Full Paper

IL-17A Induces Hypo-contraction of Intestinal Smooth Muscle via Induction of iNOS in Muscularis Macrophages

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Abstract. Intestinal inflammation causes disorder in bowel motility. Th17 cytokines are involved in intestinal inflammation. To understand the role of interleukin (IL)-17 in intestinal motility, we examined effects of IL-17A on contractile activities of organ-cultured ileum. Rat ileal smooth muscle strips were organ cultured with IL-17A. Muscle contraction was measured, and cells expressing inducible nitric oxide synthase (iNOS) were identified with immunohistochemistry. Creating Th17-transferred colitis model mice, in vivo effects of IL-17 on contractile activities, and iNOS mRNA expression in colonic smooth muscle were investigated. Treatment with IL-17A for 12 h and 3 days attenuated carbachol- and membrane depolarization-induced contractions in organ-cultured rat ileum. *N*^G-Nitro-L-arginine methyl ester (100 μ M), a nitric oxide synthase inhibitor, completely reversed the IL-17A-induced inhibition of contractile force. Ileal tissue cultured in the presence of IL-17A showed increased expression of iNOS mRNA and protein. Immunohistochemical analysis using an iNOS antibody revealed that iNOS protein was expressed on ED2-positive muscularis macrophages. The level of iNOS mRNA was also increased in inflamed colonic smooth muscle of Th17-transferred colitis model mice. In intestinal inflammation, IL-17A induces an intestinal motility disorder through iNOS expression in muscularis macrophages.

Keywords: IL-17A, macrophage, iNOS, intestinal inflammation, smooth muscle

Introduction

Recently, a new subset of helper T cells named Th17 was identified (1, 2). Th17 cytokines function as effectors in various autoimmune disease models (3 – 6). Many studies regarding Th17-mediated intestinal inflammation have also been performed. Mice deficient in interleukin (IL)-17 receptor A (IL-17RA) or wild-type mice given a neutralizing IL-17RA develop less severe colitis caused by administration of trinitrobenzene sulfonic acid (7). Another study showed that when retinoic acid receptor-related organ receptor (ROR) γ t-deficient T cells are transferred into mice, the mice fail to develop colitis.

ROR γ t is the master transcription factor involved in Th17 differentiation (8, 9). Exogenous administration of IL-17A to mice transferred with ROR γ t-deficient T cells led to development of colitis in these mice. Furthermore, adoptive transfer of IL-17F-deficient T cells to RAG1-null mice, which have no mature T lymphocytes, caused severe colitis, and this colitis was suppressed by treating them with a neutralizing anti-IL-17A antibody (10).

Intestinal inflammation causes disorders involving gastrointestinal motility (11, 12). Previously, we showed that IL-1 β induces downregulation of protein kinase C-potentiated phosphatase inhibitor protein of 17 kDa (CPI-17) expression both in vivo and in vitro (13 – 15). Because phosphorylation of CPI-17 at Thr38 inhibits the phosphatase catalytic activity of the myosin light chain in intestinal smooth muscle and increases Ca²⁺ sensitization of smooth muscle, downregulation of CPI-17

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decreases smooth muscle contraction and causes a motility disorder (16–20). This downregulation was caused by a tumor necrosis factor (TNF)- α cascade (13). The effects of Th1-type cytokines such as IL-1 β and TNF- α on inflamed intestinal motility have been shown. However, Th17-type cytokines may also be important in intestinal inflammation, but their effects on intestinal motility are unknown.

IL-17A, a member of the IL-17 cytokine family, is secreted from many types of cells including monocytes and neutrophils (21). Such cells infiltrate into the intestinal smooth muscle layer during intestinal muscularis inflammation (22), and thus, IL-17A may affect the intestinal smooth muscle contractility. In this study, we focused on intestinal smooth muscle contraction and investigated the effect of IL-17A on contraction *in vitro* using organ-cultured smooth muscle tissue. Furthermore, we investigated the effect of Th17 cytokines *in vivo* with colitis model mice that were transferred with Th17 cells.

