Increased Interleukin-10 Expression by the Inhibition of \( \text{Ca}^{2+} \)-Activated \( \text{K}^+ \) Channel \( \text{K}_{\text{Ca}3.1} \) in CD4\(^+\)CD25\(^+\) Regulatory T Cells in the Recovery Phase in an Inflammatory Bowel Disease Mouse Model

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ABSTRACT

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract arising from abnormal responses of the innate and adaptive immune systems. Interleukin (IL)-10–producing CD4\(^+\)CD25\(^+\) regulatory T (Treg) cells play a protective role in the recovery phase of IBD. In the present study, the effects of the administration of the selective \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channel \( \text{K}_{\text{Ca}3.1} \) inhibitor TRAM-34 on disease activities were examined in chemically induced IBD model mice. IBD disease severity, as assessed by diarrhea, visible fecal blood, inflammation, and crypt damage in the colon, was significantly lower in mice administered 1 mg/kg TRAM-34 than in vehicle-administered mice. Quantitative real-time polymerase chain reaction examinations showed that IL-10 expression levels in the recovery phase were markedly increased by the inhibition of \( \text{K}_{\text{Ca}3.1} \) in mesenteric lymph node (mLN) Treg cells of IBD model mice compared with vehicle-administered mice. Among several positive and negative transcriptional regulators (TRs) for IL-10, three positive TRs—E4BP4, KLF4, and Blimp1—were upregulated by the inhibition of \( \text{K}_{\text{Ca}3.1} \) in the mLN Treg cells of IBD model mice. In mouse peripheral CD4\(^+\)CD25\(^+\) Treg cells induced by lectin stimulation, IL-10 expression and secretion were enhanced by the treatment with TRAM-34, together with the upregulation of E4BP4, KLF4, and Blimp1. Collectively, the present results demonstrated that the pharmacological inhibition of \( \text{K}_{\text{Ca}3.1} \) decreased IBD symptoms in the IBD model by increasing IL-10 production in peripheral Treg cells and that IL-10\(^{\text{high}} \) Treg cells produced by the treatment with \( \text{K}_{\text{Ca}3.1} \) inhibitor may contribute to efficient Treg therapy for chronic inflammatory disorders, including IBD.

SIGNIFICANCE STATEMENT

Pharmacological inhibition of \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channel \( \text{K}_{\text{Ca}3.1} \) increased IL-10 expression in peripheral Treg cells, together with the upregulation of the transcriptional regulators of IL-10: Krüppel-like factor 4, E4 promoter-binding protein 4, and/or B lymphocyte–induced maturation protein 1. The manipulation of IL-10\(^{\text{high}} \)–producing Treg cells by the pharmacological inhibition of \( \text{K}_{\text{Ca}3.1} \) may be beneficial in the treatment of chronic inflammatory diseases such as inflammatory bowel disease.
In the recovery phase, a reduction in disease activity is accompanied by the increased production of anti-inflammatory mediators such as interleukin (IL-10).

IL-10 is the most important anti-inflammatory cytokine in the immune system, and it is produced by lymphocytes, macrophages, mast cells, and dendritic cells (Ng et al., 2013). Therefore, IL-10 is protective against a number of autoimmune responses, such as rheumatoid arthritis. IL-10–secreting CD4+CD25+ regulatory T (Treg) cells in mesenteric lymph nodes (mLN) and the lamina propria are essential for the maintenance of intestinal homeostasis and exert protective effects during the disease process of IBD. Low levels of IL-10 increase disease severity in chronic IBD, and IL-10–deficient mice exhibit severe IBD symptoms (Kennedy et al., 2000; Shah et al., 2012). IL-10 levels have been shown to markedly increase in the recovery phase of the DSS-induced IBD model but not in the induction phase, together with the expression levels of the Treg characteristic markers CD25 and Foxp3 (Bento et al., 2012). Therefore, IL-10 supplementation and Treg therapy are therapeutic regimens for the prevention of autoimmunity, including IBD. Many IL-10 transcriptional regulators (TRs) with/without the transactivation of promoters have been identified in different cell types, including T cells (Rutz and Ouyang, 2016), and some are activated by the JAK/STAT3, MAPK/ERK, and CN-NFAT signaling pathways (Martin et al., 2003; Bouhamdan et al., 2015). A member of the legume lectin family, concanavalin-A (Con-A), is a well-known T-cell mitogen that promotes differentiation into autoimmune responses, such as rheumatoid arthritis. IL-10 is protective against a number of TRs, and some are activated by the JAK/STAT3, PI3K/AKT, MAPK/ERK, and CN-NFAT signaling pathways (Martin et al., 2003; Bouhamdan et al., 2015). A member of the legume lectin family, concanavalin-A (Con-A), is a well-known T-cell mitogen that promotes differentiation into autoimmune responses, such as rheumatoid arthritis.

