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Tangeretin Inhibits IL-12 Expression and NF-κB Activation in Dendritic Cells and Attenuates Colitis in Mice

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ABSTRACT

In the preliminary study, tangeretin (5,6,7,8,4′-pentamethoxy flavone), a major constituent of the pericarp of *Citrus* sp., inhibited TNF-α, IL-12, and IL-23 expression and nuclear factor kappa-B activation in lipopolysaccharide-stimulated dendritic cells; however, it did not affect IL-10 expression. Furthermore, tangeretin (5, 10, and 20 μM) suppressed the activation and translocation of nuclear factor kappa-B (p65) into the nuclei *in vitro* by inhibiting the binding of lipopolysaccharide on dendritic cells. Oral administration of tangeretin (10 and 20 mg/kg) suppressed the inflammatory responses, such as nuclear factor kappa-B and mitogen-activated protein kinase activation and myeloperoxidase activity, in the colon of mice with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Tangeretin increased 2,4,6-trinitrobenzene sulfonic acid-suppressed expression of tight junction proteins occludin, claudin-1, and ZO-1. Tangeretin also inhibited 2,4,6-trinitrobenzene sulfonic acid-induced differentiation of Th1 and Th17 cells as well as the expression of T-bet, RORγt, interferon-γ, IL-12, IL-17, and TNF-α. However, tangeretin increased 2,4,6-trinitrobenzene sulfonic acid-suppressed differentiation of regulatory T cells as well as the expression of Foxp3 and IL-10. These results suggest that oral administration of tangeretin may attenuate colitis by suppressing IL-12 and TNF-α expression and nuclear factor kappa-B activation through the inhibition of lipopolysaccharide binding on immune cells such as dendritic cells.

Introduction

IBD, including ulcerative colitis and Crohn's disease, is a chronically relapsing inflammatory disease of the gastrointestinal (GI) tract [1]. The pathogenesis of IBD involves genetic susceptibility, host innate and adaptive immunity, and gut microbiota [2, 3]. The stimulation of commensal and infected microbes is continuously defended by the gut immune system, which consists of neutrophils, macrophages, DCs, and T cells involved in innate and adaptive immunity [2–4]. These immune cells detect microorganisms and respond to pathogen-associated molecular patterns. Activated APCs, including DCs and macrophages, present antigens, which include antigenic proteins from pathogens, to T cells involved in adaptive immunity, and stimulate the differentiation of naïve CD4+ T cells into effector T cells, such as Th1, Th17, and Tregs, by the secretion of cytokines, such as TNF-α, IL-10, and IL-12, in the immune cells, including APCs [5, 6]. TNF-α, IL-12, and IL-17 are highly expressed in the inflamed colons of mice and humans with IBD; however, IL-10 expression is downregulated, leading to colitis [7, 8]. Therefore, the downregulation of IL-12 and TNF-α expression compared to IL-10 expression may be important for the prevention and treatment of colitis.

Polymethoxy flavonoids (PMFs), including nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) and tangeretin (5,6,7,8,4′-pentamethoxy flavone), are widely distributed in the pericarp of *Citrus* sp., such as *Citrus unshiu, Citrus reticulata,* and *Citrus depressa* (Rutaceae) [9, 10]. They exhibit various biological activities, including anti-inflammatory [11–13], anticancer [10], hypolipidemic [9], antiobesity [14], and neuroprotective effects [12].

ABBREVIATIONS

APC	antigen-presenting cell
COX	cyclooxygenase
DC	dendritic cell
IBD	intestinal bowel disease
IKKβ	inhibitor of nuclear factor kappa-B kinase subunit beta
iNOS	inducible NO synthetase
IRAK1	interleukin 1 receptor-associated kinase 1
LPS	lipopolysaccharide
TAK1	transforming growth factor beta-activated kinase 1
TNBS	2,4,6-trinitrobenzene sulfonic acid
Treg	regulatory T cell

They also ameliorate scratching behavioral reactions by inhibiting the action of histamine as well as the activation of the transcription factors NF-κB and AP-1 via protein kinase C [15]. Of these, tangeretin inhibits LPS-induced expression of inflammatory mediators in RAW264.7 cells by suppressing NF-κB activity [16]. However, the anti-colitic effects of tangeretin and its anti-inflammatory mechanism in DCs have not been studied.