Materials and Methods

Tissue preparation and organ culture procedure

Animal care and treatment were conducted in conformity with the institutional guidelines of the University of Tokyo and are consistent with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Sprague–Dawley rats (age, 7–9 weeks of age) were euthanized by a sharp blow to the neck and exsanguination. A 10- to 15-cm segment of the ileum was detached from the mesentery and placed in sterile Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA). The distal ileal part was isolated from the small intestine. The isolated ileum was cut open along the mesenteric attachment, and the mucosa and submucosa were removed from the smooth muscle layer under a stereoscopic microscope. The smooth muscle sheets were then transferred to culture dishes with Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 1% penicillin–streptomycin and maintained at 37°C in an atmosphere of 95% air and 5% CO₂. In this system, intestinal muscle force was maintained at least over 3 days of culture, although the force was gradually downregulated when the muscle strip was incubated over 12 h in normal organ baths with non-sterilized normal physiological salt solution (data not shown).

Measurement of muscle tension

Measurement of circular muscle contraction was performed as described previously (23). Organ cultured ileal muscle sheets were cut along the lines of circular smooth

muscle (6-mm-wide and 15-mm-long) to make circular muscle strips. The circular muscle strips were made and suspended along the circular axis in a tissue bath filled with normal physiological salt solution of 136.9 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 23.8 mM NaHCO₃, 5.5 mM glucose, and 0.01 mM ethylenediamine tetraacetic acid (EDTA; pH 7.4). Muscle strips were maintained at 37°C in an atmosphere of 95% O₂–5% CO₂. Muscle force was measured isometrically under a resting tension of 10 mN and recorded on a pen recorder (Yokogawa, Tokyo). After equilibration for 20 min in a 10- or 20-ml bath, each strip was exposed to high K⁺ (65.4 mM) solution 3 times to stabilize the response. Relaxant effect of nitric oxide (NO) against muscle force was estimated by measuring concentration-dependent carbachol-induced contractions with or without *N*^G-nitro-L-arginine methyl ester (L-NAME). At the end of the tension measurements, the wet weight of each muscle strip was measured. Contractile force was shown in mN/mg wet weight of tissue.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described (22). The oligonucleotide primers for each target gene are listed on Table 1. Amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Shiga) using 30–35 cycles (two-cycle intervals), each consisting of 98°C for 10 s, 52°C–56°C for 30 s, and 72°C for 1 min. The products were resolved on 2% agarose gels containing 0.1% ethidium bromide. The density of detectable fluorescent bands was measured using the Gel Doc EZ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primer sets for RT-PCR and their product size (bp) are shown in Table 1.

Western blot analysis

Strips were homogenized in sample buffer containing 60 mM β -glycerophosphate, 0.5% Nonidet P-40, 0.2% SDS, 1 mM Na₃VO₄, 2 mM EGTA, 50 mM Tris-HCl, pH 8.0, and 1 mg/ml Complete (protease inhibitor cocktail). Total proteins (20 μ g per lane) prepared from the rat ileum were separated with 7.5% SDS-PAGE and transferred to a membrane. The membrane was blocked by incubating with Tris-buffered saline solution with 0.05% Tween-20 (TBST) (pH 7.4) containing 3% bovine serum albumin for 1 h at room temperature. Membranes were incubated overnight at 4°C in blocking buffer containing anti-iNOS antibody (1:1,000; BD Biosciences, San Jose, CA, USA) or anti- β -actin antibody (1:1,000, Sigma-Aldrich). After washing the membrane with TBST, secondary antibodies [horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK; 1:1,000) or anti-mouse IgG (IRDye 800; Rockland,

Table 1. Sequences and product sizes of the primers used in RT-PCR analysis

Target genes	Forward primers Reverse primers	Product size (bp)
GAPDH	5'-TACCAGCCGGGGGACCAC-3' 5'-CGAGCTGACAGAGTAGTA-3'	308
iNOS	5'-AAGGGAGTGTGTTCAGGT-3' 5'-CCACCAGCTTCTTCAAMGTG-3'	196
IL-17RA	5'-GTATCTCGAGGGTGCAGAGC-3' 5'-CAGCCAGGCACTAAGATGGT-3'	234
IL-17RC	5'-TTCTGCCCCTTCAAGAAAGA-3' 5'-ACACAGAGGTTGGGGTGTTC-3'	248
TNF- α	5'-AAATGGGCTCCCTCTCATCA-3' 5'-AGCCTTGTCCTTGAAGAGA-3'	248
IL-1 β	5'-TCCATGAGCTTTGTACAAGG-3' 5'-GGTGCTGATGACCAGTTGG-3'	246

