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Article

All-trans retinoic acid down-regulates inflammatory responses by shifting the Treg/Th17 profile in human ulcerative and murine colitis

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ABSTRACT

IBD is characterized by uncontrolled immune responses in inflamed mucosa, with dominance of IL-17-producing cells and deficiency of Treg cells. The aim of this study was to explore the effect and mechanisms of RA, the ligand of RARα, on immune responses in human and murine colitis. Colonic biopsies from patients with UC were cultured and treated with RA as the agonist of RARα or LE135 as the antagonist of RARα. Expressions of IL-17 and FOXP3 were detected by immunohistochemistry. Murine colitis was induced by intrarectal administration with TNBS at Day 1. Mice were then i.p.-treated with RA or LE135 daily for 7 days. Cytokine levels in the cultures of mouse LPMCs were measured. Expressions of FOXP3 and IL-17 in colon tissues or MLN were detected by immunohistological analysis. Body weight and colon inflammation were evaluated. RA treatment up-regulated FOXP3 expression and down-regulated IL-17 expression in colon biopsies of patients and in colon tissues and MLN of mice with colitis compared with controls. LPMCs from RA-treated mice produced lower levels of proinflammatory cytokines (TNF-α, IL-1β, IL-17) but more regulatory cytokines (IL-10, TGF-β) compared with that of untreated mice. LE135 showed the opposite effect of RA. Furthermore, RA ameliorated TNBS-induced colitis in a dose-dependent manner, as seen by improved body weight and colon inflammation. RA down-regulates colon inflammatory responses in patients with IBD in vitro and in murine colitis in vivo, representing a potential therapeutic approach in IBD treatment. *J. Leukoc. Biol.* 86: 000–000; 2009.

Introduction

IBD as well as Crohn's disease and UC are serious intestinal disorders. Although evidence indicates that genetic predisposition and environmental factors such as foods are involved [1], the pathogenesis of IBD is still unclear. Recently, it has been strongly suggested that intrinsic factors, such as dysregulated immune responses, play an important role in the development of IBD [2]. A dysregulation of intestinal mucosal immunity causes an overproduction of inflammatory cytokines, including TNF-α and IL-1β, produced mainly by macrophages and lymphocytes. These proinflammatory cytokines partake in maintaining the inflammatory response and lead to the uncontrolled intestinal inflammation seen in IBD [3]. Down-regulating the production of these proinflammatory cytokines in inflamed intestine can inhibit the established inflammatory response and ameliorate IBD successfully, as indicated by clinical and experimental research [4, 5].

Recently, imbalance of the development and function of IL-17-producing Th17 cells and $CD4^+CD25^+FOXP3^+$ Treg cells has been demonstrated to play an important role in autoimmune diseases, including IBD [6, 7]. IL-17, mainly produced by Th17 cells, is a potent inducer of various cytokines such as TNF-α and IL-1β [8] and allows the body to kill pathogens in certain infections. However, it has also been linked to the development of autoimmune disease, such as rheumatoid arthritis [9, 10], multiple sclerosis [11], systemic lupus erythematosus [12], bronchial asthma [13], and renal allograft rejection [14]. Treg cells, also known as $CD4^+CD25^+$, $FOXP3^+$ Treg cells, are involved in the maintenance of peripheral tolerance and in controlling the immune response by initiating suppressive effects on activated immune cells [15, 16]. FOXP3, a forkhead/winged-helix transcription factor, is expressed specifically in Treg cells and is a master control protein for the generation and function of those T cells [17]. The development of IBD and mechanism of experimental colitis models have been linked with an imbalance between Th17 and Treg cells in inflamed mucosa. IL-17 is expressed extensively in the mucosa and serum of IBD patients [18], and Treg cells show

Abbreviations: FOXP3=forkhead box P3, IBD=inflammatory bowel disease, LPMC=lamina propria mononuclear cell, MLN=mesenteric lymph node(s), MPO=myeloperoxidase, RA=all-trans retinoic acid, RARα=RA receptor α, RORγt=RA orphan receptor γ, TNBS=2,4,6,-trinitrobenzene sulfonic acid, Treg cell=regulatory T cell, UC, ulcerative colitis

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only moderate expansion in mucosal lymphoid tissues [19], which is far from counterbalancing the mucosal inflammation in IBD. Polarization of Th17 cells by factors such as IL-23 and IL-21 can promote the progression of IBD, as shown by in vivo and in vitro experimental studies [20, 21]. On the other hand, shift of a Th1/Th17 profile to the enhancement of Treg or inhibition of Th17 polarization is beneficial for controlling immune response and ameliorating intestinal inflammation [22, 23].

Vitamin A and its metabolites, such as RA, are biologically active agents that have a broad range of functions involving immune cell differentiation and maintaining immune homeostasis. For example, vitamin A and its metabolites are capable of ameliorating various models of autoimmunity, including rheumatoid arthritis [24], type 1 diabetes [25], and experimental encephalomyelitis [26]. Deficiency of vitamin A can lead to exacerbated experimental colitis [27], and supplementation of vitamin A results in decreased prevalence of diarrhea in children [28]. It has been reported that RA can enhance Treg growth, differentiation, and gut homing [29] and inhibit Th17 generation and function [30, 31].

The effect of RA is mediated mainly by its nuclear receptor, RARα. Activated RARα modulates the expressions of FOXP3, IL-17, and/or RORγt genes directly, thus causing induction of FOXP3 and inhibition of Th17 polarization [30, 32]. Whether RA influences the imbalance between Th17 and Treg cells in the development of IBD, however, has not been explored. In the paper, we investigated the effect of RA as the ligand of RARα or LE135 as the antagonist of RARα on the inflammatory response in colonic biopsies from patients with UC and in a TNBS-induced murine colitis model. We also investigated the immune mechanisms involved, especially in the balance of Treg/Th17.

MATERIALS AND METHODS

Patients

The UC patients were five women and five men with an age range of 21–56 years and a median age of 39 years. None of the patients received corticosteroids, immunosuppressives, or cytotoxic drugs. The diagnosis of UC had been established by clinical, endoscopic, histological, and/or radiological criteria [33]. Infection or the presence of parasites was excluded by stool culture and microscopic examination. The disease activity of active UC was determined using a grading scale including clinical and paraclinical variables. Three cases were mild, five cases were moderate, and two cases were severe colitis. Six normal subjects without any appearance of colonic inflammation or tumor under endoscopy were included as a control group. The study was approved by the Ethics Committee of Nanchang University (China).

Biopsy tissue cultures

Three mucosal biopsies within a 5-cm^2 area of colonic mucosa without macroscopic lesions were taken from each patient with UC or normal subjects using endoscopy. The biopsies were weighed, washed in sterile PBS, pH 7.4, and then incubated in RPMI 1640 supplemented with 10% FCS, 250 U/ml penicillin, 250 mg/ml streptomycin, 10 mg/ml gentamycin, and 0.625 mg/ml Fungizone for 1 h at 37°C [33]. After washing in sterile PBS, pH 7.4, three times, the biopsies were divided randomly into three groups: control, RA, and LE135. The specimens were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, and 100 U/ml penicillin/streptomycin. RA (Sigma Chemical Co., St. Louis, MO, USA) as the agonist of RARα or LE135 (Sigma Chemical Co.) as the antagonist of RARα was dissolved in DMSO 10 mM and added into the culture medium with a final concentration of 100 nM or 1 μM, respectively. All tissues were placed in a humidified 5% CO_2 chamber at 37°C for 18 h, and the supernatants were collected and stored at −70°C until analysis. Tissues were fixed in 10% buffered formalin.

