Naturally arising CD4⁺CD25⁺ regulatory T cells suppress the expansion of colitogenic CD4⁺CD44^highCD62L⁻ effector memory T cells

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Kanai, T., K. Tanimoto, Y. Nemoto, R. Fujii, S. Makita, T. Totsuka, and M. Watanabe. Naturally arising CD4⁺CD25⁺ regulatory T cells suppress the expansion of colitogenic CD4⁺CD44^highCD62L⁻ effector-memory T cells. Am J Physiol Gastrointest Liver Physiol 290: G1051–G1058, 2006. First published December 22, 2005; doi:10.1152/ajpgi.00429.2005.—Naturally arising CD4⁺CD25⁺ regulatory T (Tᵩ) cells have been shown to prevent and cure murine T cell-mediated colitis. However, their exact mechanism of controlling colitogenic memory CD4⁺ T cells in vivo systems excluding the initial process of naive T cell activation (10, 12, 17), failed to proliferate or secret cytokines in response to polyclonal or antigen-specific stimulation in the in vitro system (13, 18). CD4⁺CD25⁺ Tᵩ cells suppress the proliferation of responder CD4⁺CD25⁻ T cells in a cell contact-dependent manner (13, 18). However, their effect in vivo appears to depend in some but not all systems on IL-10 and/or TGF-β expression (1, 14). An advantage of these Tᵩ cells is their ability to act as bystander suppressors and dampen the inflammation (19), which was recently followed by the demonstration that naturally arising CD4⁺CD25⁺ Tᵩ not only prevented the development of colitis induced by the adoptive transfer of CD4⁺CD45RB^high naive T cells into severe combined immunodeficient (SCID) mice (10) but also cured the established colitis (6, 8). Although these studies suggest that manipulation of CD4⁺CD25⁺ Tᵩ cells may be beneficial in the treatment of patients with IBD, it is still unclear whether the late administration of CD4⁺CD25⁺ Tᵩ cells (10 days–4.5 wk after transfer of CD4⁺CD45RB^high cells) suppresses the pathogenic effector memory T (Tₑₘ) cells or the residual CD4⁺CD45RB^high naive T cells in the gastrointestinal system.

In this study, we have attempted to clarify the exact nature of the suppressive activity of CD4⁺CD25⁺ Tᵩ cells against pathogenic/colitogenic memory CD4⁺ T cells using the colitogenic lamina propria (LP) CD4⁺ Tₑₘ cells at the early time point (48 h after culture). Collectively, these data suggest the distinct timing of the IL-2-dependent expansion of CD4⁺CD25⁺ Tᵩ cells and the their suppressive activity on colitogenic LP CD4⁺ Tₑₘ cells.

murine colitis model; interleukin-2

THE ULTIMATE GOAL OF ANY TREATMENT for autoimmune diseases, including inflammatory bowel diseases (IBDs), is antigen- and/or site (including regional lymph node)-specific suppression of pathology. Autoaggressive lymphocytes need to be eliminated or controlled to prevent tissue damage and halt the progression of clinical disease. Although the self-antigens and commensal bacterial flora responsible for the pathogenesis of IBDs is poorly understood to date, strong evidence is emerging that the induction of regulatory T (Tᵩ) cells can suppress disease, even if the primary initiating self-antigens and/or commensal bacterial flora are unknown and inflammation is progressive (6, 8, 15).