Gilbertsville, PA, USA; 1:10,000)] were incubated for 30 min. iNOS protein was visualized with an enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences). Bands were visualized with an LAS-1000 mini luminescence imager (Fuji Film, Tokyo). β -Actin protein was detected and quantified using the Odyssey system (LI-COR Biosciences, Lincoln, NE, USA).

Detection of CPI-17 and myosin phosphatase target subunit 1 (MYPT1) phosphorylation

Western blot analysis was performed as described previously (20). Strips were stimulated with carbachol (1 μ M) for 5 min and then quickly frozen in acetone/dry ice containing 10 mM DTT, 10 mM sodium thioglycolate, and 10% (w/v) trichloroacetic acid. Strips and trichloroacetic acid/acetone/dry ice solution were then gradually warmed to 4°C, washed in water-saturated ether, and lyophilized overnight. Strips were then homogenized in sample buffer containing 60 mM β -glycerophosphate, 0.5% Nonidet P-40, 0.2% SDS, 1 mM Na₃VO₄, 2 mM EGTA, 50 mM Tris-HCl, pH 8.0, and 1 mg/ml Complete. Total proteins (20 μ g per lane) purified from rat ileum were separated with 15% (for CPI-17) or 7.5% (for MYPT1) SDS-PAGE and transferred to a membrane. The membrane was blocked by incubating in TBST containing 3% bovine serum albumin for 1 h at room temperature. Membranes were then treated with primary antibodies: anti-p-CPI-17 (Thr38) antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CPI-17 antibody [CPI-17 (N-20); 1:1,000; Santa Cruz Biotechnology], anti-p-MYPT1 (Thr696) antibody (1:1,000; Santa Cruz Biotechnology), anti-p-MYPT1 (Thr853) antibody (1:1,000; Upstate, Lake Placid, NY, USA), or anti-MYPT1 antibody [MYPT1 (N-19); 1:1,000; Santa Cruz Biotechnology] overnight at

4°C. After washing the membrane with TBST, secondary antibodies: Alexa Fluor 680 conjugated anti-goat IgG (Invitrogen, Tokyo) or anti-rabbit IgG (IRDye 800, 1:10,000; Invitrogen) were added and incubated for 30 min. Each protein was detected and quantified using the Odyssey system. We compared the ratio of phosphorylated CPI-17 and MYPT1 to the total amount of CPI-17 and MYPT1, respectively.

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as described previously (22). For immunohistochemistry, muscle strips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and processed for whole-mount preparations. Samples were incubated overnight at 4°C with anti-iNOS antibody (1:500, BD Biosciences) and/or anti-rat resident macrophage antibody (ED2, 1:500; AbD Serotec, Oxford, UK). Preparations were immunohistochemically analyzed using an LSM 510 confocal microscope (Carl Zeiss Japan, Tokyo) and a Digital Eclipse C1 (Nikon, Tokyo).

Preparation of resident peritoneal macrophages

Resident peritoneal macrophages were obtained by peritoneal lavage with cold RPMI 1640-medium (Sigma-Aldrich), followed by adherence to plastic for 2 h at 37°C. Cells were cultured with or without cytokine for 12 h at 37°C, and total RNA was extracted from cells with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). RT-PCR analysis was then performed as described in the "Quantitative RT-PCR" section.