Animals

Male BALBc mice were obtained from the Experimental Animal Center of Nanchang University and kept under specific pathogen-free conditions. The mice used in the study were 7–8 weeks old, weighing ~22 g. All mouse experiments were reviewed and approved by the Institutional Animal Care Committee of Nanchang University.

Induction of colitis and study protocol

Colitis was induced by administration of TNBS in mice at Day 1 as described previously [34]. In brief, mice were anesthetized lightly, and a 3.5-F catheter was inserted intrarectally to 4 cm proximal to the anus. To induce colitis, 100 μl 2.5 mg TNBS (Sigma Chemical Co.) in 50% ethanol was injected slowly into the lumen via the catheter. Control mice received 50% ethanol alone (100 μl). Two hours after TNBS instillation, the mice were treated i.p. with one of medium, RA (20 μg, 100 μg, or 300 μg), or 100 μg LE135. This was repeated daily until the mice were killed on Day 7. RA or LE135 was dissolved in DMSO 0.5 mg/ml. After mice were killed, the length of colon was measured, and the colonic tissue segments were frozen immediately in liquid nitrogen for cytokine determination and MPO activity measurement. A colon specimen from the middle part was fixed in 10% buffered formalin for histological analysis.

Histology

Formalin-fixed, paraffin-embedded colon tissues were stained with H&E and examined by the same investigator in a blinded manner. Histological scores were performed to grade the degree of colonic inflammation from 0 to 4 using scoring systems described previously for hapten-induced colitis [35]: 0, no signs of inflammation; 1, very low level; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, thickening of the colon wall; 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density, thickening of the colon wall.

Measurement of MPO activity

All experiments were performed within 1 week of tissue collection. MPO activity was measured according to the method described by Wallace [36]. Tissue was homogenized in hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer. Aliquots were then added to O-dianisidine hydrochloride solution. Absorbance was read at 450 nm using a microplate reader. MPO was expressed in units/milligram tissue, where 1 unit corresponds to the activity required to degrade 1 mmol hydrogen peroxide in 1 min at 24°C.

Cytokine determination by ELISA

The colonic tissues were homogenized in PBS (0.1 M) to obtain final concentrations of 10% w/v. Levels of TNF-α, IL-1β, IL-6, IL-17, IL-10, and TGF-β in the homogenates or LPMC culture supernatants of the mice and in human biopsy culture medium were determined by sandwich ELISA using the kits supplied by R&D Systems (Minneapolis, MN, USA). ELISA was performed according to the manufacturer's instructions. In short, polyclonal goat anti-mouse cytokine antibodies were used as capturing antibodies and biotinylated polyclonal rabbit anti-mouse cytokine antibodies for detection. Streptavidin-HRP and tetramethylbenzidine sulfonate were added as color indicators. Plates were read at 490 nm immediately after the color reaction was stopped with acid.

Isolation and culture of LPMCs

LPMCs were isolated from freshly obtained colonic specimens from the mice with colitis at the peak of the disease (Day 4) using a modification of

the method described previously [37]. In brief, the colonic specimens were washed in HBSS–calcium–magnesium-free solution, then cut into 5-mm pieces, and incubated in HBSS containing 0.75 mM EDTA (Sigma Chemical Co.) and 1 mM DTT (Sigma Chemical Co.) at 37°C for 30 min to remove epithelium. The tissues were digested further in RPMI 1640 (HyClone, Logan, UT, USA) containing 400 U/ml colleagenase IV (Sigma Chemical Co.) and 0.01 mg/ml DNase I (Sigma Chemical Co.) in a shaking incubator at 37°C; this step was repeated two to three times. The cells released from the tissues were layered on a 40–100% Percoll gradient (Pharmacia Biotech, Piscataway, NJ, USA) and spun at 1800 rpm for 5 min to collect the lymphocyte-enriched population at the 40–100% Percoll interface. The T cell population was enhanced by incubating LPMCs in petri dishes for 2 h at 37°C to remove adherent cells.

LPMCs were incubated in complete medium (RPMI 1640 supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 10% heat-inactivated FCS) at a concentration of 1×10^6 cells/ml for 48 h in the absence (unstimulated) or presence (stimulated) of PMA (10 ng/ml) and Con A (2 μg/ml). Cytokine production in culture supernatants was determined by ELISA.

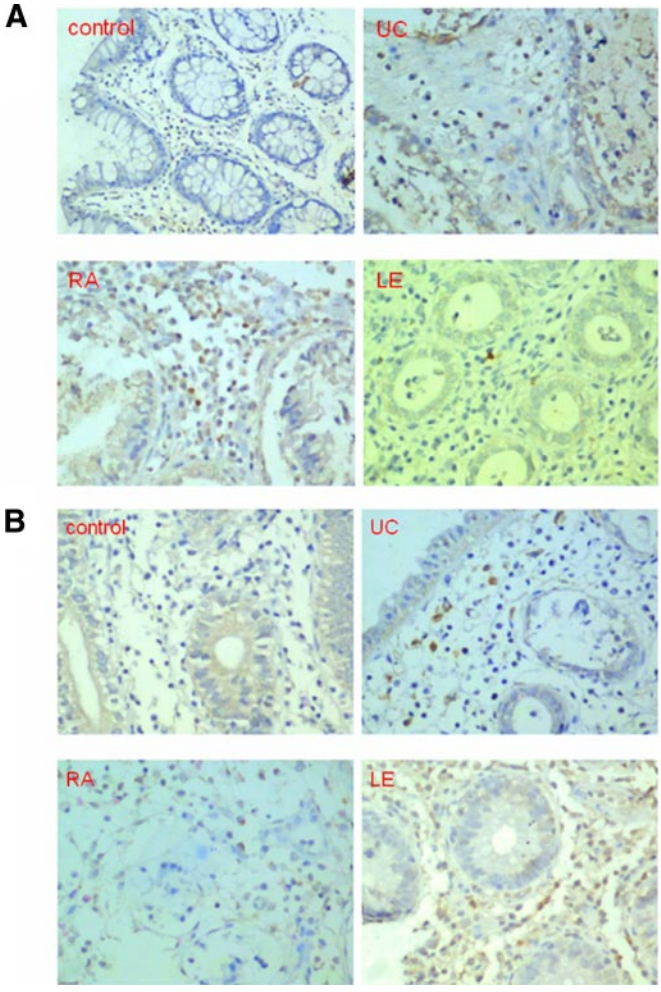
Western blot analysis

T cell-enriched LPMCs were lysed in a lysis buffer (0.1 M PBS, pH 7.4, containing 1% deoxycholic acid sodium, 0.2% SDS, and protease inhibitors). Western blot analysis was performed after protein concentrations were measured. The protein samples were separated by SDS-PAGE, then electrophoretically transferred to a polyvinylidene difluoride membrane, and processed for immunoblotting using. The first antibody included a rabbit anti-FOXP3, a rabbit anti-RORγt, or a rabbit anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubating with second antibody, the immunoreactive bands were visualized using the ECL method (Amersham Biosciences, Buckinghamshire, UK) and the rabbit anti-β-actin as inner control. Blots were scanned using the Kodak Image Station 4000MM system (Eastman Kodak, Rochester, NY, USA), and the mean intensity ratio relative to β-actin was determined by imaging software version 4.0.5f2 (Eastman Kodak).