Histologic Scoring. The damaged portion of the colon in IBD model mice was confirmed by Alcian staining as previously reported (Ohya et al., 2014). Regarding histologic assessments, a 1-cm tissue sample of the range from colon was fixed in 10% buffered formalin, embedded in a paraffin block, cut into 5-μm-thick sections, and stained with hematoxylin and eosin. Inflammation scores were determined as the multiplication of the grade of inflammation severity (grade 0–3) and its extent (grade 0–3) (Dieleman et al., 1998; Ohya et al., 2014). Data were obtained from three sections of the colon measured at least 200 μm apart per animal.

Isolation of CD4+CD25+ Treg Cell Subsets. CD4+CD25+ Treg cell subsets were isolated from mice mLN and spleen cell suspensions by Dynabeads FlowComp Mouse CD4+CD25+ Treg Cells according to the experimental protocol supplied by Thermo Fisher Scientific (Waltham, MA) (Nakakura et al., 2015). A flow cytometric analysis (FACSCanto II flow cytometers (BD Biosciences, San Jose, CA)) using antibodies labeled with different fluorophores (fluoresceinisothiocyanate–FITC, phycoerythrin–PE, allophycocyanin–APC, and peridinin chlorophyll protein–PerCP) was used for identifying cell subsets (CD4+CD25+). The CD4 and phycoerythrin-CD25 confirmed that 90% of purified T cells were CD4+CD25+.

Real-Time PCR. Total RNA extraction and cDNA synthesis from CD4+CD25+ T cells were performed as previously reported (Matsui et al., 2018). The resulting cDNA products were amplified with gene-specific primers, which were designed using Primer Express software (version 1.5; Applied Biosystems, Foster city, CA). Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa BIO, Osaka, Japan) on ABI 7500 real-time PCR instruments (Applied Biosystems) (Matsui et al., 2018). The following PCR primers were used: KCa3.1 (GenBank accession number: NM_008433, 343–452, 110 bp; IL-10 (NM_010548, 245–355), 111 bp; IL-17A (NM_010552, 165–277), 113 bp; IL-17F (NM_141856, 155–304), 120 bp; IFN-γ (NM_008337, 222–323), 102 bp; CD25 (NM_008367, 522–642), 121 bp; Foxp3 (NM_001199347, 823–942), 120 bp; nuclearide diphosphate kinase (NDPK-B (NM_008705, 467–597), 131 bp; PsK-C2B (NM_001099276, 2293–2422), 130 bp; phosphohistidine phosphatase (PHTP)-1 (NM_029293, 69–189), 121 bp; myotubularin-related protein (TMR)-5 (NM_144843, 722–832), 111 bp; HDAC2 (NM_008229, 1434–1554), 111 bp; HIF-1α (AF163668, 1309–1419), 111 bp; and β-actin (ACTB) (NM_031142, 419–519), 101 bp. Unknown quantities relative to the standard curve for a particular set of primers were calculated using the relative quantification of gene products relative to ACTB.

Materials and Methods

DSS-Induced Mouse IBD Model. Female C57BL/6J (7 to 8 weeks of age) mice were obtained from Japan SLC (Shizuoka, Japan). They were given distilled water containing 2.5% (w/v) DSS (molecular weight 36–50 kDa) (MP Biomedicals, Santa Ana, CA) ad libitum for 7 days followed by 5 days of normal drinking water (Shi et al., 2013) (Fig. 1A). Control mice were given drinking water only. The clinical assessment of inflammation included the daily monitoring of weight loss. Mice were euthanized 12 days later, tissue samples were collected, and colitis and inflammation were macroscopically assessed according to the experimental protocol supplied by Thermo Fisher Scientific (Waltham, MA) (Nakakura et al., 2015). A flow cytometric analysis (FACSCanto II flow cytometers (BD Biosciences, San Jose, CA)) using antibodies labeled with different fluorophores (fluoresceinisothiocyanate–FITC, phycoerythrin–PE, allophycocyanin–APC, and peridinin chlorophyll protein–PerCP) was used for identifying cell subsets (CD4+CD25+). The CD4 and phycoerythrin-CD25 confirmed that 90% of purified T cells were CD4+CD25+.