Th17-transferred colitis mice

The Th17-dependent experimental colitis model was developed in BALB/c mice transferred with antigen-

specific Th17 cells prepared as described previously (24). Briefly, splenic CD4⁺ T cells from DO11.10/RAG-2^{-/-} mice were cultured with X-ray-irradiated BALB/c splenocytes in DMEM/F12 medium supplemented with 10% fetal bovine serum, 0.3 μ M ovalbumin (OVA) 323-339 peptide, 10 U/ml recombinant IL-2 (BD Bioscience), 10 ng/ml IL-1 β , 20 ng/ml IL-6, 1 ng/ml transforming growth factor- β (Peprotech, Rocky Hill, NJ, USA), and 10 ng/ml IL-23 (R&D Systems, Minneapolis, MN, USA), which were added at the start of the culture together with 40 μ g/ml anti-IL-4 and 40 μ g/ml anti-IFN- γ (eBioscience, San Diego, CA, USA). Cells were harvested after 7–10 days of stimulation, and then 2×10^7 cells were transferred to each mouse. Antigen-induced colitis was induced in these mice with 4 intranal injections of 100 mg OVA once a week.

Histological analysis

The abdomen of each mouse was opened, and the colon was removed. The luminal contents of the colon were washed with normal physiological salt solution, and the colon was fixed with paraformaldehyde. Following fixation, 4- μ m-thick sections were stained with hematoxylin and eosin.

Reagents

The following chemicals were used: carbachol (Sigma-Aldrich), rat recombinant IL-17A and TNF- α (ProSpec, East Brunswick, NJ, USA), BAY 11-7082 (Santa Cruz Biotechnology), and N^G-nitro-L-arginine methyl ester (L-NAME; Enzo Life Sciences, Exeter, UK).

Statistics

Results are expressed as the means $\pm$ S.E.M. Data were statistically evaluated using unpaired Student's *t*-tests for comparisons between two groups and with one-way analysis of variance followed by Dunnett's test for comparisons among three or more groups. Values of $P < 0.05$ were considered statistically significant.

Results

To determine the effect of IL-17A on the ileal muscle contraction, we measured contractile responses of the organ-cultured ileum of the rat to carbachol for receptor stimulation and high-K⁺ for membrane depolarization. Treatment of the cultured ileum with IL-17A (10 and 100 ng/ml) for 12 h significantly attenuated contractile responses to carbachol (1 μ M) and high-K⁺ (65.4 mM) in a concentration-dependent manner (Fig. 1: a and b). Incubation with 10 ng/ml IL-17A for 12 h or 3 days significantly depressed the concentration–response curve of 0.1–1 μ M carbachol, indicating the insurmountable

inhibition of IL-17A (Fig. 1: c and e). Treatment with IL-17A for 12 h or 3 days also attenuated 65.4 mM high K⁺-induced contraction (Fig. 1: d and f).

The receptor for IL-17A is a heterodimer consisting of IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC) (25). Thus, we examined mRNA expression of IL-17RA and IL-17RC in rat ileal smooth muscles. As shown in Fig. 2, we detected mRNA of both IL-17RA and IL-17RC with RT-PCR analysis (Fig. 2a).

IL-17A activates NF- κ B signaling (26). To examine if IL-17A attenuated ileal smooth muscle contraction through NF- κ B activation, we incubated the smooth muscle layer with IL-17A and the NF- κ B inhibitor BAY 11-7082 (20 μ M) for 12 h. Results indicated that BAY 11-7082 diminished the inhibitory effect of IL-17A on carbachol-induced contraction (Fig. 2b).

Previously, we showed that IL-1 β downregulates expression of CPI-17, an endogenous myosin phosphatase inhibitory protein, via TNF- α , which in turn attenuates carbachol-induced contraction (13, 15). We examined whether IL-17A attenuates ileal smooth muscle contraction via downregulation of CPI-17. However, treatment with IL-17A for 12 h did not change CPI-17 protein levels (Fig. 3a). Unlike TNF- α , the level of CPI-17 expression was unchanged following treatment with IL-17A, even for 3 days (Fig. 3b). Inhibition of myosin light chain phosphatase via phosphorylation of CPI-17 and MYPT1, which is the large regulatory subunit of myosin light chain phosphatase, is required to maintain sufficient contraction of intestinal smooth muscle. Thus, we examined the effect of IL-17A on phosphorylation of CPI-17 at Thr38 and MYPT1 at Thr696 and Thr853. IL-17A had no effects on the phosphorylation levels of either protein (Fig. 3: c and d). Moreover, although IL-17A may induce TNF- α and IL-1 β production (27), mRNA levels of TNF- α and IL-1 β were not increased with IL-17A for 12 h in our experimental conditions (Fig. 3: e and f).