In the preliminary study, tangeretin strongly inhibited the ratio of IL-12 or TNF-α to IL-10 expression in LPS-stimulated DCs. Therefore, we investigated the anti-colitic effect of tangeretin (▶ **Fig. 1**) in mice with TNBS-induced colitis.

Results

First, we investigated the effect of tangeretin on IL-12 and TNF-α expression and NF-κB activation in LPS-stimulated DCs (▶ **Fig. 2**). The stimulation of LPS in bone marrow-derived DCs significantly increased TNF-α, IL-10, IL-12, and IL-23 expression as well as NF-κB activation. In contrast, tangeretin at a concentration of 20 µM inhibited LPS-stimulated TNF-α, IL-12, and IL-23 expression and NF-κB activation by 79, 69, 59, and 90 %, respectively; however, it did not significantly affect IL-10 expression. Thus, tangeretin inhibited the ratios of IL-12 to IL-10 and of TNF-α to IL-10 expression in LPS-stimulated DCs. Tangeretin also inhibited the activation of NF-κB activation and the expression of iNOS and COX-2 in LPS-stimulated DCs.

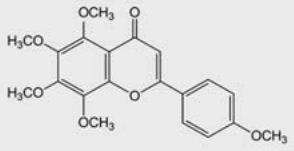
Next, to confirm the effect of tangeretin on NF-κB activation, we measured the effect of tangeretin on the translocation of NF-κB into the nucleus in LPS-stimulated DCs using a confocal microscope. The stimulation with LPS in DCs significantly increased NF-κB translocation into the nuclei. Tangeretin (5, 10, and 20 µM) significantly inhibited the translocation of NF-κB (p65). Tangeretin (20 µM) showed no cytotoxic effects against the DCs under the experimental conditions (**Fig. S1**, Supporting Information).

We next examined the inhibitory effect of tangeretin against the TLR4/NF-κB signaling pathway in LPS-stimulated DCs (▶ **Fig. 3**). Tangeretin (10 and 20 µM) inhibited LPS-stimulated phosphorylation of IKKα/β, IκBα, TAK1, and IRAK1. Nonetheless, TLR4 expression was not affected. Moreover, tangeretin inhibited LPS-stimulated activation of mitogen-activated protein kinases (ERK, JNK, and p38). Therefore, we investigated the effect of tangeretin on the binding of Alexa Fluor 488-conjugated LPS on TLR4 in DCs using a flow cytometer (▶ **Fig. 4A**). Treatment with Alexa Fluor 488-labeled LPS significantly shifted the DC population on the forward scatter. However, treatment with tangeretin at concentrations of 5 and 20 µM significantly prevented the shift of DCs by 28 and 78 %, respectively. To confirm the inhibitory effect of tangeretin on the binding of LPS to the TLR4 of DCs, we used a confocal microscope for measuring (▶ **Fig. 4B**). Tangeretin also inhibited the binding of Alexa Fluor 488-conjugated LPS to the surface of DCs.

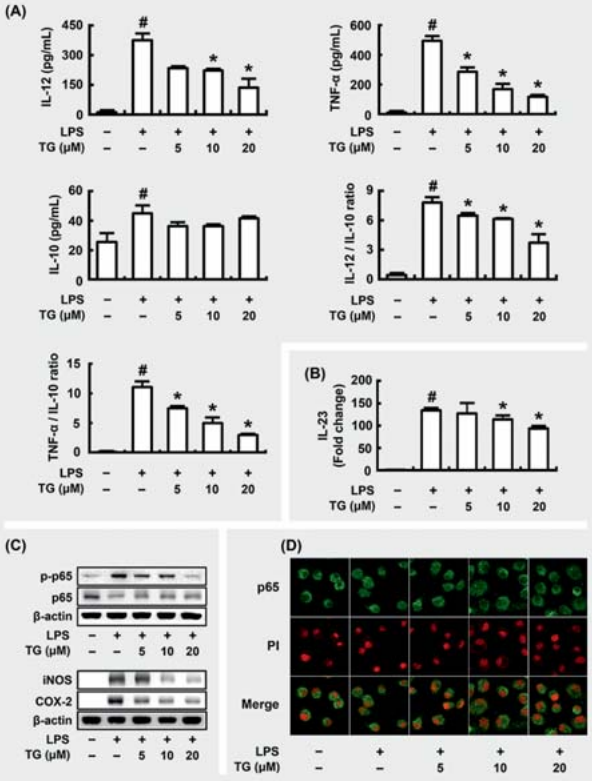
We also examined whether tangeretin could regulate MHC II and costimulatory signal molecules for the activation and survival of T cells involved in the adaptive immunity in DCs (▶ **Fig. 4C**). Tangeretin significantly inhibited LPS-induced MHC II, CD40, CD80, and CD86 expression, while the stimulation of LPS also increased these molecules.

