Original Article

Nobiletin suppresses the development of experimental autoimmune encephalomyelitis mediated by modulation of T helper 17 cell differentiation

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Multiple sclerosis is an organ-specific autoimmune disease that targets the myelin antigen in the central nervous system. Nobiletin is a dietary polymethoxylated flavonoid found in citrus fruits. In this study, we investigated how nobiletin affects the disease state and immune responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis mice. Nobiletin was administered orally from 14 days before immunization until the end of the experiment, and clinical scores were determined. The production levels of interleukin-17A and interferon-γ were measured in a culture supernatant of splenocytes stimulated with myelin oligodendrocyte glycoprotein. In addition, flow cytometric analysis was performed to examine the effect of nobiletin on T cell differentiation *in vitro*. Administration of nobiletin significantly decreased the clinical score and interleukin-17A production in splenocytes. Furthermore, *in vitro* analysis showed that nobiletin significantly suppressed Th17 cell differentiation and interleukin-17A production in a dose-dependent manner. The results suggest that nobiletin attenuates experimental autoimmune encephalomyelitis severity through modulation of Th17 cell differentiation.

***Key Words*: experimental autoimmune encephalomyelitis, nobiletin, polymethoxyflavone, Th17 differentiation, IL-17A**

Multiple sclerosis (MS) is an organ-specific autoimmune disease that targets the myelin antigen in the central nervous system (CNS). Since myelin sheaths cover axons of nerve cells in the brain and spinal cord, damage and/or demyelination of myelin proteins result in a defect in the neurotransmission pathway and limb paralysis.[1,2] Although myelin-reactive T cells have been shown to be type 1 helper T cells (Th1), recent studies have shown found that Th17 cells are crucially involved in autoimmune pathogenesis.[3] Clinically, it has been shown that interleukin (IL)-17 mRNA expression levels were increased in blood and in cerebrospinal fluid mononuclear cells in MS patients.[4,5]

Polymethoxyflavones (PMFs) are flavonoid compounds that contain more than two methoxy groups and are almost extensively found in citrus peel. Several studies have suggested that PMFs with more methoxy groups exert strong bioactivities.[6,7] Nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) is one of the PMFs with 6 methoxy groups (Fig. 1A). Nobiletin is one of the most extensively studied PMFs and has been reported to have anti-obesity,[8] anti-tumor[9] and neuroprotective effects.[10,11] In an *in vivo* study, administration of nobiletin protected the colonic mucosal layer and suppressed ulcerative colitis in a mouse model of trinitrobenzenesulfonic acid-induced inflammation.[12] In addition, we reported that nobiletin enhanced production of the antigen (Ag)-specific cytokines IL-4 and IL-10 in ovalbumin-immunized mice.[13]

Exogenous Ags are taken into Ag-presentating cells and the Ags are presented in major histocompatibility complex (MHC) class II molecules. Ag-specific naïve $CD4^+$ cells recognize MHC class II/Ag complex and differentiate into Th1, Th2, Th17 or regulatory T cells depending on the cytokine conditions. There have been many studies in which the effect of nutritional function on differentiation of Th1/Th2 cells was examined. However, there have been few studies in which the effect of nutritional function on differentiation of Th17 cells was examined.

In this study, we conducted an experiment to determine how nobiletin affects Th17 cell-related pathology in myelin oligodendrocyte glycoprotein (MOG)-immunized experimental autoimmune encephalomyelitis (EAE) model mice. Furthermore, to understand the underlying mechanism, the effect of nobiletin on differentiation of Th17 cells *in vitro* was also examined.

Materials and Methods

Mice and diets. Six-week-old female C57BL/6 mice (Japan SLC, Shizuoka, Japan) were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. The mice were given free access to water and food throughout the experiment. The mice were maintained on a control diet (No. D10012G; Research Diets Inc., New Brunswick, NJ). All studies were performed in accordance with the ethical guidelines for animal experimentation by the Institute of Biomedical Sciences, Tokushima University, Japan and were approved by the institution review board of the animal ethics committee.