In cultivated cartilage slices from osteoarthritis patients, IL-17A upregulates nitric oxide (NO) production, and experiments using human chondrocytes revealed that iNOS mRNA is induced by IL-17A (28–30). Thus, to examine whether IL-17A attenuates ileal smooth muscle contraction via NO, we applied L-NAME, which is a selective inhibitor of NO synthase, for 20 min before addition of carbachol or high K⁺. As shown in Fig. 4a, carbachol-induced dose-dependent contractions were significantly inhibited in cultured ileal tissue treated with IL-17A for 12 h. L-NAME significantly reversed the IL-17A-induced inhibition of carbachol-induced contraction close to control levels. L-NAME slightly but non-significantly enhanced the carbachol-induced contraction in control cultured tissues. Similar

Fig. 1. Time- and concentration-dependent inhibitory effects of IL-17A on organ-cultured ileal muscles contraction. a) Carbachol (1 μ M)- and b) high K⁺ (65.4 mM)-induced contraction in rat ileal smooth muscle tissue cultured with IL-17A (1 – 100 ng/ml) for 12 h. Each contraction (% of contraction) was normalized to the muscle force without IL-17A treatment. c) Carbachol (0.001 – 10 μ M)- and d) high K⁺ (65.4 mM)-induced contraction in rat ileal smooth muscle tissue cultured with IL-17A (10 ng/ml) for 12 h. Each contraction is shown as the absolute force per tissue weight (mN/mg wet wt.) e) Carbachol (0.001 – 10 μ M)- and f) high K⁺ (65.4 mM)-induced contraction in rat ileal smooth muscle tissue cultured with IL-17A (10 ng/ml) for 3 days. Each contraction is shown as the absolute force per tissue weight (mN/mg wet wt.). All results are shown as the means $\pm$ S.E.M. of 4 – 7 experiments. * P < 0.05, ** P < 0.01, compared with the control.

effects with L-NAME were obtained with high K⁺-induced contraction (Fig. 4b). L-NAME completely abolished the inhibitory action of IL-17A against high K⁺-induced contraction. L-NAME also diminished the effect of IL-17A in strips cultured for 3 days (Fig. 4: c and d). In contrast, as shown in Fig. 4, e and f, exposure to TNF- α for 3 days also inhibited carbachol- and high K⁺-induced contractions. However, these inhibitory actions by TNF- α were partially mediated by a decrease

in CPI-17 expression (Fig. 3) (13 – 15). L-NAME did not reverse the contractions induced by carbachol and high K⁺ that were attenuated by TNF- α treatment for 3 days.

For the next step, we examined the effect of IL-17A on iNOS expression levels in organ-cultured ileal tissue. As shown in Fig. 5a, iNOS mRNA expression was detected at low levels in cultured tissue in the absence of IL-17A. Treatment with IL-17A for 12 h increased iNOS mRNA. To further confirm the expression of iNOS at the protein

Fig. 2. Expression of IL-17RA and IL-17RC in ileal muscle layers and effects of an NF- κ B inhibitor on IL-17-mediated muscle relaxation. a) IL-17RA and IL-17RC were detected with RT-PCR analysis. b) Muscle strips were cultured with IL-17A (10 ng/ml) for 12 h. The NF- κ B inhibitor, BAY 11-7082 (10 and 20 μ M), was incubated with IL-17A for 12 h. Results are expressed as the means $\pm$ S.E.M. of 4–5 experiments. * P < 0.05, compared with the control. # P < 0.05, compared with IL-17A alone.

level, we performed western blotting (Fig. 5b). Inducible NOS protein was detected in control organ-cultured tissue. In ileal tissue treated with IL-17A for 12 h, iNOS protein levels were significantly increased. Interestingly, the increased iNOS protein expression was almost completely inhibited by the NF- κ B inhibitor BAY 11-7082.