Next, we investigated the anti-inflammatory effect of tangeretin in mice with TNBS-induced colitis. The intrarectal injection of TNBS caused severe colitis, including colon shortening, and an increase in colonic myeloperoxidase activity (▶ **Fig. 5**). Tangeretin suppressed TNBS-induced body weight loss and colon shortening. Tangeretin (20 mg/kg) inhibited TNBS-induced myeloperoxidase activity by 77 %. Tangeretin also inhibited TNBS-induced edema and epithelial cell disruption. Tangeretin inhibited TNBS-induced infiltration of activated APCs including DCs, which were immunostained with the anti-CD86 antibody. However, tangeretin increased TNBS-suppressed expression of tight junction proteins ZO-1, occludin, and claudin-1.

TNBS treatment increased the activation of NF-κB and MAPKs (▶ **Fig. 6**). Treatment with tangeretin (10 and 20 mg/kg) inhibited TNBS-induced phosphorylation of TAK1 and IκB-α as well as the activation of NF-κB, ERK, JNK, and p38. Furthermore, tangeretin inhibited TNBS-induced expression of iNOS and COX-2. Tangeretin inhibited this TNBS-induced expression of TNF-α, IL-12, IL-17, and IFN-γ expression in the colon; however, it increased IL-10 expression. The anti-colitic effect of tangeretin was comparable to that of sulfasalazine. Moreover, treatment with TNBS increased the differentiation of Th1 and Th17 cells and suppressed the number of Tregs in the lamina propria of the mouse colon (▶ **Fig. 7**). Treatment with tangeretin suppressed TNBS-induced differentiation of Th1 and Th17 cells; however, it increased TNBS-suppressed differentiation of Tregs. We also measured the expression levels of the Th cell differentiation markers IFN-γ, IL-10, IL-17, T-bet, RORγt, and Foxp3 by quantitative polymerase chain reaction (qPCR). Tangeretin significantly suppressed TNBS-induced expression of IFN-γ, IL-17, T-bet, and RORγt in the colon; however, it increased TNBS-suppressed expression of Foxp3 and IL-10. Therefore, to understand whether tangeretin could directly differentiate T cells, we incubated splenocytes in the absence or presence of tangeretin and measured the mRNA levels of the representative transcription factors T-bet, RORγt, and Foxp3 and cytokines IFNγ, IL-17, and IL10 of Th1, Th2, and Tregs (**Fig. S2**, Supporting Information). Tangeretin at a concentration of 20 µM



▶ **Fig. 1** The structure of tangeretin.

