stained with PerCP-conjugated anti-CD4 mAb, CD45RB high T cells and, 4 wk after transfer, they were transferred with splenic CD4+ T cells obtained from normal C57BL/6-Ly5.1 mice (Fig. 6B).

FIGURE 6. Blockade of the RANK/RANKL pathway does not affect the migration of CD4+CD25− T cells to the inflamed mucosa of colitic mice. A, CD4+ T cells (2 × 10^5) purified from normal BALB/c mice were cultured with purified SP CD11c+ DC (2 × 10^5) 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 Rl-2 or with or without 1 μg/ml anti-RANKL mAb. On day 4, cells were stained with PerCP-conjugated anti-CD4 mAb, CD45RB high T cells and, 4 wk after transfer, they were transferred with splenic CD4+ T cells obtained from normal C57BL/6-Ly5.1 mice (Fig. 6B).

FIGURE 7. Blockade of the RANK/RANKL pathway suppresses the expansion of CD4+CD25− TR 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+ T cells from normal mice were cultured with colitic LP, MLN, or SP CD11c+ DC in the presence of anti-CD3 mAb with control IgG (□) or anti-RANKL mAb (■) for 72 h, and the ratio of CD4+Foxp3+ TR cells per total CD4+ T cells (B) and the number of CD4+Foxp3+ TR cells (C) recovered from culture with colitic LP, MLN, or SP CD11c+ 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+CD4+Foxp3− 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 Treg cells in the inflamed mucosa in a RANK/RANKL-dependent manner, we evaluated the expression of RANK on CD11c+ 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 Treg cells. To this end, splenic CD4+ T cells obtained from normal mice were cultured with colitic LP, MLN, or SP CD11c+ DC in the presence of anti-CD3 mAb with or without anti-RANKL mAb for 72 h. The ratio of CD4+Foxp3+ Treg cells per the total of CD4+ T cells (Fig. 7B) and the number of CD4+Foxp3+ Treg cells (Fig. 7C) recovered from culture with colitic LP, but not MLN
or SP, CD11c<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells including CD4<sup>+</sup>CD25<sup>high</sup> TR cells and activated CD4<sup>+</sup>CD25<sup>low</sup> effector cells rather than CD4<sup>+</sup>CD25<sup>+</sup> T cells preferentially express the RANKL molecule and 2) blockade of the RANK/RANKL signaling pathway suppresses the expansion of CD4<sup>+</sup> CD25<sup>+</sup> TR cells and subsequently abolishes the TR cell-mediated suppression of colitis due to dysregulation of the cell balance between effector CD4<sup>+</sup> T cells and TR cells in the inflamed intestine. Interestingly, although activated effector CD4<sup>+</sup> T cells and inducible CD4<sup>+</sup>CD25<sup>+</sup> TR cells also express RANKL molecules in SCID mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> 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<sup>+</sup> CD25<sup>+</sup> 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 inflammation in various models remains unknown. For example, Kong et al. (26) initially reported that the blockade of this pathway by using soluble recombinant osteoprotegerin (OPG) protein at the onset of disease prevented bone and cartilage destruction, but interestingly not inflammation in a T cell-dependent model of rat adjuvant arthritis. In contrast, Ashcroft et al. (30) demonstrated that the administration of RANK-Fc protein not only reverses the bone loss in IL-2<sup>−/−</sup> mice, which is a spontaneous model of osteoporosis and colitis, but also reduces the development of colitis by blocking the interaction between RANK-expressing DC and RANKL-expressing activated CD4<sup>+</sup> T cells in the inflamed mucosa of the colon. In our colitis model induced by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into SCID mice, however, administration of neutralizing anti-RANKL mAb did not prevent the development of colitis, although inducible CD4<sup>+</sup>CD25<sup>high</sup> TR cells and previously activated CD4<sup>+</sup>CD25<sup>low</sup> T cells expressed RANKL. Consistent with this finding, Byrne et al. (31) previously demonstrated that administration of human osteoprotegerin-Fc increased bone density in this model, but had no effects on the intestinal inflammation. Several explanations have been advanced for the discrepancy, including differences in the species, the type of animal model, the type of blocking agents, and dosing regimens used. Furthermore, it has recently been demonstrated that in vivo administration of neutralizing anti-cytokine mAbs, such as anti-IL-2 mAb, enhances the corresponding cytokine activity due to the formation of cytokine/anti-cytokine mAb complexes, which are more stable and stimulatory (32). Although we previously demonstrated that our anti-RANKL mAb used in vivo successfully worked as a blocking mAb in a model of collagen-induced arthritis (21), further studies will be needed to address this issue.