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Preparation of CD4+CD25+ Treg Cells Induced In Vitro by Concanavalin-A Stimulation. Cell suspensions were prepared by pressing the spleens of female C57BL/6J (8 to 9 weeks of age) mice with frosted slide glasses in RPMI 1640 medium (FUJIFILM Wako Pure Chemicals, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO) and antibiotics (penicillin and streptomycin; FUJIFILM Wako Pure Chemicals). Splenocytes were prepared by red blood cell lysis. Isolated splenocytes were cultivated in RPMI 1640 medium supplemented with Con-A (5 μg/ml) and IL-2 (10 U/ml) for 48 hours. Various concentrations of TRAM-34 (0, 1, and 10 μM) were added. At 24 hours later, the supernatant and cells were recovered for ELISA, real-time PCR, and Western blot analyses.

Measurement of IL-10 and IL-17A Production by ELISA. Mouse IL-10 and IL-17A levels in culture supernatant samples were measured with IL-10 and IL-17A Mouse ELISA kits, respectively (Thermo Fisher Scientific), according to the experimental protocol.

Western Blotting. Protein lysates were prepared from mouse splenic CD4+CD25+ T cells using radioimmunoprecipitation assay (RIPA) lysis buffer. After the protein quantification using the DC protein assay (Bio-Rad Laboratories, Hercules, CA), protein lysates were subjected to SDS-PAGE (10%). Blots were incubated with anti–phospho-Smad2 (Ser465/467) (P-Smad2), anti–phospho-Smad3 (Ser423/425) (P-Smad3) (Cell Signaling Technology Japan, Tokyo, Japan), anti–phospho-AKT (Ser473) (P-AKT) (BioLegend, San Diego, CA), anti–phospho-STAT3 (Tyr705) (P-STAT3) (BioLegend), anti–phospho-ERK1 (Thr202/Tyr204)/ERK2 (Thr185/Tyr187) (P-ERK1/2) (R&D Systems, Minneapolis, MN), and anti–ACTB (Medical & Biologic Laboratories, Nagoya, Japan) antibodies. After staining with anti-rabbit horseradish peroxidase–conjugated IgG (Merck, Darmstadt, Germany), an enhanced chemiluminescence detection system (Nacalai Tesque, Kyoto, Japan) was used to detect the bound antibody. The images were visualized and analyzed using Amersham Imager 600 (GE Healthcare Japan, Tokyo, Japan). The light intensities of band signals were digitalized using ImageJ software (version 1.42; National Institutes of Health, Bethesda, MD). Relative protein expression levels in the control were expressed as 1.0.

Cellular Distribution of P-Smad2 and P-Smad3. Mouse splenic CD4+CD25+ T cells were fixed and permeabilized, and then cells were stained by anti–P-Smad2 and anti–P-Smad3 antibodies labeled with an Alexa Fluor 488–conjugated secondary antibody (Abcam, Cambridge, UK) and 4',6-diamidino-2-phenylindole for nucleic acid staining (Matsui et al., 2019). Fluorescence images were visualized using a confocal laser scanning microscope system (A1R; Nikon, Tokyo, Japan).

Chemicals. TRAM-34 was purchased from Santa Cruz Biotechnology (Dallas, TX); KN-62 was from Medchemexpress (Monmouth Junction, NJ); ciclosporin A was from FUJIFILM Wako Pure Chemicals; 5,15-diphenylporphyrin was from Abcam (Cambridge, UK); and AZD5363, everolimus, LY364947, and SCH772984 were from Cayman.
Chemical (Ann Arbor, MI). All other chemicals used in the present study were from Sigma-Aldrich, FUJIFILM Wako Pure Chemicals, or Nacalai Tesque unless otherwise stated.

**Statistical Analysis.** Statistical analyses were performed with the statistical software XLSTAT. To assess the significance of differences between two groups and among multiple groups, the unpaired/paired Student’s t test with Welch’s correction or Tukey’s test was used. Data that were not normally distributed were analyzed using Mann-Whitney’s U test. Results with a P value of less than 0.05 or 0.01 were considered to be significant. Data are presented as means ± S.E.M.