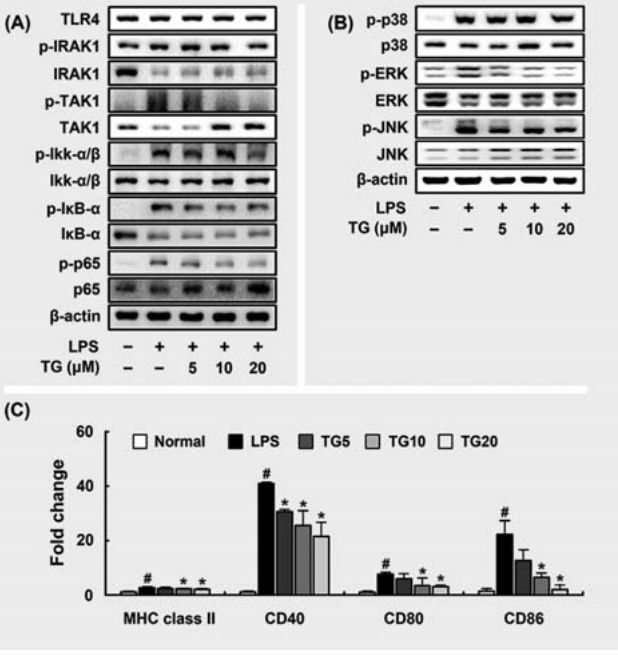


▶ **Fig. 2** Anti-inflammatory effect of tangeretin in LPS-stimulated bone marrow-drived DCs. **A** Effect on TNF-α, IL-10, and IL-12 expression, using ELISA. **B** Effect on the ratios of TNF-α or IL-12 to IL-10 expression. **C** Effect on IL-23 expression, using qPCR. **D** Effect on NF-κB activation and iNOS and COX-2 expression. **E** Effect on NF-κB (p65) nuclear translocation. DCs were incubated with or without LPS [200 ng/mL, in the absence or presence of tangeretin (TG, 5, 10, or 20 μM)]. All values are the mean ± SD (n = 4). #P < 0.05 vs. normal control group; *p < 0.05 vs. LPS alone-treated group.

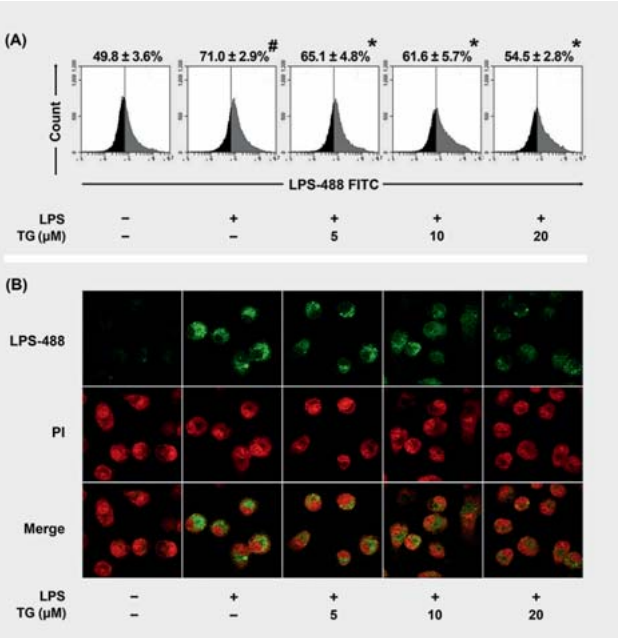
weakly increased Foxp3 and IL-10 expression and suppressed RORγt expression.

Discussion

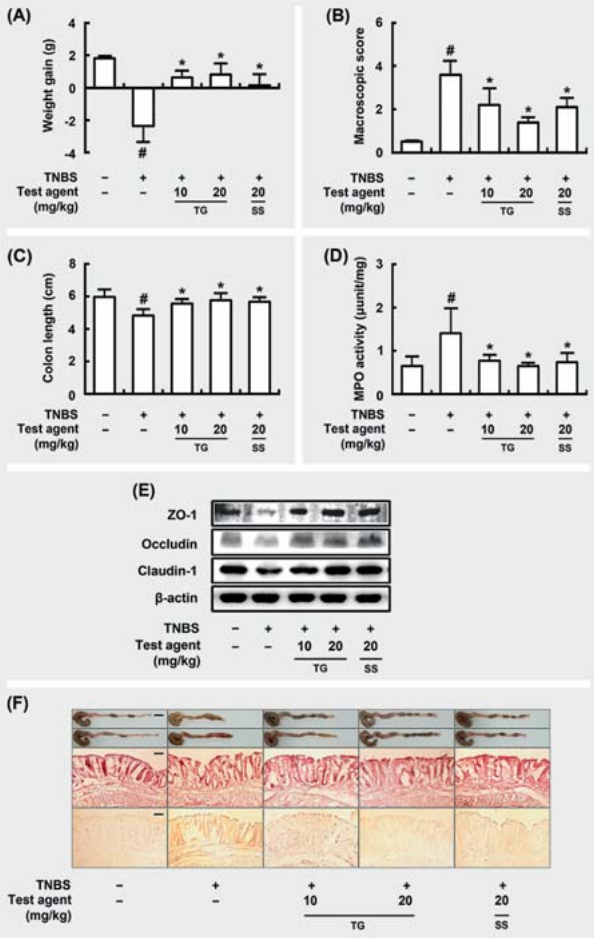
DCs are activated by the stimulation of pathogen infections or tissue injuries [5]. Activated DCs stimulate the adaptive immune response, including the differentiation of Th17 cells and Tregs,



▶ **Fig. 3** Inhibitory effect of tangeretin on NF-κB and MAPK signal pathways in LPS-stimulated DCs. **A** Effect on the TLR4/NF-κB signaling pathway. **B** Effect on the MAPKs signaling pathway. DCs were stimulated with LPS in the absence or presence of tangeretin (5, 10, and 20 μM). Proteins were analyzed by immunoblotting.