Induction of EAE in mice. Complete Freund's Ajuvant (Sigma Chemical Co., St. Louis, MO) containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra (DIFCO Lab, Detroit, MI) and 1 mg/ml MOG peptide (MEVGWYRSPFSRVVHLRNGK, Invitrogen, Carlsbad, CA) solution dissolved in phosphate buffered saline were mixed at a ratio of 1:1. Then 100 μl of the mixture was injected to the back of each mouse. Thereafter, the mice were injected intraperitoneally with 300 ng of pertussis toxin. At 48 h after the treatment, the mice were intraperitoneally

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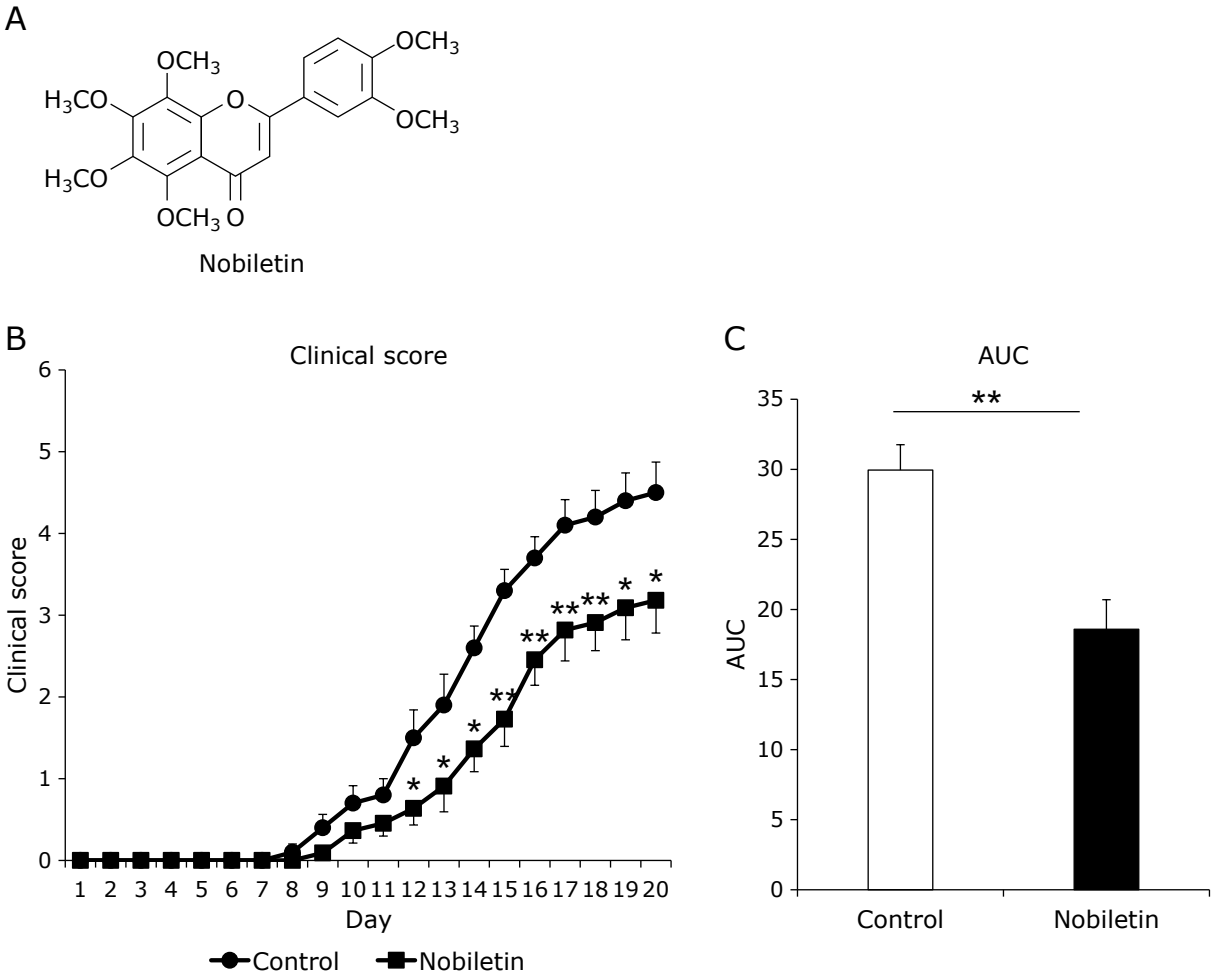