To clarify which type of cells in ileal smooth muscle layer expressed iNOS protein, we performed immunohistochemical analysis using an iNOS antibody and a whole-mount preparation of organ-cultured ileal tissue. In control tissue, we did not detect iNOS-positive cells (Fig. 6a, upper panel), but we detected many ramified formed resident macrophages, which were stained with the ED2 antibody. In contrast, as shown in Fig. 6a, lower panel, many iNOS-positive cells were detected in the muscle layer as a red signal. The iNOS-positive cells completely overlapped with ED2-positive cells. To confirm that iNOS was activated in macrophages by IL-17A, we prepared rat resident peritoneal macrophages, which were also stained with the ED2 antibody (data not shown). As shown in Fig. 6b, peritoneal macrophages also expressed iNOS mRNA following IL-17A treatment for 12 h.

To examine the effect of Th17 cytokines on intestinal motility via iNOS induction in vivo, we injected OVA

peptide-sensitive Th17 cells into BALB/c mice and induced Th17-dependent colitis. The length of the colon was shortened by the OVA challenge in mice transferred with Th17 cells (data not shown), indicating colonic inflammation (14, 31). Histological analysis showed leukocyte infiltration into the mucosal layer of the colon of mice transferred with Th17 cells (Fig. 7: a and b). In Th17-transferred mice, carbachol- or high K^+ -induced contractions of colonic smooth muscles showed a trend toward inhibition (Fig. 7: c and d). The level of iNOS mRNA was significantly increased in the colon of mice transferred with Th17 cells (Fig. 7e).

Discussion

IL-17A attenuated ileal smooth muscle contraction as assessed with organ-cultured tissue in the present study. However, there was a possibility that other induced cytokines such as TNF- α and IL-1 β may attenuate ileal smooth muscle contractility because IL-17A could induce TNF- α and IL-1 β production (26, 27). We therefore examined mRNA levels of TNF- α and IL-1 β , and these levels were not increased in organ-cultured tissue treated of IL-17A for 12 h. We previously showed that the Th1 cytokine IL-1 β downregulates CPI-17 protein expression through TNF- α production in intestinal smooth muscles (13). In this study, we also confirmed that TNF- α inhibited CPI-17 expression (Fig. 4b). On the other hand, treatment with IL-17A for 12 h or 3 days did not change the CPI-17 protein level. IL-1 β decreases carbachol-induced contraction but not high K^+ -induced contraction (15), indicating that high K^+ -induced contraction is not mediated by CPI-17 phosphorylation (20). IL-17A nonselectively inhibited both carbachol- and high K^+ -induced contraction to the same degree. These differences may be due to different inhibitory mechanisms of contraction that are mediated by IL-17A but not through IL-1 β and/or TNF- α . Taken together, IL-17A inhibits the signaling pathways of the smooth muscle contractile machinery, which may be different from the mechanisms involving TNF- α .

To clarify the mechanism of attenuation of the contraction by IL-17A, we investigated the effects of IL-17A on phosphorylation of CPI-17 and MYPT1 via muscarinic receptor stimulation. Phosphorylated CPI-17 at Thr38 inhibits dephosphorylation of the myosin light chain and maintains contraction of smooth muscle (16, 17). In addition to CPI-17, phosphorylation of MYPT1 at Thr696 and Thr853 also maintains contraction by inhibiting myosin light chain phosphatase activity (32, 33). In intestinal smooth muscle, all of these phosphorylation events involving CPI-17 and MYPT1 are essential for decreasing the activity of myosin light chain phos-