CD4⁺CD25⁺ T cells have been shown to be potent Tᵩ cells in a number of murine models as well as in rats and humans (12). Functional analysis of murine CD4⁺CD25⁺ Tᵩ cells has shown that those cells, which constitutively express inhibitory cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), and Foxp3 transcription factor (Forkhead box Foxp3 transcriptional isoform) (10, 12, 17), failed to proliferate or secret cytokines in response to polyclonal or antigen-specific stimulation in the in vitro system (13, 18). CD4⁺CD25⁺ Tᵩ cells suppress the proliferation of responder CD4⁺CD25⁻ T cells in a cell contact-dependent manner (13, 18). However, their effect in vivo appears to depend in some but not all systems on IL-10 and/or TGF-β expression (1, 14). An advantage of these Tᵩ cells is their ability to act as bystander suppressors and dampen the inflammation (19), which was recently followed by the demonstration that naturally arising CD4⁺CD25⁺ Tᵩ not only prevented the development of colitis induced by the adoptive transfer of CD4⁺CD45RB^high naive T cells into severe combined immunodeficient (SCID) mice (10) but also cured the established colitis (6, 8). Although these studies suggest that manipulation of CD4⁺CD25⁺ Tᵩ cells may be beneficial in the treatment of patients with IBD, it is still unclear whether the late administration of CD4⁺CD25⁺ Tᵩ cells (10 days–4.5 wk after transfer of CD4⁺CD45RB^high cells) suppresses the pathogenic effector memory T (Tₑₘ) cells or the residual CD4⁺CD45RB^high naive T cells in the gastrointestinal system.

In this study, we have attempted to clarify the exact nature of the suppressive activity of CD4⁺CD25⁺ Tᵩ cells against pathogenic/colitogenic memory CD4⁺ T cells using the colitogenic lamina propria (LP) CD4⁺ Tₑₘ cells at the early time point (48 h after culture). Collectively, these data suggest the distinct timing of the IL-2-dependent expansion of CD4⁺CD25⁺ Tᵩ cells and the their suppressive activity on colitogenic LP CD4⁺ Tₑₘ cells.

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MATERIALS AND METHODS

Mice. Female BALB/c, C.B-17 scid/scid (SCID), C57BL/6-Ly5.2, and C57BL/6 recombinase-activating gene (RAG)-2-knockout mice (RAG-2-KO; Ly5.2) were purchased from Japan Clear (Tokyo, Japan). C57BL/6-Ly5.1 mice were obtained from The Jackson laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used at 6–12 wk of age according to the guidelines of the Institutional Committee on Animal Research in Tokyo Medical and Dental University and were approved by the committee.

Antibodies. The following MAbs were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: Fc-γ (CD16/CD32)-blocking MAb (2.4G2); FITC-, phenoylephrine (PE), and CyChrome-conjugated anti-mouse CD4 (RM4-5); FITC- and PE-conjugated anti-mouse CD25 (7D4); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); purified anti-murine CD3e (145-2C11); purified anti-murine CD28 (37.51); purified anti-murine IL-2 (JES6-5H4); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG.

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Induction of colitis induced by the adoptive transfer of CD4^+CD45RB^{high} T cells. Colitis was induced in SCID mice by adoptive transfer of CD4^+CD45RB^{high} T cells as described previously (9). Briefly, CD4^+ T cells were isolated from splenocytes from BALB/c mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched CD4^+ T cells were labeled with PE-conjugated anti-mouse CD4 MAb and FITC-conjugated anti-CD45RB MAb and sorted into CD45RB^{high} (highest staining 30%) and CD45RB^{low} (lowest staining 30%) fractions on a FACS Vantage (Becton Dickinson, Sunnyvale, CA). Each SCID mouse underwent intraperitoneal injection with 3 x 10^5 CD4^+CD45RB^{high} T cells. The colitic CD4^+CD45RB^{high} T-cell-transferred SCID mice were killed 6–8 wk after transfer to induce the colitogenic LP CD4^+ T cells (20). The entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+} - and Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington Biochemical) for 2 h. The cells were pelleted twice through a 40% isotonic Percoll gradient solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%-75%). Enriched CD4^+ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells analyzed using FACS Calibur contained >96% CD4^+ cells.

Flow cytometry. Flow cytometric two-color analysis was performed as described previously (4). Isolated LP mononuclear cells (LPMCs) from colitic CD4^+CD45RB^{high} cell-transferred SCID mice or age-matched normal BALB/c mice were preincubated with Fe^{3+} receptor-blocking MAb for 20 min, followed by incubation with CyChrome-conjugated anti-mouse CD4 MAb and PE-conjugated anti-CD45RB, anti-CD44, anti-CD62L, or anti-CD69 MAb for 30 min on ice. After cells were stained, flow cytometry and data analysis were performed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA).