Detection of FOXP3 and IL-17 using histological analysis

The expressions of FOXP3 and IL-17 in colon or MLN of the mice at the peak of the disease or in biopsies of the UC patients were detected by immunohistochemistry or immunofluorescence.

The murine colon tissues and MLN and the cultured biopsies of UC patients were fixed in 10% buffered formalin and embedded in paraffin; 4 μm sections were cut. After blocking inner peroxidase, the sections were incubated sequentially with rabbit anti-FOXP3 or rabbit anti-IL-17 antibodies (Santa Cruz Biotechnology), followed by three washes in pH 7.4 PBS. The sections of MLN tissues were then incubated in goat anti-rabbit IgG conjugated to FITC, and images were captured by using a Zeiss microscope and Axioviewer image analysis software (Carl Zeiss, Jena, Germany). The sections of murine colon or the cultured biopsies of UC patients were incubated in goat anti-rabbit IgG conjugated to peroxidase-labeled polymer,



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	Normal subjects	UC patients		
		Treated with PBS	with RA	with LE
FOXP3	undetectable	3.2%± 1.5% *	7.5%± 2.3%	1.8%± 1.2% **
IL-17	undetectable	15.7%± 3.6% *	10.6%± 3.8%	19.2%± 4.9% **

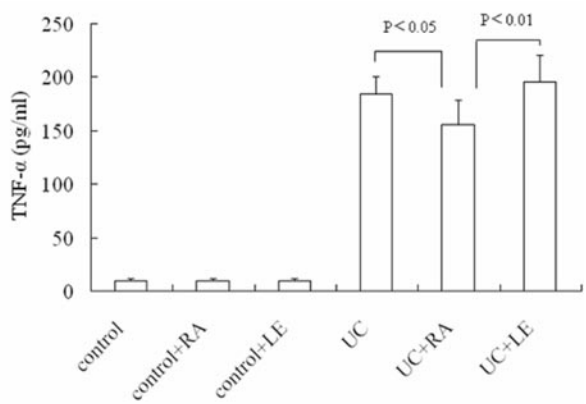

Figure 1. RA inhibits the colonic inflammatory responses of UC. Colonic mucosal biopsies from UC patients (n=10) and normal subjects (n=6) were cultured in the presence of 100 nM RA or 1 μM LE135 (LE), and then the expressions of FOXP3 and IL-17 were detected by immunohistochemistry. The supernatants were collected for TNF-α determination by ELISA. (A) FOXP3 expression was assessed by immunochemical staining of the colonic mucosa of UC patients and normal subjects. (B) IL-17 expression was detected by immunohistochemistry. All images are at the same original magnification (×200). (C) Percentages of FOXP3- and IL-17-positive cells in colonic tissues (*, P<0.05; **, P<0.01, compared with RA). (D) The concentration of TNF-α in the supernatants was determined by ELISA.

colored using a diaminobenzidine reaction, and counterstained with hematoxylin. The sections were evaluated using light microscopy, and 100 LPMCs/high-power field (magnification, ×400) were calculated for statistical analysis. Negative controls were established by omitting the primary antibodies.

Statistical analysis

All data in the text and figures are expressed as mean ± SD. Comparisons of more than two groups were made with a one-way ANOVA with post hoc Tukey's test. Comparison with two groups was made using Student's *t*-test for unpaired data when appropriate. Differences were considered statistically significant if P was <0.05.

RESULTS

RA in vitro inhibits colonic inflammatory response of UC

To investigate the effect of RA on the colonic inflammatory response of UC, we cultured the colonic biopsies from UC patients and normal subjects for 18 h and examined by immunohistochemistry the expressions of FOXP3 and IL-17. FOXP3 is the key transcription factor for the development and function of $CD4^+CD25^+$ Treg cells, and IL-17 is the main functional cytokine of Th17 in colonic mucosa of UC. There was no FOXP3 or IL-17 expression in normal colonic tissues, but under intestinal inflammation, a few LPMCs (~3.2%) expressed FOXP3, and many LPMCs (15.7%) showed IL-17 expression. When the tissues were treated by RA for 18 h, FOXP3 expression was up-regulated and detectable in 7.5% LPMCs, and IL-17 was down-regulated and detectable in 10.6% LPMCs, as shown in **Figure 1, A–C**. However, treatment with LE135, the antagonist of RARα, showed the reverse effect of RA, with 1.8% FOXP3-positive and 19.2% IL-17-positive LPMCs.

Next, we determined the concentration of TNF-α in the supernatants by ELISA. Acute UC is characterized by high production of proinflammatory cytokines secreted by LPMCs, such as TNF-α. As shown in Figure 1D, RA down-regulates the concentration of TNF-α in the supernatants of biopsy cultures from UC patients as compared with those without treatment ($P<0.05$), whereas LE135 up-regulates TNF-α content ($P<0.01$) compared with that of RA. There was no difference among the