**Results**

**Effects of the Subcutaneous Administration of the KCa3.1 Blocker TRAM-34 on IBD Symptoms in DSS-Induced IBD Model Mice.** In the IBD model, loose and bloody feces were observed 3 days after exposure to 2.5% DSS in drinking water. A previous study reported that the plasma concentration of TRAM-34 was more than 10-fold higher than the in vitro IC50, even 4 days after the single subcutaneous administration of 1 mg/kg TRAM-34 (Ohya et al., 2014). In the present study, to investigate the therapeutic effects of KCa3.1 inhibition, TRAM-34 was administered twice on days 5 and 9 (Fig. 1A). At 12 days after the exposure to 2.5% DSS for 7 days followed by 5 days of normal water, the effects of the repeated administration of TRAM-34 on macroscopic (body weight change, diarrhea and visible fecal blood, colon thickening) and microscopic (crypt damage and colonic inflammation) IBD symptoms were assessed as previously reported (Ohya et al., 2014; Nakakura et al., 2015). Figure 1B showed the changes in body weight during the induction and recovery phases in vehicle- and TRAM-34–administered (closed circles and open circles, respectively) IBD model mice (n = 9 for each). By drinking water for 5 days (from days 8 to 12), body weight loss was partially recovered. The recovery rate of body weight loss was relatively large in TRAM-34–administered mice compared with vehicle-administered mice; however, no significant differences were found between both groups (Fig. 1B). In vehicle-administered IBD model mice, the average scores of diarrhea and visible fecal blood on day 12 were 2.67 ± 0.17 and 0.89 ± 0.20 (n = 9 for each), respectively, and the fecal blood score was lower than that on day 7 (2.83 ± 0.17, n = 6). In IBD model mice, both scores were significantly reduced by the inhibition of KCa3.1: 1.89 ± 0.20 (P = 0.0092) and 0.11 ± 0.11 (P = 0.0054) (n = 9 for each), respectively (Fig. 1, C and D). The colon was thicker and the mice mLN and spleen were larger in vehicle-administered IBD model mice than in untreated mice, and they were not affected by the inhibition of KCa3.1 (Fig. 1, E–G) because of the decrease in these parameters on day 12 compared with those on day 7: 1.52 ± 0.12, 7.16 ± 0.56 mg/g and 7.92 ± 0.38 mg/mm (n = 8 for each) in mLN weight, spleen weight, and colon thickness. Colonic inflammation and crypt damage were assessed by histologic visualization (Fig. 2, A–C) and scoring (Fig. 2, D and E). In vehicle-administered IBD model mice, average scores were 15.33 ± 0.73 and 14.56 ± 0.67 (n = 9 for each), respectively. Both scores were significantly reduced by the inhibition of KCa3.1: 11.33 ± 1.01 (P = 0.0058) and 9.78 ± 0.78 (P = 0.0002) (n = 9 for each), respectively (Fig. 2, D and E). Preliminarily, we examined the effects of subcutaneous administration of 0.1 mg/kg TRAM-34 on the scores of diarrhea and visible fecal blood. No significant improvement in IBD symptoms was found after administration of 0.1 mg/kg TRAM-34. Unexpectedly, improvement of IBD symptoms was not observed after administration of 10 mg/kg TRAM-34. In 10 mg/kg TRAM-34–administered IBD model mice, the average scores of diarrhea and visible fecal blood were 2.78 ± 0.15 and 0.78 ± 0.15 (n = 9 for each), respectively, and no significant changes were found between vehicle- and 10 mg/kg TRAM-34–administered groups (P = 0.1732 and 0.3861, respectively) (Supplemental Fig. 1, A and B). Also, in 10 mg/kg TRAM-34–administered IBD model mouse, the average scores of colonic inflammation and crypt damage were 15.44 ± 1.02 and 15.11 ± 0.81 (n = 9 for each), respectively, and no significant changes were found between vehicle- and 10 mg/kg TRAM-34–administered groups (P = 0.2046 and 0.1229, respectively) (Supplemental Fig. 1, C and D). A recent study by Süss et al. (2020) suggested that KCa3.1 may have a protective role in epithelial barrier in IBD. Therefore, the administration of 10 mg/kg TRAM-34 may deteriorate IBD symptoms by dysregulating epithelial barrier function. In untreated mice, the administration of 1 mg/kg TRAM-34 on days 5 and 9 did not affect any IBD symptoms (body weight loss, colon thickening, diarrhea, and visible fecal blood) (n = 6). In mLN CD4+CD25+ T cells, the expression levels of proinflammatory cytokines (IFN-γ and IL-17F) were significantly increased in the induction phase; however, they are recovered to basal levels in the recovery phase, and no significant changes in them were found by the administration of 1 mg/kg TRAM-34 (Supplemental Fig. 2).

