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

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-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 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].
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 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 nuclei 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 proteinsZO-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...
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,
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 and MAPKs 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).

**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+).
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 rmCSF. 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 H2O2 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.
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.

References

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.


Noursargh S, Alon R. Leukocyte migration into inflamed tissues. Immunity 2014; 41: 694–707