In vitro proliferation assay. As antigen-presenting cells (APCs), CD4^-cells were prepared from BALB/c splenocytes by depleting CD4^+ cells with anti-CD4 MACS beads and treated with 50 μg/ml mitomycin C (MMC) for 45 min at 37°C. To obtain splenic CD4^+CD25^+ T cells, enriched CD4^- splenocytes were stained with PE-conjugated anti-mouse CD4 MAb and FITC-conjugated anti-CD4 MAb and sorted into CD4^+CD25^+ and CD4^+CD25^- subpopulations on a FACS Vantage. In coculture experiments, splenic CD4^+CD25^- or CD4^+CD25^- T cells (0, 1, 0, 2, or 4 x 10^5 as T or control non-T cells) were cultured with splenic CD4^+CD25^- T cells (1 x 10^5 as control responders) or colitogenic memory CD4^+ T cells (1 x 10^5 as responders) and MMC-treated CD4^- cells (1 x 10^5 as APCs) in round-bottomed 96-well plates in RPMI 1640 medium containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (Complete medium) supplemented with soluble anti-CD3 MAb (145-2C11, 50 ng/ml colitogenic memory LP CD4^+ cells, 5 μg/ml CD4^+CD25^- cells; BD Pharmingen). In some experiments, blocking MAb against IL-2 was added at 0, 2, or 10 μg/ml from the beginning of culture. To determine proliferation, each well was pulsed with 1.0 μCi of [3H]thymidine (NEN, Boston, MA) for the last 24-96 h culture. [3H]thymidine incorporation in [3H]thymidine at proliferation assays were collected and analyzed by a specific ELISA (R&D Systems, Minneapolis, MN) or mouse Th1/Th2 cytokine CBA kit (IL-2, IL-4, IL-5, TNF-α, and IFN-γ; BD Biosciences, San Jose, CA) according to the manufacturer’s recommendations.

Statistical analysis. The results are expressed as means ± SD. Groups of data were compared using the Mann-Whitney U-test. Differences were considered statistically significant at P < 0.05. RESULTS

Interaction between CD4^+CD25^- T cells and colitogenic TEM cells. Efforts to delineate TR cell population have revealed that CD4^+CD25^- T cell populations in mice and humans retain TR function (12). However, the mechanism by which naturally arising CD4^+CD25^- TR cells control pathogenic CD4^+ TEM cells in autoimmune diseases is not fully understood. To clarify this mechanism, we assessed the in vivo TR activity of CD4^+CD25^- T cells from normal BALB/c spleen against the isolated colitogenic memory CD4^+ TEM cells. To study CD4^+ TEM cells, we first isolated LP CD4^+ T cells of Th1-mediated colitic C.B-17 SCID mice by in vivo uptake of FCSE~CD4~ T cells (9).
As shown in Fig. 1, flow cytometric analysis revealed that the colitic LP CD4+ TEM cells were CD44highCD69lowCD62L−, indicating that they were activated TEM cells. In contrast, normal splenic CD4+ T cells express CD44high−CD69−CD62L+.

We next sought to determine whether splenic CD4+CD25+ T<sub>R</sub> cells suppress the proliferation of colitic LP CD4+ TEM cells in vitro. In a 48-h coculture assay, CD4+CD25+ cells significantly suppressed the proliferation of normal splenic CD4+CD25− responders at 0.5-to-4.0 T<sub>R</sub>-to-responder ratios, but they did not suppress that of colitic LP CD4+ TEM cells at any ratio (Fig. 2A). Interestingly, [3H]thymidine uptake of coculture with CD4+CD25+ T cells and the colitic LP CD4+ TEM cells was conversely increased in parallel with the increased numbers of splenic CD4+CD25+ T<sub>R</sub> cells. In the late assay performed 96 h after culture, however, CD4+CD25+ cells significantly suppressed both the proliferation of splenic CD4+CD25− cells at 0.5-to-4.0 responder-to-T<sub>R</sub> ratio and the colitic LP CD4+ cells at 1.0-to-4.0 T<sub>R</sub>-to-responder ratio (Fig. 2B).