**Increased IL-10 Expression by TRAM-34 Administration in mLN CD4+CD25+ Treg Cells of IBD Model Mice.** Bento et al. (2012) showed that IL-10 expression was markedly increased in inflamed colon segments in the recovery phase in the DSS-induced IBD model (“recovery phase”) but not in the inflammation induction phase (“induction phase”). Our preliminary experiments also showed the upregulation of IL-10 and Treg cell markers (CD25 and Foxp3) in mLN at the end of the recovery phase (day 12) and no changes in the induction phase (day 7) (Supplemental Fig. 3). A fluorescent dual color dot plot of fluorescein isothiocyanate-CD4 versus phycoerythrin-CD25 was analyzed in living mLN cells using flow cytometry. The percentage of the CD4+CD25− subset within the whole CD4+ T-cell population in the mLN was significantly higher in IBD model mice than in untreated mice (n = 4 for each, P = 0.0046) (Supplemental Fig. 4A); however, no significant changes were observed in the mLN of TRAM-34–administered IBD model mice (Supplemental Fig. 4B). Furthermore, no significant differences were observed in the expression levels of CD25 transcripts in mLN Treg cells between vehicle- and TRAM-34–administered IBD model mice (Supplemental Fig. 4C).

No significant changes were observed in the expression levels of IL-10 transcripts in mLN CD4+CD25+ Treg cells in the induction phase in IBD model mice (n = 4 for each) (Fig. 3A); however, significant increases in the expression levels of IL-10 transcripts were noted in the recovery phase (n = 4 for each, P = 0.0006) (Fig. 3B). An approximately 5-fold increase in the expression levels of IL-10 transcripts was observed in these mice after the administration of TRAM-34: 0.49 ± 0.006 and 0.254 ± 0.052 (in arbitrary units) in the vehicle- and TRAM-34–administered groups, respectively (n = 5 for each, P = 0.006) (Fig. 3C).
We previously reported the increased expression of KCa3.1 in the CD4+CD25+ T cells of acute IBD model mice (Ohya et al., 2014). We investigated the expression levels of KCa3.1 and its regulatory molecules in mLN Treg cells during the recovery phase in IBD model mice. No significant changes were observed in the expression levels of KCa3.1 in Treg cells in the recovery phase between IBD model and untreated mice \((n=4\) for each\) (Supplemental Fig. 5A). Matsui et al. (2018) showed that the inflammation-associated upregulation of the histone deacetylases HDAC2 and HDAC3 enhanced KCa3.1 transcription in the inflammatory CD4+CD25+ T cells of acute IBD model mice. However, in the recovery phase in IBD model mice, no significant changes were found in the expression levels of their transcripts \((n=4\) for each\) (Supplemental Fig. 5, B and C). Additionally, no significant changes were noted in the expression levels of the positive and negative regulators of KCa3.1 (NDPK-B, PI3K-C2B, PHPT-1, and MTMR-6) in Treg cells in the recovery phase in IBD model mice (Supplemental Fig. 5, D–G). Hypoxia is involved in intestinal inflammation in patients with IBD (Westendorf et al., 2017), and the activation of HIF-1α has been shown to promote Treg activity (Colgan and Taylor, 2010). The inflammation-associated induction of HIF-1α expression was observed in Treg cells in IBD model mice \((n=4\) for each, \(P=0.0088\)) (Fig. 4D).

Molecular Identification of Possible TRs Contributing to the Upregulation of IL-10 by the Inhibition of KCa3.1 in mLN Treg Cells of IBD Model Mice. A number of positive and negative TRs of IL-10 have been detected in lymphoid and myeloid cell subsets, including Treg cells (Neumann et al., 2019). To identify the TRs potentially contributing to the KCa3.1 inhibition–induced upregulation of IL-10 in the mLN Treg cells of IBD model mice, the transcriptional expression levels of 13 candidates (Foxp3, cMAF, E4BP4, KLF4, TRIM33, RoRγt, BATF, GATA3, AGR2, Blimp1, IL-27, JunB, and HIF-1α) (Rutz and Ouyang, 2016; Neumann et al., 2019) were assessed by real-time PCR. As shown in Supplemental Fig. 6, A, C–E, no significant differences were observed in the expression levels of four candidates (Foxp3, RoRγt, BATF, and GATA3) between the Treg cells of the vehicle- and TRAM-34–administered groups \((n=5\) for each), and AGR2, IL-27, and JunB were rarely expressed (under 0.002 in arbitrary units) in both groups. Consistent with the upregulation of IL-10, the expression of the three positive TRs, E4BP4, KLF4, and Blimp1, was increased by the inhibition of KCa3.1 in the mLN Treg cells of IBD model mice \((n=5\) for each, \(P=0.0023, 0.0000,\) and 0.0000, respectively\) (Fig. 4, E–G). A negative correlation was observed between changes in the expression levels of positive TRs (cMAF, HIF-1α) and negative TR (TRIM33) \((n=5\) for each, \(P=0.0011, 0.0433,\) and 0.0000 in cMAF, HIF-1α, and TRIM33, respectively) (Fig. 4H; Supplemental Fig. 6, B and F). The expression levels of E4BP4 and KLF4, but not Blimp1, were significantly higher in Treg cells in the recovery phase than in those from untreated mice \((n=4\) for each, \(P=0.0006\) and 0.0001 in E4BP4 and KLF4, respectively\) (Fig. 4, A–C).
Therefore, the activation of E4BP4 and/or KLF4 may be the mechanism underlying the KCa3.1 inhibition–induced upregulation of IL-10 in the peripheral Treg cells of IBD model mice.