Fig. 1. Clinical score and area under the curve (AUC) in mice with EAE induced by MOG peptide. Chemical structure of nobiletin is shown (A). C57BL/6 female mice with EAE induced by MOG peptide-immunization. The mice were administered 100 mg/kg body weight of nobiletin daily by gavage during the experimental period. Clinical score (B) and AUC (C) in EAE mice. Statistical differences were analyzed between the control and nobiletin groups of 9 or 10 mice/group were analyzed. Data are shown as means ± SEM. *p<0.05 vs control group. **p<0.01 vs control group.

injected with 300 ng of pertussis toxin again. EAE was evaluated according to clinical scores of 0–6: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limp paralysis; 4, compete hind limb paralysis; 5, forelimb paralysis or moribund; 6, death.[14] The clinical score for each mouse was recorded every day during the experimental period.

Nobiletin treatment. Nobiletin was provided by Ushio-Chemix Co. (Shizuoka, Japan). The purity of nobiletin used in this study was >98%. For oral administration, nobiletin was dissolved in 0.5% sodium carboxyl methylcellulose. The mice were administered 500 µl of nobiletin solution containing 100 mg/kg body weight by gavage for 34 days. Control mice were treated with 500 µl of 0.5% sodium carboxyl methyl-cellulose solution.

Cytokine production. Splenocytes (2.5×10^6 cells/well) from EAE mice were stimulated with 20 µg/ml MOG in a 48-well flat-bottom plate at 37°C under 5% CO_2 for 72 h. After the culture, culture supernatants were collected and stored at −30°C until used. Interferon (IFN)-γ, IL-17A, and IL-10 in the supernatants were quantified using mouse IFN-γ, IL-10, and IL-17A enzyme-linked immunosorbent assay (ELISA) kits (Biolegend, San Diego, CA) according to the instructions of the manufacturer.

Antigen-specific T cell response. Spleens of EAE mice were collected and single cells were prepared. Splenocytes (5×10^5/well) were stimulated with 20 µg/ml MOG in a 96-well flat-bottom plate at 37°C under 5% CO_2 for 72 h. For the last 8 h of culture, 37 kBq of [^{3}H]thymidine deoxyribose (TdR) was added to the wells, and the amount of [^{3}H]TdR incorporated was measured by a scintillation counter (Aloka, Tokyo, Japan).

Effect of nobiletin to Th17 cell differentiation. Splenocytes (1×10^6 cells/well) from C57BL/6 mice were cultured in an anti-CD3 monoclonal antibody (Ab) (clone 145-2C11)-bound plate in the presence of IL-1β (10 ng/ml), IL-6 (20 ng/ml) and transforming growth factor (TGF)-β1 (2 ng/ml) for 18 h. Then 2 µM or 10 µM of nobiletin was added and the cells were cultured in a 48-well flat-bottom plate at 37°C under 5% CO_2 for 48 h. At the same time, an equal amount of dimethyl sulfoxide (DMSO) was added to the culture in the control group. After the culture, the proportion of Th17 cells was confirmed by flow cytometry, and IL-17A production in the culture supernatant was confirmed by ELISA.