To determine which type of cocultured cells proliferate and which type is suppressed, we next conducted a per-cell proliferation assay using CFSE labeling. After normal splenic C57/BL6J-Ly5.2 CD4+CD25+ T<sub>R</sub> cells and the colitic LP memory CD4+ TEM cells obtained from colitic C57/BL6J-Ly5.1 CD4+CD45R<sup>B</sup>high T cell-transferred mice were labeled with CFSE, one or two subpopulations were stimulated for 120 h with anti-CD3 MAb and unlabeled, MMC-treated C57/BL6J-Ly5.2 CD4− APCs. Colitic LP CD4+ TEM cells did proliferate in the absence of CD4−CD25+ T<sub>R</sub> cells (Fig. 3A, top). In contrast, CD4+CD25+ T<sub>R</sub> cells did not proliferate in the absence of colitic CD4+ TEM cells (Fig. 3A, middle). Importantly, CD4+CD25+ T<sub>R</sub> cells expanded in the presence of colitic CD4+ TEM cells, whereas the proliferation of colitic LP CD4+ T cells conversely was suppressed in the presence of CD4+CD25+ T<sub>R</sub> cells compared with colitic LP CD4+ TEM cells cultured without CD4+CD25+ cells (Fig. 3A, bottom). These data indicate that CD4+CD25+ T<sub>R</sub> cells not only suppressed the expansion of colitic LP CD4+ TEM cells but also expanded by themselves via factors produced by the colitic LP CD4+ T cells or through intercellular interaction between CD4+CD25+ T<sub>R</sub> cells and colitic LP CD4+ TEM cells.

IL-2 produced by colitic CD4+ TEM cells at early phase extensively promoted proliferation of CD4+CD25+ T<sub>R</sub> cells. We further attempted to investigate why CD4+CD25+ T<sub>R</sub> cells proliferated in the presence of the colitic LP CD4+ T cells. We measured IL-2 concentrations in culture supernatants at the different time points, because it is well known that 1) CD4+CD25+ T<sub>R</sub> cells themselves are anergic to in vitro T cell receptor (TCR) stimulation, 2) the anergic state of CD4+CD25+ T<sub>R</sub> cells by in vitro TCR stimulation is broken by exogenously added IL-2, and 3) abrogation of the anergic state in the presence of IL-2 results in simultaneous loss of T<sub>R</sub> suppressive activity (12). As shown in Fig. 3B, IL-2 concentrations in supernatants cultured with colitic LP CD4+ TEM cells alone, normal splenic CD4+CD25− T cells alone, or colitic LP CD4+ TEM cells with CD4+CD25− T<sub>R</sub> cells in the same culture conditions shown in Fig. 2 were high at 48 h after culture but gradually decreased at 72 h and significantly decreased at 96 h, respectively. In contrast, IL-2 concentrations in supernatants cultured with normal splenic CD4+CD25− T cells with CD4+CD25+ T<sub>R</sub> cells were undetectable at any time point (48, 72, or 96 h after coculture), indicating that CD4+CD25+ T<sub>R</sub> cells suppressed both the proliferation (Fig. 2A) and the expansion of CD4+CD25− T cells, which might be caused by endogenous IL-2 production. These findings confirm that per-cell proliferation of CD4+CD25− T cells depends on the relative abundance of T<sub>R</sub> cells and IL-2 concentrations in the coculture assay.
CD4+CD25+ T cells inhibit development of TEM cell-mediated colitis. To investigate the suppressor activity of splenic CD4+CD25+ TR cells in a TEM cell-mediated chronic colitis model, we first transferred CD4+ TEM cells obtained from various tissues of colitic CD4+CD45RBhigh T cell-transferred SCID mice into new SCID mice (Fig. 4A). SCID mice that underwent transfer of colitic spleen (SP), mesenteric lymph node (MLN), or LP CD4+ T cells developed severe wasting diseases (Fig. 4, B and C) 5 wk after transfer, in contrast to normal SCID mice that were administered PBS. Histological examination showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells in the LP of the colon in SCID mice that had undergone transfer with colitic SP, MLN, or LP CD4+ T cells, but not in SCID mice that did not undergo cell transfer (Fig. 4D). This difference was confirmed by histological scoring of multiple colon sections (Fig. 4E). A further quantitative evaluation of CD4+ T cell infiltration was performed by isolating LPL from resected bowels. Only a few CD4+ T cells were recovered from colonic tissue of SCID mice that had not undergone transfer compared with SCID mice that had undergone transfer with colitic SP, MLN, or LP CD4+ T cells (Fig. 4F).