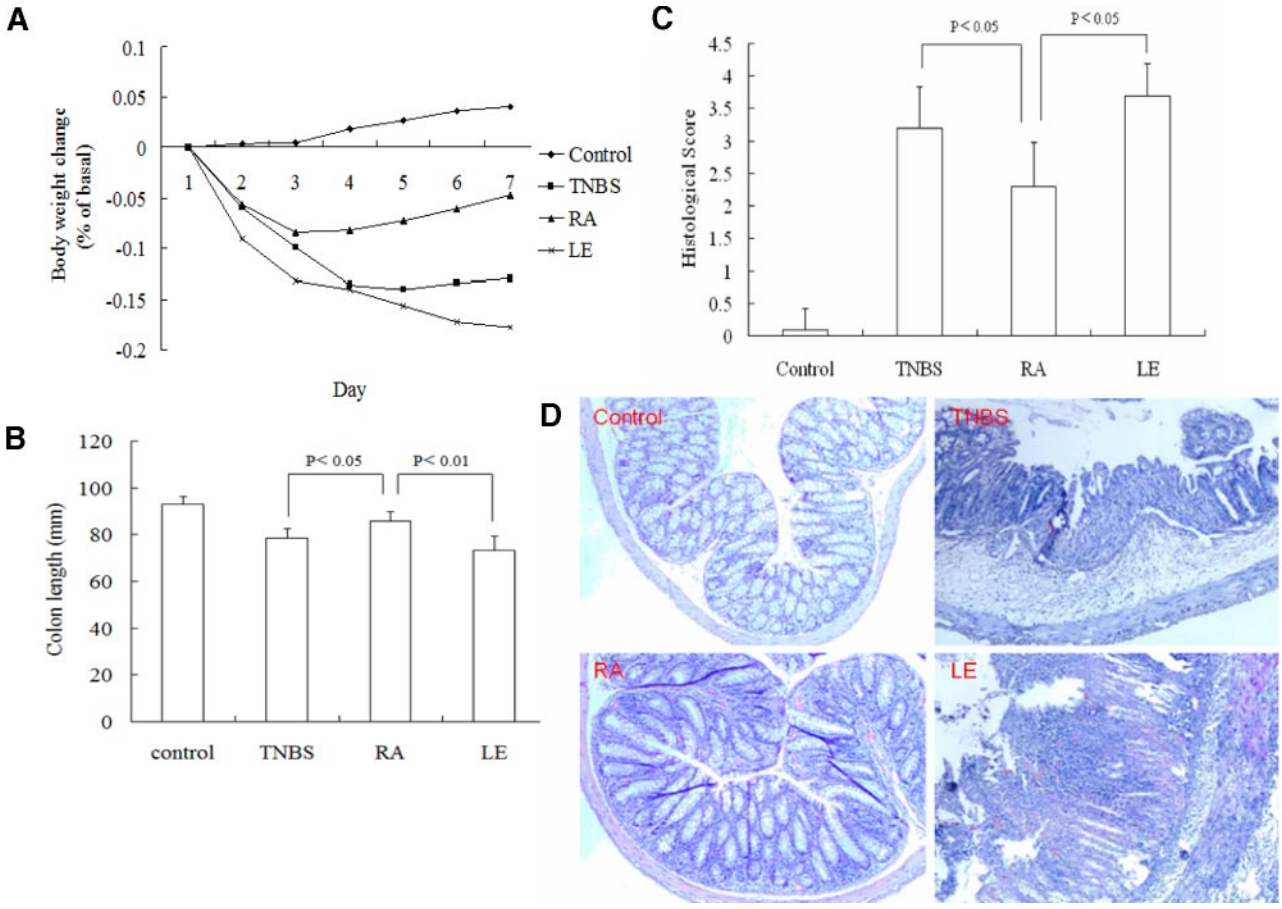

Figure 2. RA treatment after initiation of colitis inhibits the progression of TNBS-induced disease. Colitis was induced by intracolonic administration of 2.5 mg TNBS. RA (300 μg) or 100 μg LE135 was given i.p. 2 h after TNBS instillation and repeated daily until the mice were killed on Day 7. Mice treated with ethanol alone were used as a control. (A) Body weight changes of the mice of each group after colitis induction; n = 12–16 mice/group. *, $P < 0.05$, compared with the LE135 group or TNBS mice. (B) Length of colon in different groups on Day 7. (C) Colonic inflammation was scored by histological analysis at the end of the experiment. (D) H&E staining of colonic tissues of four groups of mice. All images are at the same original magnification (×40).

tissues from normal subjects treated by RA, LE135, and just vehicle.

Together, the results indicate that at least partly by modulating IL-17-producing T cell and Treg cell balance, activating RARα by the ligand is able to down-regulate the production of proinflammatory cytokines and inhibit the inflammatory response of active UC in vitro.

Treatment with the ligand of RARα ameliorates TNBS-induced murine colitis

Clinical symptoms and mortality. To investigate the therapeutic effect of RARα activation on TNBS-induced colitis, we induced colitis by administration of TNBS and then treated the mice with RA (RARα agonist) or LE135 (RARα antagonist). Mice receiving TNBS developed severe colitis characterized by bloody diarrhea and rectal prolapse accompanied by extensive wasting syndrome and a sustained weight loss, resulting in a mortality of 27.8% (5/18) by Day 7 (**Fig. 2A**). Mice treated with RA 2 h after TNBS colitis induction and repeated the treatment daily showed a mortality rate of 11.1% (2/18) on Day 7 and recovered the lost body weight rapidly, whereas those given LE135 had the highest mortality rate (36.8% or 7/19) and extensive body weight loss. The reduced inflammation in the RA group was also evidenced by the colon length, which was significantly longer than those in TNBS and LE groups (Fig. 2B).

Colon histological changes. Histological examination of the distal colon of mice with TNBS colitis showed patchy ulceration, epithelial cell loss, reduction of the density of tubular glands, focal loss of crypts, inflammatory cells infiltrate composed of macrophages, lymphocytes, and neutrophils, named LPMCs in the LP, and transmural inflammation involving all layers of the bowel wall (Fig. 2D). When the mice received RA treatment, these histological signs were much improved with significant reduction of inflammatory activity and neutrophil infiltration. Semiquantitive analysis showed that the inflammation score in the RA group was significantly lower than those in TNBS and LE135 groups (Fig. 2C). The mice with LE135 administration showed the highest histological score with severe mucosal tissue damage, extensive immune cell infiltration, and the highest MPO activity (**Figs.** 2D and **3A**).

MPO and cytokine levels in colon tissues. MPO is an enzyme found in neutrophils, which can be used as an index for tissue inflammation. MPO activity was significantly higher in the RA group than those in TNBS and LE groups (Fig. 3A). We also evaluated the effect of the agonist or antagonist of RARα on the production of inflammatory cytokines involved in TNBS-induced colitis, such as TNF-α, IL-1β, and IL-6. As shown in Figure 3, B–D, RA treatment strikingly reduced the production of inflammatory cytokines in colonic homogenates such as TNF-α (1152.8 pg/g), IL-1β (601.8 pg/g), and IL-6 (350.5 pg/g) when compared with untreated colitis mice (1412.9 pg/g, 760.9 pg/g, and 503.9pg/g, respectively). Conversely, LE135 administration up-regulated the production of TNF-α (1540.4 pg/g), IL-1β (844.1 pg/g), and IL-6 (594.8