▶ **Fig. 4** Effect of tangeretin on the binding of LPS to TLR-4 on DCs. DCs were incubated with LPS-488 binding (FITC-FL1 fraction) in the absence or presence of tangeretin (TG, 5, 10, or 20 μM) and detected by a flow cytometer (**A**) and a confocal microscope (**B**). All values are the mean ± SD (n = 3). #P < 0.05 vs. normal control group; *p < 0.05 vs. LPS alone-treated group.



▶ **Fig. 5** Effects of tangeretin and sulfasalazine on body weight (**A**), macroscopic disease (**B**), colon length (**C**), myeloperoxidase (MPO) activity (**D**), tight junction proteins (**E**), and histological examination and immunostaining (**F**) in mice with TNBS-induced colitis. Mice was treated with or without TNBS (normal control group) and subsequently treated with saline, tangeretin (TG, 10 or 20 mg/kg), or sulfasalazine (SS, 20 mg/kg). Bars in (**F**) indicate 1 cm (top) and 0.1 mm (middle and bottom). All data are the mean ± SD (n = 6). $^{\#}P < 0.05$ vs. the normal control group; $^{*}p < 0.05$ vs. the TNBS alone-treated group.

through antigen presentation and cytokine secretion of TNF-α, IL-1β, and IL-12 [6, 8]. The differentiated Th17 cells secrete IL-17 and IL-22. IL-17 increases the recruitment of monocytes and neutrophils to the site of inflammation, stimulates Th17 cell differentiation, and acts synergistically with proinflammatory cytokines [7, 8]. Therefore, DCs play an important role in chronic inflammatory diseases such as IBD. This has been supported by reports that the inhibitors of NF-κB activation, such as aminosalicylates and prednisolone, ameliorate IBD [16, 17].

In the present study, we found that tangeretin inhibited the ratio of IL-12 or TNF-α to IL-10 by inhibiting IL-12 and TNF-α expression and NF-κB activation in activated DCs. Moreover, tangeretin inhibited LPS-induced IL-23 expression. In previous studies, tangeretin was shown to inhibit LPS-induced TNF-α, IL-1β, and IL-6 production in microglia cells [18], as well as LPS-induced NO production in RAW264.7 cells [19]. Tangeretin also inhibits LPS-induced activation of NF-κB and MAPKs (ERK, JNK, and p38) in microglial cells [20]. Tangeretin has been shown to inhibit LPS- and IgE-induced NF-κB and AP1 activation in mast cells and RBL-2H3 cells (basophils) [15, 21]. These results suggest that tangeretin may inhibit TNF-α and IL-12 expression by inhibiting NF-κB activation.

Tangeretin potently attenuated colitis parameters such as colon shortening, myeloperoxidase activity, and NF-κB and MAPKs activation in the colon of mice as well as IFN-γ, TNF-α, IL-17, COX-2, and iNOS expression. Tangeretin also inhibited TNBS-induced differentiation of Th1 and Th17 cells and increased TNBS-suppressed differentiation of Tregs. Furthermore, tangeretin inhibited T-bet and RORγt expression; however, it increased TNBS-suppressed Foxp3 and IL-10 expressions. Jang et al. reported that tangeretin inhibited histamine- and compound 48/80-induced NF-κB and AP-1 in mice [15]. Xu et al. reported that tangeretin inhibited NF-κB activation in respiratory syncytial virus-infected mice [22]. Choi et al. reported that cirus extract, which contains nobiletin and tangeretin, inhibited TNF-α expression in mice with ethanol-induced liver injury [23]. These results suggest that tangeretin may attenuate colitis by regulating the innate immune responses. Additionally, tangeretin inhibited LPS-induced expression of MHC II, an antigen-presenting molecule for Th cells, and costimulatory signaling molecules CD40, CD80, and CD86. However, tangeretion weakly inhibited the Th17 transcription factor RORγt, not Th1 transcription factor T-bet, in splenocytes, while tangeretion weakly increased Treg transcription factor Foxp3 expression. These results suggest that tangeretin may suppress IL-12 and TNF-α expression in immune cells such as DCs involved in innate immunity rather than T cell differentiation involved in adaptive immunity, resulting in the suppression of Th1 and Th17 cell differentiation involved in adaptive immunity.