We next assessed whether naturally arising CD4+CD25+ TR cells suppress the development of colitogenic LP CD4+ TEM cell-mediated colitis (Fig. 5A). Because the mice that underwent transfer with colitogenic LP CD4+ TEM cells developed rapid wasting disease and their percentage decrease in original body weight reached 10% (Fig. 5B), the mice were killed 4 wk after undergoing transfer. In contrast, mice that were cojected with CD4+CD25+ TR cells appeared to remain healthy, with gradual increases in body weight and without diarrhea during the entire observation period (Fig. 5B). Four weeks after being transferred, the colons from mice that had undergone transfer with colitogenic LP CD4+ TEM cells alone, but not with both LP CD4+ TEM cells and CD4+CD25+ TR cells, were enlarged and had greatly thickened walls (Fig. 5C). Histological examination showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells in LP of the colon obtained from mice that had undergone transfer with LP CD4+ TEM cells alone (Fig. 5D). In contrast, glandular elongation was mostly abrogated and only a few mononuclear cells were observed in LP of the colon from mice that had undergone transfer with both LP CD4+ TEM cells and CD4+CD25+ TR cells (Fig. 5D). This finding was confirmed on the basis of histological scores (Fig. 5E). As shown in Fig. 5F, the cotransfer of CD4+CD25+ TR and LP CD4+ TEM cells significantly inhibited the expansion and infiltration of LP CD4+ TEM cells compared with those of LP CD4+ TEM cells alone.

We also examined the cytokine production by CD4+ LPL in mice that underwent transfer with LP CD4+ TEM cells or with both LP memory CD4+ TEM cells and CD4+CD25+ TR cells. As shown in Fig. 5G, LP CD4+ T cells from mice that underwent transfer with both LP CD4+ TEM cells and CD4+CD25+ TR cells produced significantly less IFN-γ, IL-2, and TNF-α upon in vitro anti-CD3/CD28 MAb stimulation compared with production of these cytokines in mice that underwent transfer with LP CD4+ TEM cells alone. In contrast, the production of IL-4 or IL-10 was not significantly affected (Fig. 5G). These results suggest that naturally arising CD4+CD25+ TR cells prevented the development of colitis
primarily by inhibiting the expansion and infiltration of colitogenic LP CD4+ T<sub>EM</sub> cells in the colon and secondarily by inhibiting the development of pathogenic Th1 cells producing IFN-γ, IL-2, and TNF-α.

**DISCUSSION**

In the present study, we have demonstrated that naturally arising CD4+CD25<sup>+</sup> T<sub>REG</sub> cells suppressed the development of colitogenic CD4+ T<sub>EM</sub> cell-mediated chronic colitis. Furthermore, in vitro suppression assay revealed that CD4+CD25<sup>+</sup> T<sub>REG</sub> cells proliferated extensively in response to a high dose of IL-2 produced by colitogenic LP CD4+ counterpart T<sub>EM</sub> cells at the early time point (48 h after coculture) but suppressed the proliferation of colitogenic memory CD4+ T<sub>EM</sub> cells at the late time point (96 h) in accordance with the decreased production of IL-2. These results may indicate the exact strategy of CD4+CD25<sup>+</sup> T<sub>REG</sub> cells against CD4+ T<sub>EM</sub> cells in autoimmune disorders.