Upregulation of IL-17A by the Administration of TRAM-34 in mLN CD4⁺CD25⁺ Treg Cells of IBD Model Mice. Increased numbers of IL-17A–producing CD4⁺CD25⁻Foxp3⁺ Treg cells, which are protective against IBD, have been reported in patients with IBD (Li and Boussiotis, 2013) and a chronic IBD mouse model (Hovhannisyan et al., 2011). In the present study, increased expression levels of IL-17A transcripts in mLN Treg cells were detected in the recovery phase in IBD model mice (n = 4 for each, P = 0.0096) (Fig. 5B) but not in the induction phase (Fig. 5A) (n = 4 for each). Similar to the marked increase observed in IL-10 expression levels, an approximately 6-fold increase in the expression levels of IL-17A transcripts was noted after the administration of TRAM-34: 0.005 ± 0.002 and 0.027 ± 0.006 (in arbitrary units) in vehicle- and TRAM-34–administered IBD model mice, respectively (n = 5 for each, P = 0.0028) (Fig. 5C).

Six candidates, cMAF, KLF4, RoRγt, BATF, HIF-1α, and GATA3, are common positive TRs of IL-10 (Capone and Volpe, 2020). TRIM33 is a positive TR of IL-17A, in contrast to IL-10 (Tanaka et al., 2018). TRIM33 is a positive TR of IL-17A, in contrast to IL-10 (Tagishi et al., 2016). As shown in Fig. 6, A and B, IL-10 transcription and secretion in Treg cells were both increased by the treatment with 10 μM TRAM-34 for 24 hours (n = 4 for each). IL-10 secretion was increased approximately 1.5-fold by the inhibition of KCa3.1 for 24 hours in Treg cells (Fig. 6B). Similarly, IL-17A transcription and secretion were increased by the treatment with TRAM-34 for 24 hours (n = 4 for each) (Fig. 6C, D). IL-17A secretion was increased approximately 1.4-fold by the inhibition of KCa3.1 for 24 hours in Treg cells (Fig. 6D). Consistent with the results obtained on the in vivo effects of the administration of TRAM-34 on IL-10 and/or IL-17A TRs in the mLN Treg cells of IBD model mice, the transcription of E4BP4, KLF4, TRIM33, and Blimp1...
Discussion

The production of IL-10 is impaired by human T-cell subsets in patients with IBD, and the therapeutic potential of IL-10 is currently being evaluated in clinical trials for the treatment of IBD (Roncarolo et al., 2018). Recent studies have focused on the development of Treg therapy for IBD (Soukou et al., 2018; Clough et al., 2020). KCa3.1 is a pivotal regulator of cytokine expression; however, its contribution to IL-10 production in Treg cells has not yet been elucidated. The main results of the present study are as follows: 1) the disease activity of IBD was suppressed by the in vivo administration of a KCa3.1 inhibitor (1 mg/kg, s.c.) to IBD model mice (Figs. 1 and 2); 2) the in vivo administration of the KCa3.1 inhibitor induced an increase in IL-10 expression in Treg cells in the recovery phase in IBD model mice (Fig. 3), with the upregulation of positive IL-10 TRs (Figs. 6 and 7); and 3) IL-10 production was increased by an in vitro treatment with the KCa3.1 inhibitor in peripherally induced Treg cells, which was consistent with the upregulation of IL-10 TRs (Figs. 6 and 7). The upregulation of KCa3.1 in proinflammatory CD4+CD25+ T cells in acute IBD model mice was recovered, together with the attenuation of the pathogenesis of IBD (Ohya et al., 2014), and KCa3.1 was post-translationally...
regulated by the histone deacetylases HDAC2 and HDAC3 in these cells (Matsui et al., 2018). The upregulation of KCa3.1, HDAC2, and HDAC3 in the mLN Treg cells of IBD model mice in the recovery phase was not detected in the present study (Supplemental Fig. 5, A–C). Since the population of CD4+CD25+ subsets was almost 10%–15% of the CD4+ subset (less than 3% of all mLN cells) (Supplemental Figure 4), difficulties were associated with examining the in vitro effects of the KCa3.1 inhibitor on IL-10 secretion using the isolated Treg cells of IBD model mice. Accordingly, to elucidate the transcriptional mechanism enhancing IL-10 expression and secretion by the inhibition of KCa3.1, CD4+CD25+ Treg cells peripherally induced in vitro by a lectin stimulation were used.