The cells that differentiated into Th17 cells in the presence of nobiletin were restimulated with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 µg/ml) and monensin (5 µg/ml) for 4 h at 37°C under 5% CO_2. After the culture, the cells were collected and stained with fluoresceinisothiocyanate-labeled anti-mouse

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CD4 Ab (Biolegend) for cell surface staining on ice for 30 min. After that, the cells were fixed with 2% paraformaldehyde for intracellular staining. Furthermore, the cell membrane was permeated with a cell permeation buffer containing 0.5% saponin and 0.5% bovine serum albumin in phosphate buffered saline and stained with an allophecocyanin-labeled IL-17A Ab (Biolegend). Flow cytometric analysis was performed on Guava easyCyte using Guava Incyte software (Merck Millipore, Darmstadt, Germany).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from spleen cells using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany). Two μg of the extracted total RNA was transcribed with a PrimeScript RT Master Mix kit (Takara Bio, Siga, Japan). Real-time PCR was performed by using specific primers and SYBR green dye (Takara Bio) in ABI StepOnePlus™ (Applied Biosystems, CA) according to the manufacturer's instructions. The primers used were 5'-AGC TGGACCACCACATGAAT-3' (sense) and 5'-AGCATCTTCTCG AACCCTGAA-3' (antisense) for IL-17A, 5'-GAAGGCAAATAC GGTGGTGT-3' (sense) and 5'-GGGCAATCTCATCCTCAGAA-3' (antisense) for RORγτ, and 5'-CTGACCCTGAAGTACCCC ATTGAACA-3' (sense) and 5'-CTGGGGTGTTGAAGGTCT CAAACATG-3' (antisense) for β-actin.

Statistics. Data were analyzed using Student's *t* test for the EAE clinical score experiment and using ANOVA followed by post-hoc comparison tests for the *in vitro* Th17 cell differentiation experiment. Data are expressed as means ± SEM. Differences were considered significant at $p<0.05$.

Results

Nobiletin attenuates clinical symptoms in EAE mice. To examine the effect of nobiletin on EAE in mice, the mice were orally administered 100 mg of nobiletin per kg body weight daily from 14 days before MOG immunization and clinical scores were determined. Both groups of mice began to develop EAE symptoms about 8 days after MOG immunization. From 12 days after immunization, a significant difference in the clinical scores was found between the two groups, and mild symptoms were confirmed in the nobiletin group. The area under the curve (AUC) of clinical scores also showed that nobiletin attenuated the disease (Fig. 1B and C).

Nobiletin suppresses MOG-specific IL-17A production in EAE mice. We examined the levels of IFN-γ, IL-17A, and IL-10 production. When splenocytes were stimulated with MOG, the levels of IL-17A production in the nobiletin group were decreased compared to those in the control group, whereas the levels of IFN-γ and IL-10 production were the same in the two groups (Fig. 2A–C). Although no significant difference was found, the concentration of IL-17A in serum tended to be lower in the nobiletin group than in the control group (Fig. 2D).

Nobiletin suppresses MOG-specific cell proliferation. Splenocytes from EAE mice were stimulated with MOG *in vitro* and proliferative responses were determined. The MOG-specific T cell responses were significantly different in the control group and nobiletin group. The level of proliferation response in the nobiletin group was about 2-times lower than that in the control group (Fig. 3).

Nobiletin inhibits Th17 cell differentiation *in vitro*. To examine the effect of nobiletin on Th17 cell differentiation *in*