IL-2 is required for in vivo and in vitro activation of CD4+CD25<sup>+</sup> T<sub>REG</sub> cells and to sustain their CD25 expression (3). Because CD4+CD25<sup>+</sup> T<sub>REG</sub> cells do not produce IL-2 (12, 18) (Fig. 3B), their proliferation seems to depend on IL-2 produced by their target T cells or activated dendritic cells. Until recently, CD4+CD25<sup>+</sup> T<sub>REG</sub> cells were described as refractory to stimulation through the TCR and as nonproliferative in in vitro culture (13). In addition, under circumstances in which T<sub>REG</sub> cells were made to proliferate in vitro by the addition of exogenous IL-2, they lost their suppressive capacity (12). How

![Fig. 4. Severe combined immunodeficient (SCID) mice that underwent transfer of SP, mesenteric lymph node (MLN), and LP CD4+ T cells obtained from mice that had undergone classic CD4+-CD45RB<sup>hi</sup> colitic cell transfer developed colitis. A: C.B-17 SCID mice underwent intraperitoneal injection with normal splenic CD4+CD45RB<sup>hi</sup> T cells. Six weeks after transfer, CD4+ T cells were isolated from SP, MLN, and LP and were injected into new SCID mice (1.5 × 10<sup>7</sup> cells/mouse). As a negative control, PBS without cells was injected into SCID mice. n = 5 mice in each group. All mice were killed 5 wk after undergoing transfer. B: mice that underwent transfer with the colitic SP, MLN, and LP CD4+ T cells did not gain weight. *P < 0.05. C: mice that underwent transfer with colitic SP, MLN, and LP CD4+ T cells showed signs of severe clinical colitis. *P < 0.05. Data are means ± SD of 5 mice in each group. *P < 0.05. D: histopathological comparison of distal colon from the mice injected with the colitic SP, MLN, or LP CD4+ T cells, or PBS. Original magnification, ×10. E: histograms were obtained to calculate histological scores determined 5 wk after mice underwent transfer as described in MATERIALS AND METHODS. Data are means ± SD from 4 mice in each group. *P < 0.05. F: LP CD4+ T cells were isolated from SCID mice that underwent injection with colitic SP, MLN, or LP CD4+ T cells or with PBS 5 wk after transfer, and the number of LP CD4+ cells was determined using flow cytometry. Data are means ± SD of 4 mice in each group. *P < 0.05.
is IL-2 involved in suppressing IL-2-producing responder T cells by CD4\(^+\)CD25\(^-\) TR cells that cannot produce IL-2 in the intestine under inflammatory conditions?

At the early time point (48 h after in vitro coculture), CD4\(^+\)CD25\(^+\) TR cells proliferated vigorously in response to a high amount of IL-2 produced by the colitogenic LP CD4\(^+\) TEM cells. Indeed, anergy-breaking CD4\(^+\)CD25\(^+\) TR cells cannot suppress colitogenic LP CD4\(^+\) TEM cells directly by intercellular interaction at this stage; however, CD4\(^-\)CD25\(^+\) TR cells may compete for IL-2 with LP CD4\(^+\) TEM cells. The constitutive expression of all three chains of the high-affinity IL-2 receptor might be enabling CD4\(^+\)CD25\(^+\) TR cells to take up IL-2 preferentially rather than LP CD4\(^+\) TEM cells, which rarely express high levels of IL-2R\(\alpha\) (CD25). Consistent with this hypothesis, it has been demonstrated in vitro and in vivo that competition for and/or consumption of IL-2 indeed occurs (5). During coculture of CD4\(^+\)CD25\(^-\) TR cells with responder cells, CD4\(^+\)CD25\(^+\) TR cells upregulate CD25, whereas induction of CD25 expression in responder T cells is conversely suppressed (5). Furthermore, upregulation of CD25 on CD4\(^+\)CD25\(^+\) TR cells is inhibited by the addition of anti-IL-2 antibody, whereas the addition of IL-2 conversely restores CD25 expression in responder T cells, demonstrating that the differential expression of CD25 is regulated by IL-2 (2). Thus competition for and/or consumption of IL-2 by CD4\(^+\)CD25\(^+\) TR cells may be boosted by the positive feedback loop of IL-2 uptake and CD25 upregulation. The same inverse regulation of CD25 expression on CD4\(^+\)CD25\(^+\) TR cells vs. responder T cells has been observed in vivo upon adoptive cotransfer of TR cells and responder cells (5). Although CD4\(^+\)CD25\(^+\) TR cells do not suppress the proliferation of the LP CD4\(^+\) TEM cells directly, IL-2 consumption by
CD4+CD25+ T<sub>R</sub> cells might lead to an increased ratio of T<sub>R</sub> to responder cells and inactivation of LP CD4<sup>+</sup> TEM cells because of the lack of IL-2 followed by decreased IL-2 production by LP CD4<sup>+</sup> TEM cells.