Based on these findings, tangeretin may attenuate colitis by inhibiting IL-12 and TNF-α expression and NF-κB activation in DCs, thereby signifying its potential in augmenting IBD.

Materials and Methods

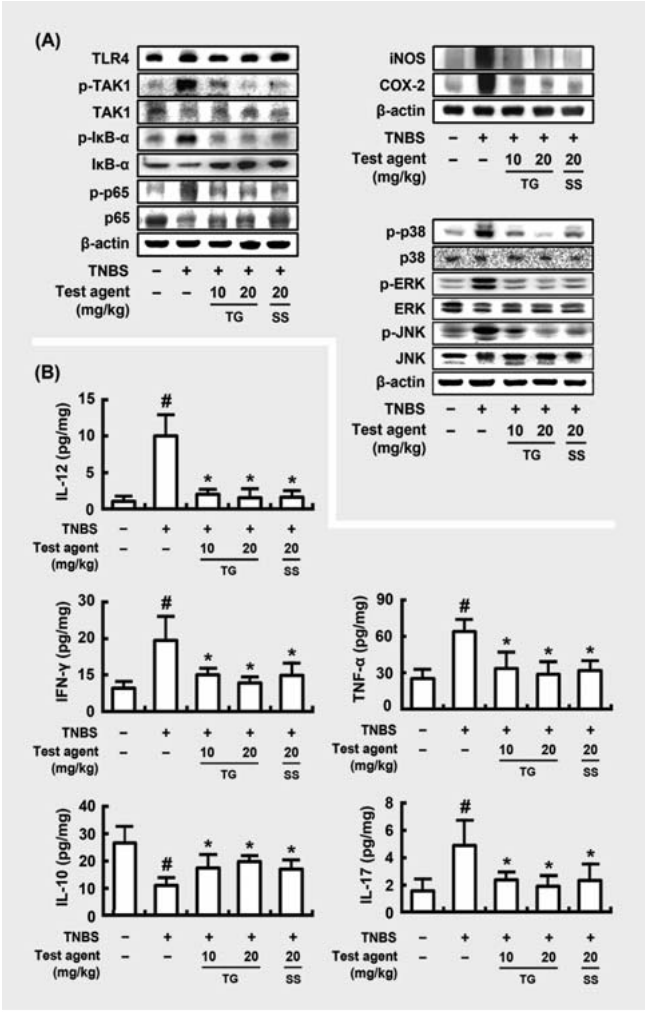
Materials

TNBS, LPS purified from *Escherichia coli* O111:B4, collagenase type VIII, RPMI 1640, radioimmunoprecipitation assay (RIPA) buffer, and tetramethyl benzidine were purchased from Sigma-Aldrich. Antibodies for immunoblotting were purchased from Cell Signaling Technology. FBS was purchased from PAN Biotech. ELISA kits were purchased from R&D Systems. The mRNA isolation kit was purchased from Qiagen. Other chemicals used were of the highest grade available.

Isolation of tangeretin

Tangeretin was isolated from the dried fruit peels of *Citrus tachibana* (1 kg) according to the previously reported method of Jang et al. [15].

Tangeretin (purity > 95 %) – light yellow needles; m. p. 153–154 °C; EI-MS, *m/z* 372 (M+).



▶ **Fig. 6** Effects of tangeretin and sulfasalazine on the activation of NF-κB and MAPKs, expression of iNOS and COX-2 (**A**), and levels of inflammatory cytokines (**B**) in mice with TNBS-induced colitis. Mice was treated with or without TNBS (normal control group) and subsequently treated with saline, tangeretin (TG, 10 or 20 mg/kg), or sulfasalazine (SS, 20 mg/kg). iNOS, COX-2, and NF-κB and MAPKs signaling molecules were determined by immunoblotting. TNF-α, IL-10, IL-12, IL-17, and interferon-γ (IFN-γ) were determined by ELISA. All values are the mean ± SD (n = 6). $^{\#}P < 0.05$ vs. the normal control group; $^{*}p < 0.05$ vs. the TNBS alone-treated group.