Fig. 3. Effects of IL-17A on expression of CPI-17 and phosphorylation of CPI-17 and MYPT1 in the presence of carbachol stimulation. CPI-17 protein expression was quantified by western blotting of tissue cultured with a) IL-17A (10 ng/ml) for 12 h or b) IL-17A (10 ng/ml) or TNF- α (10 ng/ml) for 3 days. Phosphorylation levels of c) CPI-17 at Thr38 and d) MYPT1 at Thr696 or Thr853 were quantified with western blotting in tissue cultured with IL-17A (10 ng/ml) for 12 h. The phosphorylation level was measured 5 min after carbachol stimulation. Band density was normalized to that of control tissue. Although the signal ratio of the objective band to reference band differed from 1, signal ratio of bands were normalized by regarding the ratio of control tissue as 1. Results are expressed as the means $\pm$ S.E.M. of 4–12 experiments. * $P < 0.05$, compared with the control. e) TNF- α and f) IL-1 β mRNA quantified with RT-PCR analysis in tissue cultured with IL-17A (10 ng/ml) for 12 h. Results are expressed as the means $\pm$ S.E.M. of 4 experiments.

phatase (20). We detected phosphorylation of CPI-17 at Thr38 and MYPT1 at Thr696 and Thr853 5 min after carbachol stimulation, but IL-17A did not affect these phosphorylation events. These results indicated that IL-17-mediated inhibitory action against muscle contractility is not caused by regulatory systems of myosin phosphatase activity of smooth muscle contractile machinery.

In RAW264.7 cells, it has been reported that IL-17A activates the iNOS gene to produce NO (7), one of the

major inhibitory compounds for smooth muscle contraction (34). In the present study, a selective inhibitor of NO synthase, L-NAME, diminished the inhibitory effect of IL-17A on smooth muscle contraction, indicating that IL-17A may have decreased the contraction by inducing NO. In contrast, the inhibitory effect on contraction caused by TNF- α was not changed by L-NAME. Upregulation of iNOS mRNA and protein were also induced by IL-17A. These results suggest that IL-17A upregulated

Fig. 4. Effects of L-NAME on carbachol- and high K⁺-induced contraction in organ-cultured rat ileum with or without IL-17A. The effect of L-NAME (100 μ M) on a) carbachol (0.001 – 10 μ M)- and b) high K⁺ (65.4 mM)-induced contraction in rat ileal smooth muscle tissue cultured with IL-17A (10 ng/ml) for 12 h. c) and d) show the results of the same protocol seen in panels a and b when muscle strips were cultured for 3 days. The effect of L-NAME (100 μ M) on e) carbachol (0.001 – 10 μ M)- and f) high K⁺ (65.4 mM)-induced contraction in rat ileal smooth muscle tissue cultured with TNF- α (10 ng/ml) for 3 days. Results are expressed as the means $\pm$ S.E.M. of 5 – 14 experiments. * P < 0.05, ** P < 0.01, compared with the control. # P < 0.05, ## P < 0.01, compared with IL-17A alone. \$ P < 0.05, \$\$ P < 0.01, compared with L-NAME alone.

Fig. 5. Effects of IL-17A on expression of iNOS. Change in iNOS mRNA expression (a) and protein expression (b) following IL-17A (10 ng/ml) stimulation for 12 h in organ-cultured tissue. Each upper picture shows the typical results of 10–12 independent experiments. Effect of BAY 11-7082 (20 μ M) on IL-17A-induced iNOS protein expression was also investigated. Band density was normalized to that of control tissue. Results are expressed as the means $\pm$ S.E.M. of 10–12 experiments. ** P < 0.01, compared with the control. ## P < 0.01, compared with IL-17A alone.

Fig. 6. Expression of iNOS mRNA in the organ-cultured rat ileum and peritoneal macrophages with or without IL-17A for 12 h. a) Representative results of whole-mount immunohistochemistry. iNOS-positive cells (red) appeared following IL-17A treatment, and these cells were also stained with the anti-ED2 antibody (green). b) iNOS mRNA quantified with RT-PCR analysis in rat peritoneal macrophages cultured with IL-17A (10 ng/ml) for 12 h. Band density was normalized to that of the control. Results are expressed as the means $\pm$ S.E.M. of 10 experiments. * P < 0.05, compared with the control.