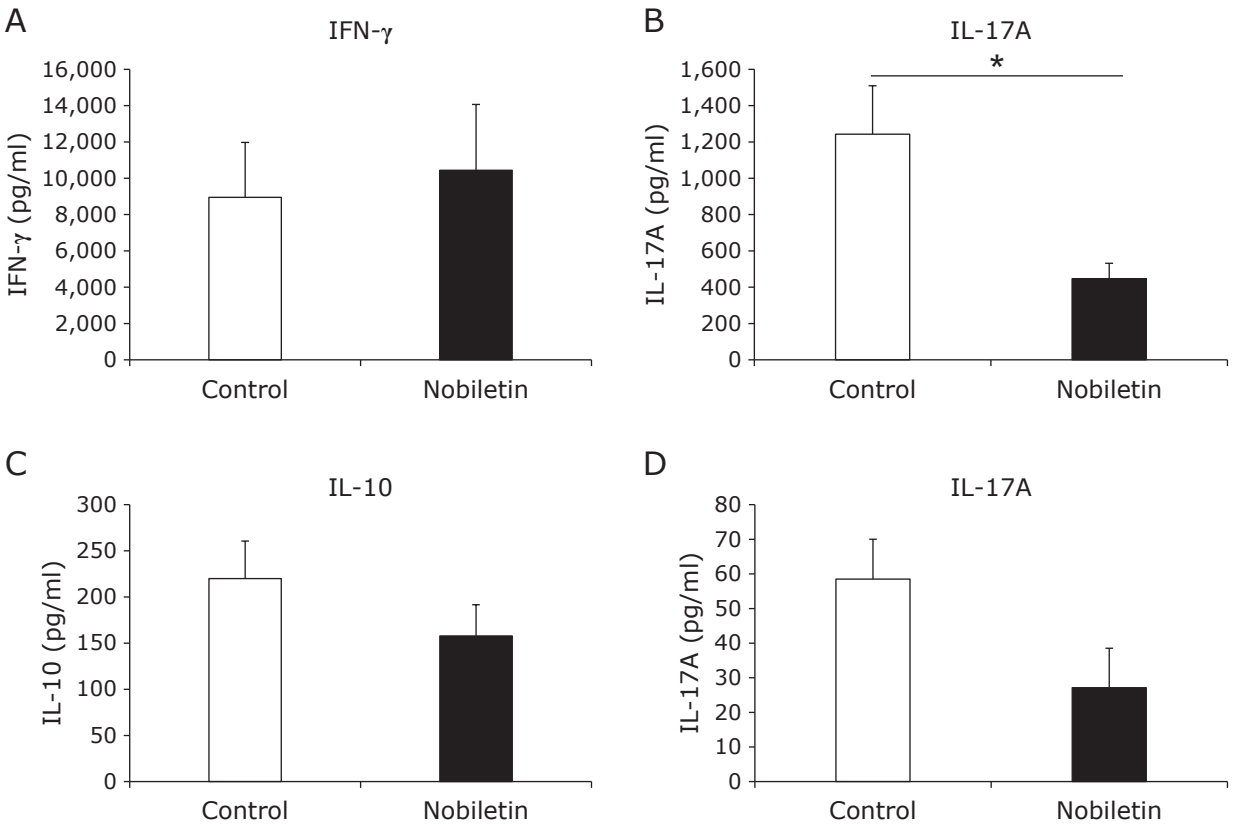

Fig. 2. Effect of nobiletin on production of Ag-specific cytokines. Splenocytes from EAE mice that had been treated with nobiletin were stimulated with 20 μg/ml MOG peptide for 72 h. Concentrations of IFN-γ (A), IL-17A (B), and IL-10 (C) in the culture supernatant and IL-17A in serum of EAE mice (D) were determined by ELISA. Statistical differences between the control and nobiletin groups of 9 or 10 mice/group were analyzed. Data are shown as means ± SEM. *$p<0.05$ vs control group (white bar).

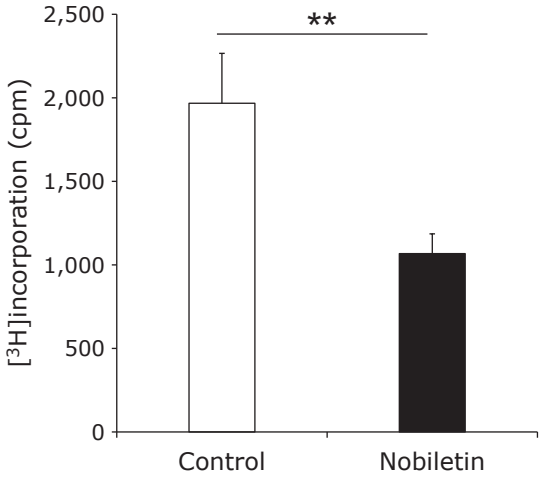

Fig. 3. Nobiletin suppresses MOG-specific T cell induction. Splenocytes of EAE mice that had been treated with nobiletin were stimulated with 20 μg/ml MOG peptide for 72 h. Proliferative response was determined by incorporation of [3H]TdR. Statistical difference between the control and nobiletin groups was analyzed of 9 or 10 mice/group. Data are shown as means ± SEM. **p<0.01 vs control group (white bar).