Recent studies reported that IL-17A plays a protective role against IBD (Bellemore et al., 2015; Gagliani et al., 2015), and in DSS-induced IBD model mice, protective IL-17A reflects the chronic stage of disease (O’Connor et al., 2009). A large population of IL-17A–producing CD4+CD25+Foxp3+ Treg cells (also referred to as Th17-like Treg) cells has been reported in patients with IBD and an IBD mouse model (Li and Boussiotis, 2013; Hovhannisyan et al., 2011; Kryczek et al., 2011). Th17-like Treg cells have also been found in patients with rheumatoid arthritis, multiple sclerosis, and severe psoriasis (Kryczek et al., 2011; Pandiyan and Zhu, 2015). The present study showed that IL-17A was also upregulated by the inhibition of KCa3.1 in the Treg cells of IBD model mice and Con-A–differentiated Treg cells (Figs. 5 and 7). During the resolution of inflammation, Th17 cells transdifferentiate into Treg cells (Bellemore et al., 2015). Therefore, the inhibition of KCa3.1 may be crucial for differentiation into IL-17A–expressing Treg cells, which play a protective role in the recovery phase of IBD.

A number of the TRs of IL-10 and IL-17A have been identified in lymphoid and myeloid cells, including Treg cells (Jung et al., 2017; Capone and Volpe, 2020; Fang and Zhu, 2020). As shown in Figs. 4 and 7, the TRs promoting IL-10 and/or IL-17A expression in various T-cell subsets—namely, E4BP4, KLF4, and Blimp1—are commonly upregulated by TRAM-34 in Treg cells in the recovery phase in IBD model mice and Con-A–differentiated Treg cells. KLF4 has been identified as a positive TR for IL-10 and IL-17A in macrophages and Th17 cells (Liu et al., 2007; An et al., 2011), and KLF4 was found to be upregulated in goblet cells from patients with Crohn disease (Gersemann et al., 2009). Fujimoto et al. (2019) recently showed that the ablation of KLF4 resulted in the hyperphosphorylation of SMAD2 and elevated nuclear localization of P-SMAD2/3. Smad2 regulates CD4+ T-cell differentiation into induced Treg cells and Th17 cells (Malhotra et al., 2010). However, discrepancies exist in the findings reported—namely, KCa3.1 blockers and activators both attenuated Smad2/3 phosphorylation (Yu et al., 2014; Roach et al., 2015; Matsui et al., 2019). One hypothesis to explain the KCa3.1 inhibitor–induced increase in IL-10 expression is that the inhibition of KCa3.1 enhances KLF4-mediated SMAD2 phosphorylation in Treg cells. However, the inhibition of KCa3.1 did not affect the phosphorylation of SMAD2/3 (Fig. 8, F and G). We previously reported that the nuclear localization of P-SMAD2 was regulated by KCa3.1; however, in the present study, no significant changes in the nuclear localization of P-SMAD2/3 were found after TRAM-34 treatment in Con-A–differentiated Treg cells.
T\textsubscript{reg} cells (Supplemental Fig. 9). The MAPK-ERK, PI3K-AKT, and CN-NFAT signaling pathways are generally regulated in a Ca\textsuperscript{2+}-dependent manner. Therefore, intracellular Ca\textsuperscript{2+} influx by the inhibition of K\textsubscript{Ca3.1} attenuates these signaling pathways. A previous study reported that K\textsubscript{Ca3.1} inhibition-induced increases in intracellular K\textsuperscript{+} concentrations may be attenuated by the AKT signaling pathway (Eil et al., 2016). KLF4 expression may be positively and negatively regulated by the AKT and ERK signaling pathways (Riverso et al., 2017; Liu et al., 2017; Dong et al., 2019). However, the inhibition of K\textsubscript{Ca3.1} did not affect the phosphorylation of ERK1/2 or AKT (Fig. 8), and their inhibitors did not alter the secretion of IL-10 or IL-17A (Supplemental Fig. 8). Although KLF4 is also activated by the JAK-STAT3 signaling pathway, the inhibition of K\textsubscript{Ca3.1} did not affect the phosphorylation of STAT3 (Fig. 8), and a STAT3 inhibitor did not alter the secretion of IL-10 or IL-17A (Supplemental Fig. 8). Further studies are needed to elucidate the mechanisms underlying the K\textsubscript{Ca3.1} inhibition–induced upregulation of KLF4 in IL-17A–expressing T\textsubscript{reg} cells in the recovery phase in IBD model mice.