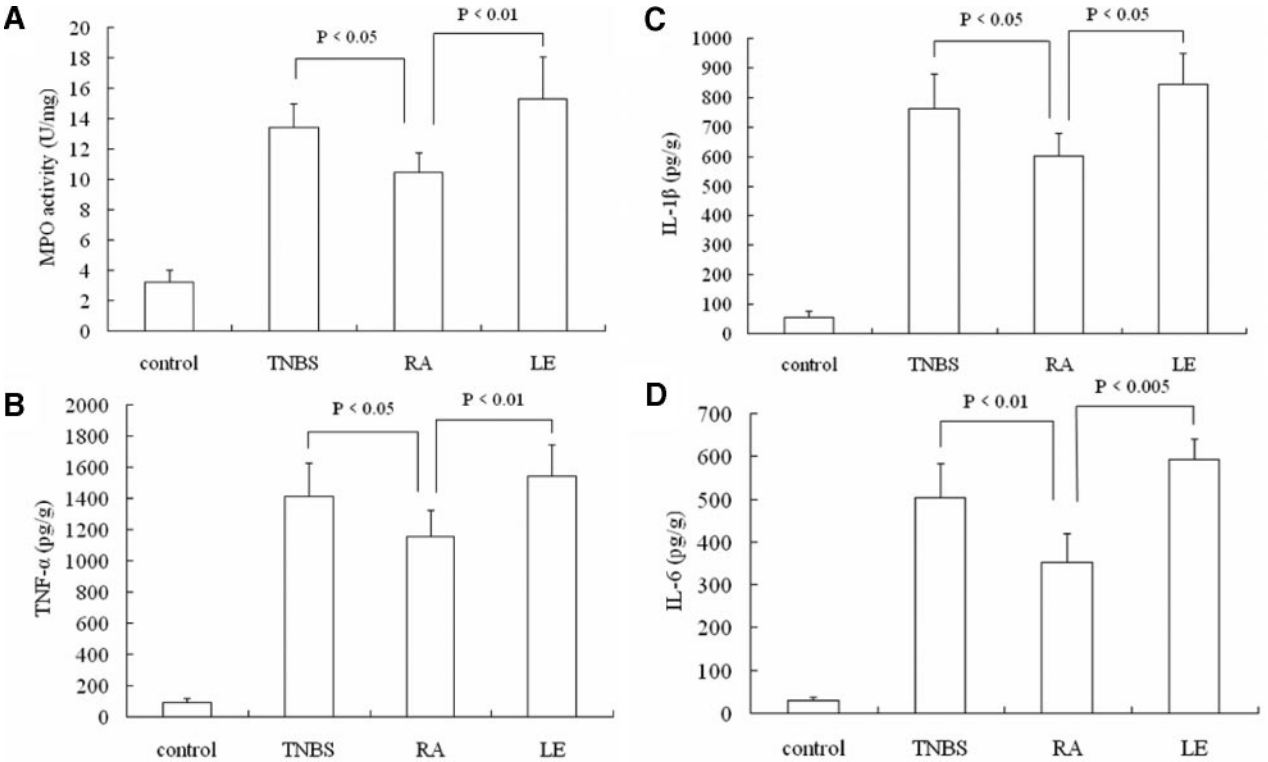

Figure 3. RA management inhibits the inflammatory responses of TNBS-induced colitis. The MPO activities (A) in colonic tissues and the concentrations of TNF-α (B), IL-1β (C), and IL-6 (D) in colonic homogenates of each group of mice were determined by MPO activity assay and specific sandwich ELISA kits, respectively; $n = 8$.

pg/g; Fig. 3, B–D). These results suggest that activating RARα by the ligand RA is able to turn off an established in vivo inflammatory response.

RARα activation regulates inflammatory response by shifting the Treg/Th17 profile in TNBS-induced colitis

Imbalance between Th17 and Treg cell in local tissues triggers the progression of TNBS-induced colitis. To investigate the development of Th17 and Treg cells in the progress of TNBS-induced colitis, we detected the expressions of FOXP3 and IL-17, two key functional molecules of Treg and Th17 cells, respectively, in colonic mucosa and MLN of active colitis at the peak of the disease using immunohistological methods. There was no detectable FOXP3 or IL-17 in normal colonic tissues, but under intestinal inflammation, some FOXP3 (2.8%±0.8%)- or IL-17 (8.0%±1.0%)-positive LPMCs were scattered within the inflamed colonic mucosa of TNBS-induced colitis, as detected by immunohistochemistry (**Fig. 4, A** and **B**). RA treatment induced more LPMCs to express FOXP3 (4.6%±1.1%; $P<0.05$ compared with the TNBS group) in the nucleus and inhibited IL-17 expression (5.2%±1.6%; $P<0.05$ compared with TNBS) in colonic mucosa, and LE135 management showed the opposite effect of RA with inhibited expression of FOXP3 (1.6%±0.9%; $P<0.005$ compared with the RA group) and increased detection of IL-17 (9.8%±2.3%; $P<0.01$ compared with RA) in LPMCs.

We also detected the expression of FOXP3 and IL-17 in MLN by immunofluorescence. As shown in Figure 4, C and D, little FOXP3 and no IL-17 are detectable in the cells of normal MLN, but colonic inflammation induced the expressions of FOXP3 and IL-17. RA treatment induced more cells to express nuclear FOXP3 and inhibited IL-17 expression in the cells of MLN, whereas LE135 management showed the reverse effect of RA with decreased numbers of FOXP3-positive cells and increased IL-17-positive cells in MLN.

TNBS-induced colitis is characterized by a high production of proinflammatory cytokines such as TNF-α and IL-1β and the Th17-type cytokine IL-17. To evaluate the effect of