Animals

Male C57BL/6 (20–22 g, 6 weeks) were supplied from RaonBio, Inc. and acclimatized for 7 days before the experiments. All animals were housed in wire cages at 20–22 °C and 50 ± 10 % humidity, and fed standard laboratory chow and water *ad libitum*.

All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals in the Kyung Hee University [January 28, 2015; IRB No. KHUASP(SE)-15–098] and performed in accordance with the Kyung Hee University guidelines for Laboratory Animals Care and Usage.

Preparation of bone marrow dendritic cells

Bone marrow cells were isolated from the femurs and tibias of mice and washed with RPMI 1640 according to the modified method described by Lutz et al. [24]. Briefly, for differentiation of the bone marrow cells into DCs, the cells (2×10^6 cells/well) were seeded in a 12-well plate and cultured in RPMI 1640 containing 10 % FBS, 1 % antibiotic-antimycotic, 150 µg/mL gentamicin, and 20 ng/mL rGM-CSF. To examine the anti-inflammatory effect of tangeretin, the DCs were fed with the medium on days 3 and 6. The DCs were stimulated with 200 ng/mL of LPS in the absence or presence of tangeretin (5, 10, and 20 µM) for 90 min (for NF-κB and MAPKs) or 24 h (for IL-10, IL-12, and TNF-α) on day 8.

Preparation of experimental colitic mice

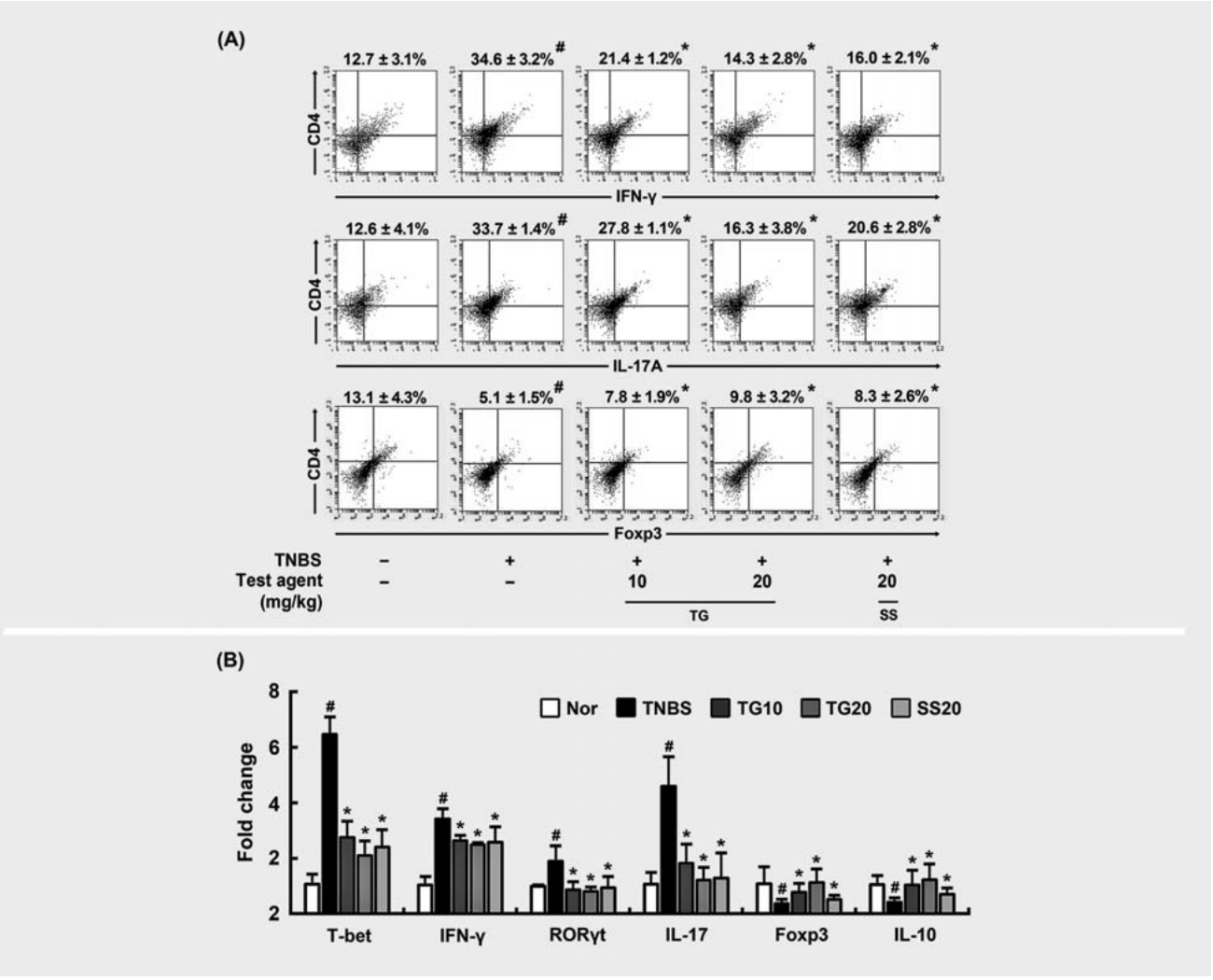
After acclimation for seven days, the mice were randomly divided into six groups: one normal control group and four TNBS-induced colitis groups treated with vehicle, tangeretin (10 or 20 mg/kg), or sulfasalazine (20 mg/kg). Each group consisted of six mice. Colitis was induced by the intrarectal administration of 2.5 % (w/v) TNBS solution (100 µL, dissolved in 50 % ethanol) into the colon [25]. The normal group was treated with saline instead of TNBS. To evenly distribute TNBS within the colon, the mice were held in a vertical position for 30 s after the TNBS administration. Saline, tangeretin, or sulfasalazine dissolved in 2 % Tween 20 was administered once a day for 3 days after treatment with TNBS by oral gavage. Mice were sacrificed 18 h after the final administration of tangeretin or vehicle. The colon was removed and opened up longitudinally. The colitis grade (0 to 5) was macroscopically scored, as previously reported [25]. The colons were gently washed with ice-cold PBS and were stored at − 80 °C until used in the experiment.