In contrast, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells might gain original suppressive activity to responder T cells in accordance with the decreased IL-2 concentrations at the late stage. In fact, [3H]thymidine uptake was significantly suppressed in a culture with both CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells and colitogenic LP CD4<sup>+</sup> TEM cells at 96 h but not at 48 h after culture. This suppression might depend on intercellular contact but would also require IL-2 competition and/or consumption. Altogether, we hypothesize that the suppression of IL-2-producing LP CD4<sup>+</sup> TEM cells by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells occurs via the following two steps: 1) IL-2 consumption followed by the expansion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells and the adjustment of the T<sub>R</sub>-to-responder ratio and 2) regained suppressive activity via intercellular contact.

Most recently, two groups of researchers have demonstrated that naturally arising CD4<sup>+</sup>CD25<sup>+</sup> TR cells cured the established colitis induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (6, 8). Although these reports suggest that manipulation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells may be beneficial in view of clinical checkpoints for the treatment of IBDs, whether delayed administration of CD4<sup>+</sup>CD25<sup>+</sup> TR cells suppress pathogenic CD4<sup>+</sup> TEM cells or residual CD4<sup>+</sup>CD45RB<sup>high</sup> naive T cells in their systems because of relatively early administration of CD4<sup>+</sup>CD25<sup>+</sup> T cells (10 days–4.5 wk after transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> cells) is still unclear. Importantly, using TEM-cell adoptive transfer experiments, we have demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells suppress the expansion of pathogenic CD4<sup>+</sup> TEM cells as well as the development of chronic colitis without having any impact on naive T cells.

However, the question arises whether CD4<sup>+</sup>CD25<sup>+</sup> TR cells suppress the pathogenic CD4<sup>+</sup> TEM cells in vivo. Because approximately two-thirds of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells express CD62L (12), which is essential for homing to lymph nodes, these CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells appear to function in lymph nodes, mesenteric lymph nodes in this case, to suppress the activation and proliferation of naive T cells. However, it is also known that not one-third of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells obtained from normal spleen do not express CD62L (19) but also that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells can express integrin α<sub>L</sub>β<sub>2</sub> (16), which is a homing receptor for gut. Mottet et al. (8) recently demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells acted in the intestine to regulate the effector function or the expansion of pathogenic TEM cells as well as acting in the secondary lymph nodes in a murine colitis model induced by the adoptive transfer of normal CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into SCID mice. In fact, our group (7) has identified CD4<sup>+</sup>CD25<sup>+</sup> bright T cells with a regulatory phenotype in human colon obtained from normal individuals and from patients with IBD. Furthermore, LP CD4<sup>+</sup> TEM cells have a characteristic of the TEM phenotype and tend to recirculate through nonlymphoid tissues (11). Collectively, it is possible that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells act in the gut in this setting. However, we have demonstrated that the transfer of colitic SP and MLN CD4<sup>+</sup> T cells obtained from mice that underwent CD4<sup>+</sup>CD45RB<sup>high</sup> T cell transfer into new SCID mice induced colitis as well as colitic LP CD4<sup>+</sup> T cells. The evidence suggests that the SP and the MLN may play an important role as a reservoir for colitogenic CD4<sup>+</sup> TEM cells that can recirculate into the gut. Thus it might be possible that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells prevent the recruitment of recirculating colitogenic CD4<sup>+</sup> TEM cells in the SP and the MLN. Further studies are needed to assess this issue using splenectomized lymph node-null mice to exclude the role of lymph nodes.

In summary, we have demonstrated herein that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells suppress the expansion of pathogenic CD4<sup>+</sup> TEM cells as well as the development of chronic colitis without any impact on naive T cell activation on the basis of TEM cell adoptive transfer experiments. Although many critical checkpoints remain to be passed, this study indicates that cell therapy using T<sub>R</sub> cells as living immunosuppressants offers hope for the treatment of patients with autoimmune diseases such as IBDs.

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