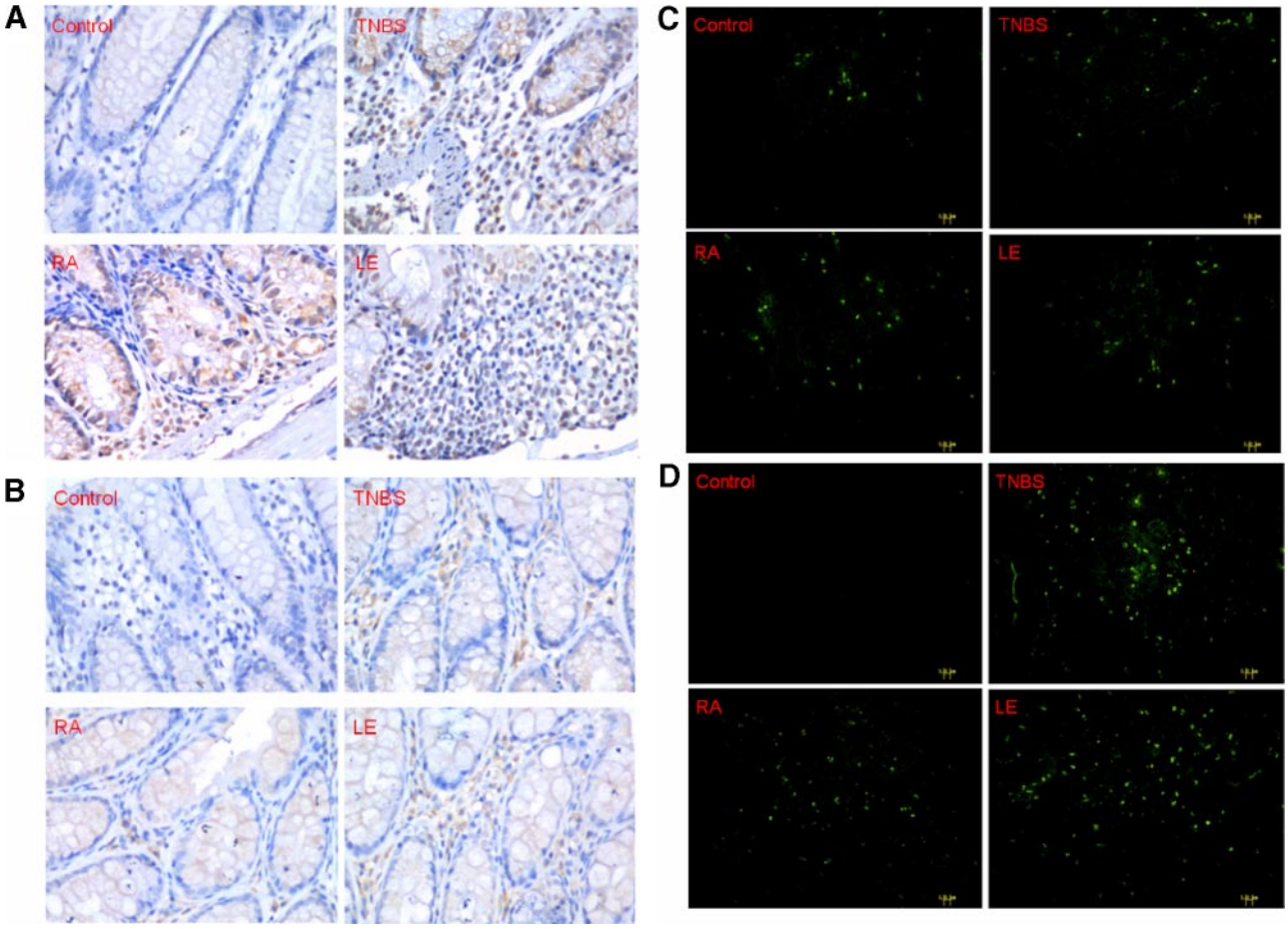

Figure 4. The expressions of FOXP3 and IL-17 in colonic tissues and MLN. TNBS colitis was induced at Day 1, and 300 μg RA or 100 μg LE135 was administered i.p. daily until the mice were killed. Seven mice in each group were killed on Day 4, and the colonic tissues and mesenteric lymph nodes were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm were cut for immunohistological study. The expressions of FOXP3 (A) and IL-17 (B) in colonic tissues of different groups were assessed by immunohistochemistry, and the expressions of FOXP3 (C) and IL-17 (D) in MLN were detected by immunofluorescence. All images are at the same original magnification (×200).

activating or inhibiting RARα on the production of proinflammatory cytokines and regulatory cytokines in TNBS-induced colitis, we isolated LPMCs from different groups with TNBS colitis, made T cell-enriched LPMCs by removing adherent cells, and cultured the cells in the absence (unstimulated) or presence (stimulated) of PMA and Con A for 48 h. As shown in **Figure 5**, the LPMCs obtained from TNBS colitis mice produced high concentrations of proinflammatory cytokines such as TNF-α, IL-1β, and IL-17, and the LPMCs from the mice given RA treatment induce the production of significantly more regulatory cytokines such as IL-10 and TGF-β and lower amounts of TNF-α, IL-1β, and IL-17. In contrast, LE135 administration resulted in LPMCs, secreting the highest levels of those proinflammatory cytokines and the least production of IL-10 and TGF-β, opposite to the effect of RA. Stimulating the cells with PMA and Con A emphasized the above results obtained without stimulation (Fig. 5). These results indicate that activating RARα by the ligand suppresses the production of proinflammatory cytokines and the Th17 cytokine response and inhibits an established in vivo inflammatory response, at least partly by modulating the Th17 and Treg cell balance. This is opposite to the effect of the antagonist of RARα, which facilitates the inflammatory response and down-regulates the production of regulatory cytokines such as IL-10 and TGF-β.

We next detected the expressions of FOXP3 and RORγt in T cell-enriched LPMCs by Western blot, the key transcriptional factors of Treg cell and Th17 differentiation [17, 30]. As shown in **Figure 6**, RA treatment inhibits the expression of RORγt but enhances FOXP3 expression in LPMCs, and LE135 management shows the reverse effect of RA, as RORγt expression is up-regulated, and FOXP3 expression is inhibited. The results indicate that by modulating the expressions of FOXP3 and RORγt in T cell-enriched LPMCs, the ligand of RARα suppresses the productions of proinflammatory cytokine and Th17 cytokine responses and drives the Th17 response toward a Treg cell-predominant profile, and the antagonist of RARα facilitates the inflammatory response by enhancing Th17 responses.

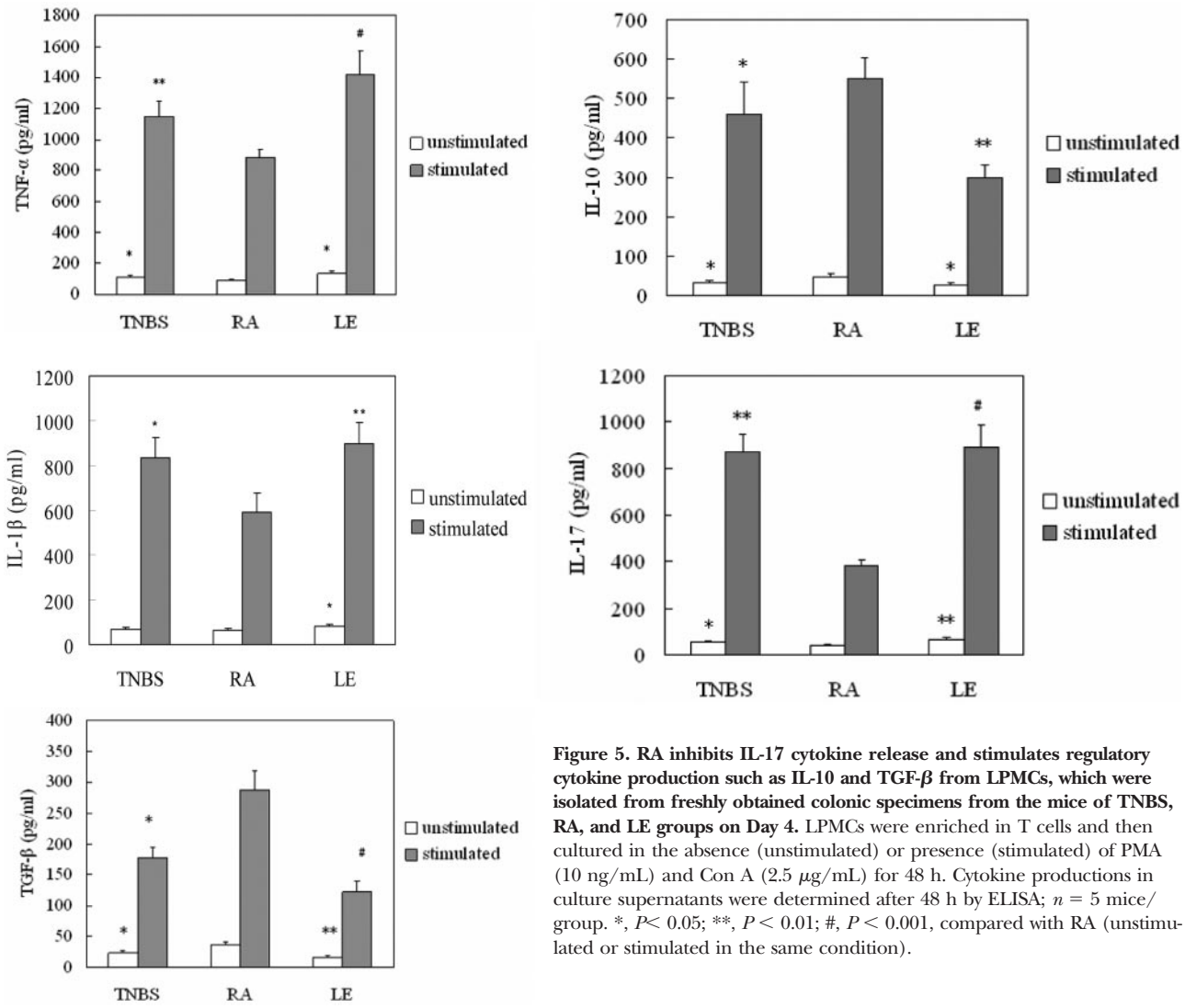

Figure 5. RA inhibits IL-17 cytokine release and stimulates regulatory cytokine production such as IL-10 and TGF-β from LPMCs, which were isolated from freshly obtained colonic specimens from the mice of TNBS, RA, and LE groups on Day 4. LPMCs were enriched in T cells and then cultured in the absence (unstimulated) or presence (stimulated) of PMA (10 ng/mL) and Con A (2.5 μg/mL) for 48 h. Cytokine productions in culture supernatants were determined after 48 h by ELISA; $n = 5$ mice/group. *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$, compared with RA (unstimulated or stimulated in the same condition).