Fig. 7. Effect of IL-17A on colonic inflammation, colonic contraction, and iNOS gene expression in Th17 cell-transferred colitis model mice. Administration of OVA-caused colitis and induced iNOS mRNA in Th17-transferred mice. a) Representative change in the length of the gastrointestinal tract. b) Representative hematoxylin and eosin staining of the colon. c) Carbachol (0.1–10 μ M)- and d) high K^+ (65.4 mM)-induced contraction in mouse colonic smooth muscle tissue. Results are expressed as the means $\pm$ S.E.M. of 7 experiments. e) iNOS mRNA quantified with RT-PCR analysis in colonic smooth muscle tissue. Band density was normalized to that of the control. Results are expressed as the means $\pm$ S.E.M. of 7 experiments. * P < 0.05, compared with the control.

the iNOS gene which in turn produced NO, and this resulted in inhibition of ileal smooth muscle contraction. We further identified the iNOS-expressing cells in the intestinal muscle layer. Many resident macrophages exist in the myenteric plexus region of the intestinal muscle layer (35). The muscularis resident macrophages can be activated by lipopolysaccharide, which results in production of NO to inhibit contraction of intestinal smooth muscles (36). Immunohistochemical analysis revealed that iNOS protein was expressed by these resident macrophages stained with the ED2 antibody in cultured ileal tissue treated with IL-17A. In addition, ED2-positive peritoneal macrophages also expressed the iNOS gene following IL-17A treatment. Taken together, IL-17A may stimulate muscularis macrophages to induce iNOS.

In the present study, the NF- κ B inhibitor BAY 11-7082 diminished iNOS gene induced by IL-17A. The NF- κ B pathway appeared to be involved in the induction of iNOS in rat ileal smooth muscle tissue by IL-17A as well as in human osteoarthritic chondrocytes (30). In human osteoarthritic chondrocytes, in addition to the NF- κ B pathway, extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase also activate the transcription of iNOS (37–39). Furthermore, Th1-like immunoactivation drives iNOS expression through NF- κ B, signal transducer and activator of transcription, and cAMP signals (40, 41). In intestinal resident macrophages, cyclooxygenase-2-derived prostaglandin E₂ induces iNOS expression through cAMP/extracellular signal-regulated kinase pathways by activating prostaglandin E₂ receptor, EP2 and EP4 (42). In muscularis macrophages, as seen above, IL-17A may activate various signaling pathways to induce iNOS gene. However, in this study, treatment of BAY 11-7082 suppressed iNOS protein expression induced by IL-17, and the inhibitory effect of ileal smooth muscle contraction was also abolished by BAY 11-7082. These results suggest that NF- κ B appears to be the major pathway to induce iNOS by IL-17A in muscularis macrophages in the small intestine.

Finally, to determine if Th17 cytokines also attenuate intestinal contraction *in vivo*, we transferred Th17 cells into mice and induced colitis in accordance with our previous methods (24). In intestinal inflammation model animals with treatment of 2,4,6-trinitrobenzene sulfonic acid, IL-17 may be produced (7), but in these models, other cytokines such as IL-1 β and TNF- α are also produced (43), and it makes difficult to know the effect of IL-17 on the intestinal smooth muscle contraction. The main advantage of the Th17-transferred colitis model is that inflammation is mediated only by Th17 cell-induced cytokines, and thus, we can estimate the

pathophysiological function of Th17 cytokines in colitis without the influence of other types of cytokines such as Th1. Transferred Th17 cells differentiate from CD4⁺ T cells of DO11.10 mice, which are transgenic mice with T-cell receptors that recognize OVA residues 323–339. Thus, treatment with OVA peptide (323–339) activated Th17 cells, and Th17-dependent colitis was induced in mice. In OVA-treated, Th17-transferred mice, inflammation was accompanied by slightly decreased contraction, and iNOS mRNA levels were increased. Thus, we demonstrated that Th17 cytokines induced iNOS, which in turn produced NO, causing colonic dysmotility *in vivo*.

In conclusion, IL-17A attenuated intestinal contraction by NO production via induction of iNOS in muscularis macrophages. Th17 cytokines may partly contribute to intestinal motility disorder that occurs in intestinal inflammation.

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Conflicts of Interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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