Assay of myeloperoxidase activity

Mouse colons were homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 % hexadecyl trimethyl ammonium bromide, and centrifuged (20 000 × *g*, 4 °C for 10 min) [25]. The supernatant (50 µL) was added to the reaction mixture containing 0.1 mM H_2O_2 and 1.6 mM tetramethyl benzidine, incubated at 37 °C for 3 min, and then the absorbance was monitored at 650 nm for 5 min. The myeloperoxidase activity was calculated as the quantity of enzyme degrading 1 µmol/mL of peroxide, and expressed in unit/mg protein.

Quantitative polymerase chain reaction

Reverse transcription was performed with total RNA (2 µg) isolated from the colon according to the method described by Lim et al. [25]. Real-time PCR for IFN-γ, IL-10, IL-17, Foxp3, RORγt, T-bet, and GAPDH was performed as described previously [25, 26], utilizing a Takara thermal cycler, which used SYBER premix agents. Thermal cycling conditions were as follows: activation of DNA polymerase at 95 °C for 5 min, followed by 32 cycles of amplification at 95 °C for 10 s and at 60 °C for 30 s. The normalized expression of the assayed genes, with respect to β-actin, was computed for all samples by using a Microsoft Excel data spreadsheet.



▶ **Fig. 7** Effects of tangeretin and sulfasalazine on the differentiation of Th cells into Th17 and Treg cells and expression of their transcription factors and cytokines in mice with TNBS-induced colitis. **A** Effects on Th1, Th17, and Treg cell differentiation. **B** Effects on the expression of Th cell cytokines and their transcription factors. Mice were treated with or without TNBS (normal control group) and subsequently treated with saline, tangeretin (TG, 10 or 20 mg/kg), or sulfasalazine (SS, 20 mg/kg). Th1, Th17, and Treg cells were then analyzed by flow cytometry. IL-10, IL-17, IFN-γ, T-bet, RORγt, and Foxp3 were determined by qRT-PCR. All values are the mean ± SD (n = 6). $^{\#}P < 0.05$ vs. the normal control group; $^{*}p < 0.05$ vs. the TNBS alone-treated group.

Statistical analysis

Data were analyzed using SPSS statistical software version 23.0 produced by SPSS, Inc. All data are indicated as the mean ± standard deviation (SD), with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test ($p < 0.05$).

Supporting information

Methods for flow cytometry and confocal microscopy, flow cytometry of Th1, Th17, and Tregs in the lamina propria of mouse colons, histological examination, ELISA, and immunoblotting are available as Supporting Information.

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Conflict of Interest

The authors have declared no conflict of interest.

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