Although we could not detect a suppressive effect of neutralizing anti-RANKL mAb on the development of colitis in SCID mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone, we found that this treatment induced colitis in mice transferred with CD4<sup>+</sup> CD45RB<sup>high</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> TR cells at a ratio of 3:1, while mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and CD4<sup>+</sup> CD25<sup>+</sup> TR cells at the same ratio and given control IgG did not develop colitis. This strongly suggested that the target cells for anti-RANKL mAb are CD4<sup>+</sup>CD25<sup>+</sup> TR cells rather than CD4<sup>+</sup> CD45RB<sup>high</sup> T cells or the differentiated effector CD4<sup>+</sup> T cells. Consistent with this notion, we found that RANKL was expressed on CD4<sup>+</sup>CD25<sup>high</sup> TR cells, but not on CD4<sup>+</sup>CD25<sup>+</sup> cells (Fig. 1). In an in vitro coculture assay to further evaluate the role of RANKL on CD4<sup>+</sup>CD25<sup>+</sup> TR cells in modulating the TR activity of CD4<sup>+</sup>CD25<sup>high</sup> cells in vitro, however, the addition of anti-RANKL mAb or anti-RANK mAb produced no detectable reduction of TR<sub>T</sub> activity, suggesting that the direct interaction between RANKL-expressing CD4<sup>+</sup>CD25<sup>+</sup> TR cells or activated CD4<sup>+</sup> T cells and RANK-expressing APCs including DC is not essentially important for abolishing TR<sub>T</sub> activity at least in vitro. Since in vitro assays do not always reflect the TR<sub>T</sub> function in vivo, we next performed another adoptive transfer experiment using Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells and Ly5.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> TR cells to evaluate the possibility that blockade of the RANK/RANKL signaling pathway affects the recruitment and expansion of specific populations in our model. In this experiment, we found that the ratio of the Ly5.2-derived CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cell population per total CD4<sup>+</sup> T cells in anti-RANKL mAb-treated mice was significantly decreased in the LP, but not in the MLN, spleen, or liver, as compared with the ratio in control-IgG-treated mice. This finding suggests three points. First, it is possible that the migration of CD4<sup>+</sup>CD25<sup>+</sup> TR cells to the inflamed mucosa is regulated by the interaction between RANKL-expressing TR cells and possibly RANK-expressing endothelial cells. Consistent with this hypothesis, it has been reported that RANK is expressed on murine and human endothelial cells (24). However, this is unlikely because our short-term in vivo adoptive transfer experiment (Fig. 7) demonstrated that treatment of anti-RANKL mAb did not affect the recovered cell number of CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells in the inflamed mucosa. Second, it is possible that the in vivo expansion of RANKL-expressing CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells or colitogenic CD4<sup>+</sup> effector/memory T cells is modulated by RANK-expressing DC in the inflamed mucosa. Consistent with this hypothesis, we found that 1) the expression of RANK on colitic LP DC is significantly increased as compared with that on normal LP DC and 2) the ratio of CD4<sup>+</sup>Foxp3<sup>+</sup> TR<sub>T</sub> cells to total CD4<sup>+</sup> T cells after stimulation with colitic LP, but not MLN or splenic, CD11c<sup>+</sup> DC was significantly suppressed by the addition of anti-RANKL mAb. Thus, it is possible that the interaction of RANKL-expressing CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells and RANK-expressing activated DC in the inflamed mucosa plays an important role in the maintenance of TR<sub>T</sub> cells. Third, not only the first suppression of T cell priming in draining lymph nodes by CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells, but also the second line of suppression in the inflamed mucosa by these cells is critically involved in the intestinal homeostasis to suppress the development of colitis. Consistent with our finding, Green et al. (33) previously reported that the blockade of the RANK/RANKL pathway resulted in a decreased frequency of CD4<sup>+</sup>CD25<sup>+</sup> TR cells in the draining lymph nodes and pancreas in the NOD mouse (33). However, it remains unknown why the ratio of CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells in MLNs was unchanged in our adoptive transfer model. The exact mechanism of the function of CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</sub> cells in the inflamed mucosa warrants further investigation.

Finally, the suppressive site of colitis should be discussed. Denning et al. (34) previously demonstrated that integrin ββ<sub>1</sub>-deficient (β<sub>2</sub>−/−) CD4<sup>+</sup>CD25<sup>+</sup> TR<sub>T</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 integrin α<sub>i</sub>β<sub>i</sub> and α<sub>G</sub>β<sub>i</sub> molecules, are capable of preventing intestinal inflammation, suggesting that TR<sub>T</sub> accumulation in the intestine is dispensable for the protection of this colitis model. In their protection protocol, indeed, it is possible
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(high) 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 $\alpha_6\beta_7$. 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$^+$CD25bright and mouse CD4$^+$CD25$^+$ T cells reside in the intestinal LP, express CTLA-4, GITR, and Foxp3 and possess Treg activity in vitro (11, 12). We also found that the clinical score in SCID mice transferred with CD4$^+$CD45RB(high) T cells and intestinal LP CD4$^+$CD25$^+$ T cells at a ratio of 3:1 was significantly decreased as compared with that in SCID mice transferred with CD4$^+$CD45RB(high) T cells alone (12), indicating that the murine intestinal LP CD4$^+$CD25$^+$ T cells maintain intestinal homeostasis to suppress the development of colitis. Having evidence that the murine intestinal LP CD4$^+$CD25$^+$ T cells suppressed the development of colitis induced by the adoptive transfer of CD4$^+$CD45RB(high) T cells, 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 transferred with CD4$^+$CD45RB(high) 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 cotransferred with Ly5.2-derived CD4$^+$CD45RB(high) 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