vitro, naïve T cells in splenocyte of C57BL/6 mice were cultured in the presence of IL-6, IL-1β, and TGF-β with or without nobiletin. Flow cytometric analysis showed that treatment with nobiletin decreased the percentage of $CD4^+IL\text{-}17A^+$ cells in a dose-dependent manner (Fig. 4A). Furthermore, it was shown that IL-17A production in the culture supernatant was also decreased by nobiletin in a dose-dependent manner (Fig. 4B). The mRNA expression levels of *IL-17A* and *RORγτ* in cultured cells were also significantly reduced when 10 μM nobiletin was added to the culture (Fig. 4C and D).

Discussion

In this study, we examined how nobiletin affects disease severity and pathogenic Th17 immune responses in a mouse EAE model. Furthermore, to understand the mechanism underlying the action of nobiletin, the differentiation to Th17 cells was also examined in an *in vitro* model. The main findings of this study are that the natural flavonoid nobiletin attenuated the clinical score of EAE disease and the production of IL-17 in mice as well as the *in vitro* differentiation of naïve Th cells to Th17 cells. To our knowledge, this study is the first study showing that a polymethoxy flavonoid regulates the differentiation of Th17 cells and Th17 cell-mediated autoimmune disease.

In patients with autoimmune multiple sclerosis, IL-17 expression is augmented in mononuclear cells of the blood