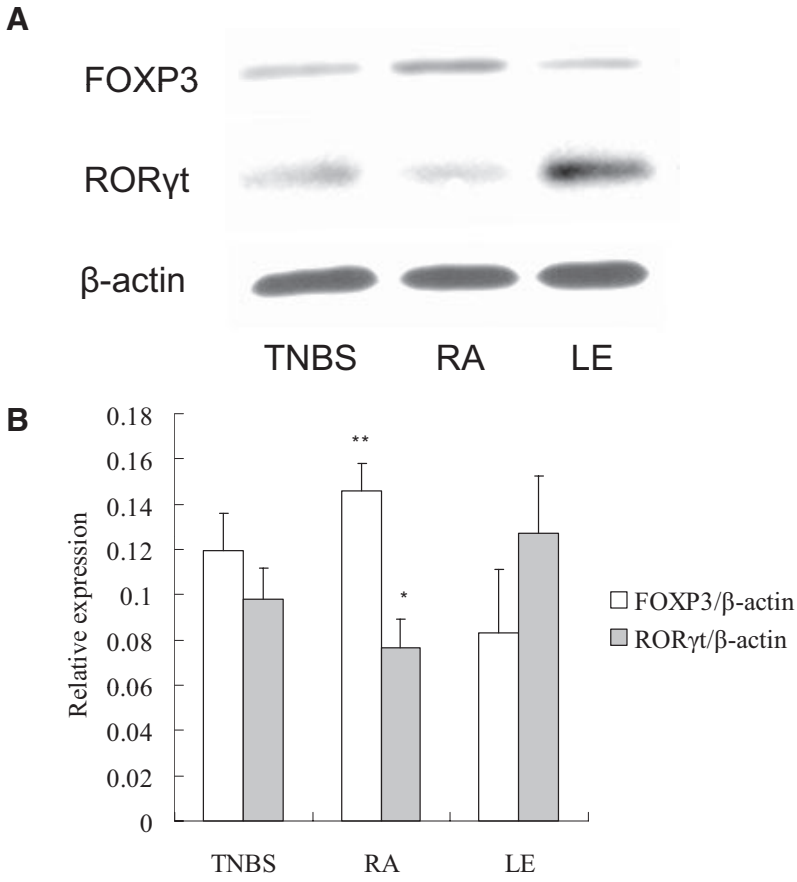

Figure 6. The expressions of FOXP3, RORγt, and β-actin in LPMCs detected by Western blot. (A) Total cell lysates of LPMCs were prepared to detect the expressions of FOXP3, RORγt, and β-actin. (B) The expressions of FOXP3 and RORγt detected by Western blot were analyzed statistically relative to β-actin expression by densitometry (n=4). *, $P < 0.05$; **, $P < 0.01$, compared with TNBS and LE135.

RA ameliorates TNBS-induced colitis in a dose-dependent manner

To investigate further the beneficial effects of varying doses of RA on TNBS-induced colitis, we induced colitis by intrarectal administration of 2.5 mg TNBS in 50% ethanol and then treated the mice with different doses of RA (20 μg, 100 μg, or 300 μg) i.p. 2 h after TNBS instillation and repeated the treatment daily until the mice were killed on Day 7. Treatment with 100 μg or 300 μg RA succeeds in reducing body weight loss of the mice (**Fig. 7A**). This treatment also reduced TNBS-induced colon inflammation, as seen by increased colon length and decreased inflammatory score, MPO activity, and TNF-α concentration when compared with the control group (Fig. 7, B–E). Furthermore, the therapeutic effect of RA was dose-dependent, showing maximal effects at doses between 100 μg and 300 μg for each mouse with TNBS-induced colitis.

DISCUSSION

Recently, a subset of T cells, Th17 cells, has been well studied and shown to have a crucial role in the induction of autoimmune tissue injury [38, 39]. In contrast, another subset of T cells, Treg cells, inhibits autoimmunity and protects against tissue injury [15, 16]. The differentiation of naïve Th cells into Th17 or Treg cells is driven by several factors. For example, TGF-β is a critical differentiation factor for the generation of Treg cells [40] and also directs FOXP3 expression, which is a specific marker in Treg cells and is responsible for the function of these cells [17, 41]. On the other hand, TGF-β, acting together with IL-6, induces the differentiation of pathogenic Th17 cells from naive T cells [42, 43]. Other cytokines, such as IL-21 and IL-23, can also drive Th17 cell differentiation [20, 21]. As well, Th cell differentiation is manipulated by activation of some nuclear factors. For example, FOXP3 directs Treg cell differentiation and induces the production of regulatory cytokines such as TGF-β and IL-10 [17, 44], and RORγt dominates Th17 cell formation and IL-17 production [45, 46]. Other factors, such as RARα, manipulate FOXP3 and RORγt expression. When activated by the ligand, RARα can modulate the expressions of FOXP3, IL-17, and/or RORγt genes directly, thus causing induction of FOXP3 and inhibition of Th17 polarization [30, 32]. By activating the receptor RARα, RA has been reported to promote Treg cell growth, differentiation, and gut homing [29] and inhibit the formation of Th17 cells [30, 31]. However, in inflammatory responses such as IBD, the role of the agonists of RARα in modulating Th17 and Treg cell differentiation has not been explored thoroughly. Here, we show that in the progress of UC and the development of TNBS-induced colitis, RA, the agonist of RARα, succeeds in favoring FOXP3 expression and inhibiting IL-17 production, and LE135, the antagonist of RARα, has the opposite effect. This implies that the rehabili-