The transcription of E4BP4 is activated through the MAPK-ERK, PI3K-AKT, CN-NFAT, TGF-β-SMAD, and CaMK signaling pathways (Nishimura and Tanaka, 2001; Motomura et al., 2011; Kim et al., 2019). The attenuation of intracellular Ca\textsuperscript{2+} influx by the inhibition of K\textsubscript{Ca3.1} was shown to prevent the Ca\textsuperscript{2+}-dependent CaMK signaling pathway in addition to the ERK, AKT, and CN-NFAT signaling pathways. In contrast, Simma et al. (2014) reported that its inhibition activated the ERK, AKT, and CN-NFAT signaling pathways, resulting in an increase in IL-10 expression in activated B cells. The present study revealed no significant changes in IL-10 and IL-17A secretion (Supplemental Fig. 8) after the pharmacological blockade of the ERK, CN-NFAT, and CaMK signaling pathways in the absence and presence of TRAM-34, thereby strongly supporting the lack of involvement of these signaling pathways in the K\textsubscript{Ca3.1} inhibition–induced upregulation of E4BP4. Moreover, Blimp1 was shown to increase IL-10 expression in various T-cell subsets, and its expression was modulated by the MAPK-ERK, PI3K-AKT, and TGF-β-SMAD signaling pathways (Sciortino et al., 2017; Setz et al., 2018). Blimp1 was not upregulated in T\textsubscript{reg} cells in the recovery phase in IBD model mice (Fig. 4). In this study, we did not examine the effects of TRAM-34 on the activities of TR candidates possibly involved in IL-10 and/or IL-17A expression in T\textsubscript{reg} cells. Further studies will be needed to determine which TRs are major contributors of IL-10/IL-17A transcription. It is also important to clarify the mechanisms underlying the K\textsubscript{Ca3.1} inhibition–induced upregulation of TRs in T\textsubscript{reg} cells in the recovery phase in IBD model mice.

Hypoxia is involved in intestinal inflammation in patients with IBD, and the activation of HIF-1α promotes T\textsubscript{reg} cell activity, such as IL-10 expression, without altering Foxp3 expression (Colgan and Taylor, 2010; Sakaki-Yumoto et al., 2013; Westendorf et al., 2017). As shown in Fig. 4D, the expression level of HIF-1α was high, possibly as a result of enhanced oxygen consumption in the diseased area. On the
other hand, improvement of IBD symptoms by the administration of TRAM-34 suppressed the expression level of HIF-1α (Fig. 4H), possibly as a result of escape from hypoxic condition. HIF-1α enhances IL-10 expression by directly binding the promoter and also enhances IL-17A through the RORγt-mediated activation of the promoter (Flück and Fandrey, 2016; Westendorf et al., 2017). However, HIF-1α expression was negatively regulated by the in vivo administration of the Kc3.1 inhibitor (Fig. 4H), suggesting that HIF-1α did not contribute to the Kc3.1 inhibition–induced upregulation of IL-10 and IL-17A. In conclusion, the present results strongly suggest the potential of Kc3.1 in peripherally induced Treg cells as a target for more efficient Treg therapy for chronic inflammatory disorders and also suggest that treatments with Kc3.1 inhibitor in Treg cells may effectively increase IL-10 production. Protective effects during the process of disease IBD may involve peripherally induced IL-10–producing Treg cells (Mayne and Williams, 2013; Meng et al., 2018), and recombiant IL-10 therapy was previously shown to ameliorate established T-cell transfer colitis (Mizoguchi et al., 2020). Recent clinical studies focused on the development of Treg cell therapy for Crohn disease to provide a continuous source of anti-inflammatory cytokines, such as IL-10, and thus achieve a meaningful therapeutic outcome. The manipulation of IL-10ββ high Treg cells by the inhibition/knockdown of Kc3.1 may be beneficial in the treatment of chronic inflammatory diseases.

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Participated in research design: Ohya, Matsui. Conducted experiments: Ohya, Matsui, Kajikuri, Endo, Kito. Performed data analysis: Ohya, Matsui, Kajikuri, Endo, Kito. Wrote or contributed to the writing of the manuscript: Ohya, Matsui, Kajikuri, Endo, Kito.

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