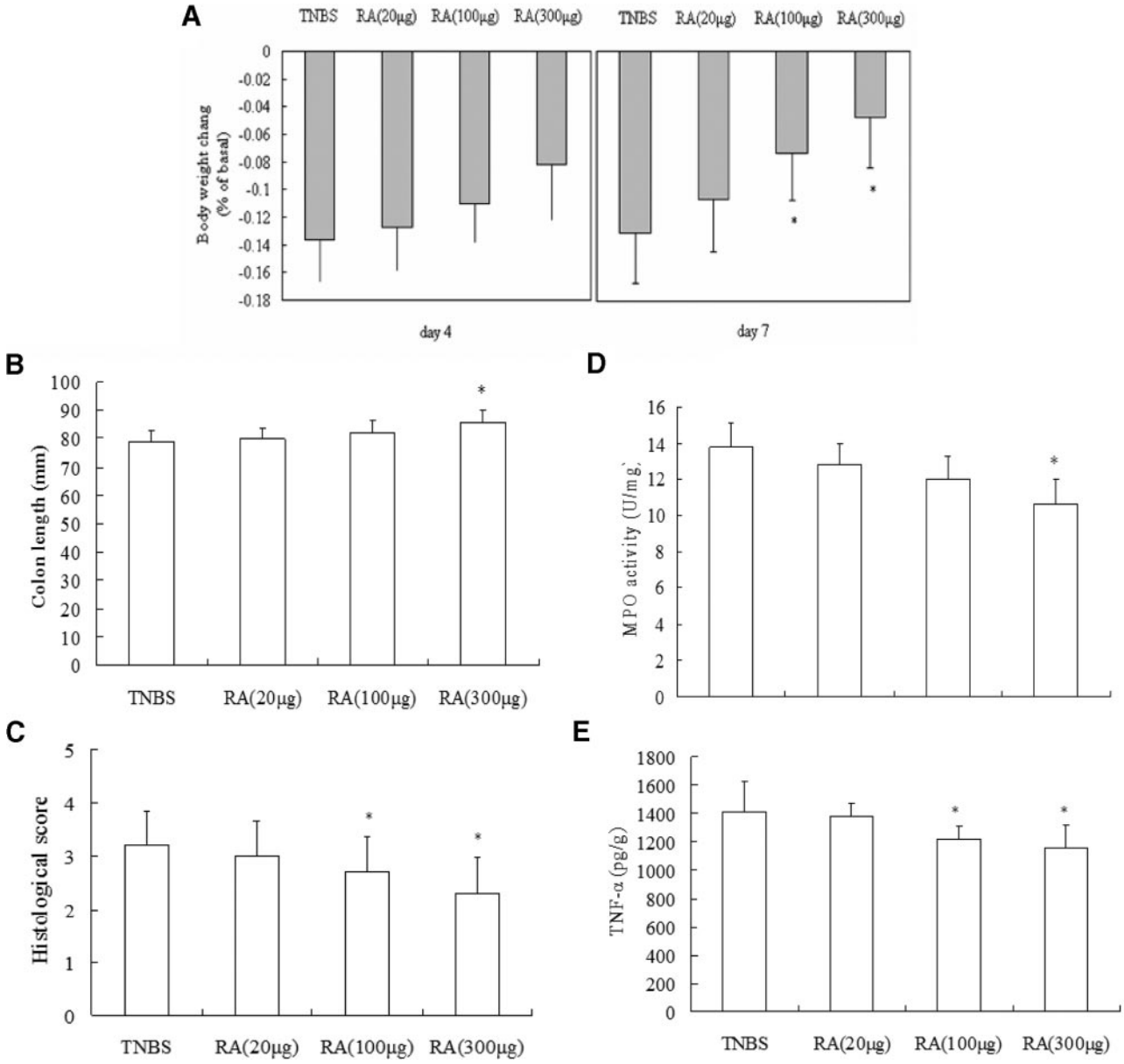

Figure 7. Treatment with different doses of RA protects the mice against TNBS-induced colitis, which was induced in mice by intrarectal administration of 2.5 mg TNBS in 50% ethanol. Different doses of RA (20 μg, 100 μg, or 300 μg) were administered i.p. 2 h after TNBS instillation and repeated daily until the mice were killed on Day 7. Control mice received 50% ethanol alone; $n = 9$ mice/group. Disease severity was monitored by body weight change at Days 4 and 7 (A), the length of whole colon (B), and the histological score (C). Colonic tissues were homogenated and MPO activity and TNF-α content determined as described in Materials and Methods. *, $P < 0.05$, compared with TNBS mice.

tating effect of the agonist of RARα in IBD works through restoring the balance between Th17 and Treg differentiation.

To explore the regulation of cytokine production by the agonist or antogonist of RARα, we cultured T cell-enriched LPMCs from different groups of mice with TNBS-induced colitis and determined the concentration of proinflammatory cytokines such as TNF-α, IL-1β, and IL-17 and regulatory cytokines such as IL-10 and TGF-β. LPMCs from the mice with RA treatment secreted lower concentrations of proinflammatory cytokines and produced higher concentrations of regulatory cytokines compared with those cells from the untreated colitis group. Conversely, LE135 administration resulted in LPMCs producing the highest levels of proinflammatory cytokines and the lowest levels of regulatory cytokines. When LPMCs were stimulated by PMA and Con A, the difference in production of cytokines among the groups was emphasized. As IL-17 is produced mainly by Th17 cells and regulatory cytokines by Treg cells, the in vitro study indicates that the agonist of RARα directs the production of regulatory cytokines and abrogates the production of IL-17 in the progress of experimental colitis, in accordance with the expressions of FOXP3 and IL-17 in mesenteric lymph and colonic tissues. The results demonstrate that

in intestinal inflammation, RARα activation by RA favors Treg function and inhibits the formation and production of Th17 cells.

Proinflammatory cytokines, such as TNF-α, contribute positively to the progression of IBD and experimental colitis in animal models of IBD [47–49], and blockade of TNF-α bioactivity by specific antibodies such as infliximab can down-regulate the inflammatory response and limit the tissue damage of IBD and experimental colitis [50, 51]. As the production of TNF-α in inflamed tissues is driven by IL-17 but inhibited by the regulatory cytokines [8, 52], we detected in vitro the effects of the agonist and antagonist of RARα on the production of TNF-α in colonic mucosa of active UC. RA, the ligand of RARα, down-regulated the production of TNF-α, whereas LE135, the antagonist of RARα, facilitated TNF-α expression. Next, we tested the therapeutic efficacy of RA in ameliorating TNBS-induced colitis in mice. The beneficial, therapeutic effect was observed in the murine model of colitis, including decreased weight loss and improvement in the colitis histological score. This was in accordance with the down-regulated production of TNF-α in inflamed colonic mucosa. The colitic mice given the antagonist of RARα showed the highest histological score, with severe mucosal tissue damage, intensive immune cell infiltration, as well as the highest MPO activity and TNF-α concentration in inflamed colonic mucosa. Furthermore, the therapeutic effect of RA was dose-dependent, showing maximal effects at doses between 100 μg and 300 μg for each mouse with TNBS-induced colitis. The results provide evidence of the therapeutic effect of RA on IBD and experimental colitis and indicate that activation of RARα by its agonists is able to down-regulate the production of proinflammatory cytokines, thereby turning off the immune response of active UC and experimental colitis.

In summary, we have reported that by modulating the expression of FOXP3 and IL-17 in inflamed tissues, RA, as the ligand of RARα, can down-regulate the inflammatory response of active UC in vitro and ameliorate acute TNBS-induced colitis in mice. This suggests that RARα ligand-based pharmaceutical strategies may offer a promising alternative to our current approaches of managing IBD.

AUTHORSHIP

A. B. is the guarantor of the article and planned the study and executed the study design, data analysis, and writing of the article. N. L. planned the study and completed data collection. Y. G. performed laboratory analyses and interpretation of the data. J. C. fulfilled laboratory analyses. Z. P. implemented laboratory analyses, planned the study, and completed the final review of the manuscript.

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DISCLOSURE

There are no competing interests.

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