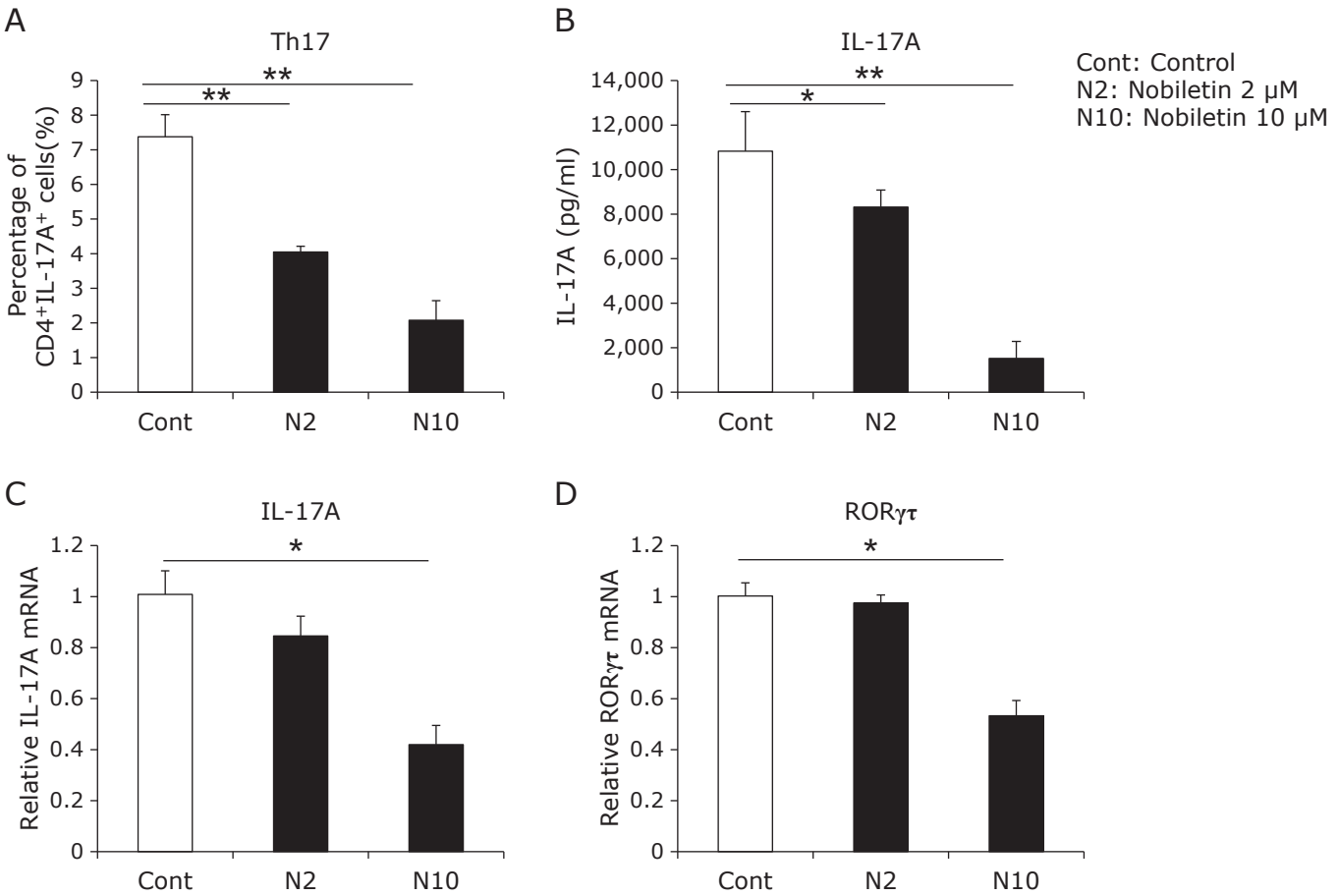

Fig. 4. Nobiletin suppresses differentiation of Th17 cells *in vitro*. Splenocytes from C57BL/6 female mice were pretreated with IL-1β, IL-6, and TGF-β for 18 h and then stimulated with anti-CD3 Ab with and without nobiletin for 48 h. At the same time, an equal amount of DMSO was added in the control group. After the culture, the percentage of $CD4^+IL17A^+$ cells was determined by FACS (A) and production of IL-17A in the culture supernatant was determined by ELISA (B). The expression levels of IL-17A (C) and RORγτ (D) mRNA were determined by RT-qPCR and the values were corrected by the expression of β-actin mRNA. The levels are shown as relative values of the control group. Data are shown as means ± SEM (n = 3). All data are representative of three independent experiments. *p<0.05 vs control group (white bar). **p<0.01 vs control group (white bar).

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and cerebrospinal fluid and at the site of lesions.[15,16] IL-17 expression is correlated with disease activity. IL-17 and IL-22 promote blood-brain barrier disruption and CNS inflammation by inducing chemokines in endothelial cells and by downregulating tight junction proteins.[17] We focused on the effect of nobiletin on Th17 cell differentiation in a mouse model of EAE because there has been little study on Th17 cell differentiation in contrast to studies on Th1/Th2 cell differentiation. Nobiletin suppressed MOG-specific T cell response and IL-17 production but not production of inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 (Fig. 2 and 3). Although we did not obtain direct evidence that suppression of Th17 cell differentiation by nobiletin causes prevention of EAE symptoms *in vivo*, Th17 cells have been shown to crucially contribute to autoimmune pathology in a mouse EAE model.[18]

It has been shown that TGF-β and IL-6, two cytokines with opposing effects, synergize to induce orphan nuclear receptor RORγτ, which orchestrates the expression of IL-17A and IL-17F in naïve T cells.[19,20] In an *in vitro* Th17 cell differentiation model, 10 μM of nobiletin suppressed Th17 cell differentiation and IL-17 production (Fig. 4). It cannot be discussed here whether the concentration of 10 μM of nobiletin in an *in vitro* study is relevant to the concentration in an *in vivo* study. We verified that 10 μM of nobiletin did not affect T cell viability and Th differentiation into both Th1 and Th2 *in vitro* (data not shown).

There is increasing experimental evidence of a pivotal role of epigenetic modifications in autoimmune disease. RORγτ has been identified as a Th17-specific transcription factor that induces Th17 cell differentiation.[21] It has been shown that TNF-α inhibitors suppress RORγτ protein and mRNA expression in human Th17-polarized cells, and these suppressive effects might be mediated through the downregulation of histone H3 and H4 acetylation.[22] Some studies have shown that flavonols and flavones regulate activities of histone deacetylases.[23] There is a possibility that nobiletin affects the activities of histone deacetylases and/or transferases, resulting in RORγτ mRNA expression.

Nobiletin has been shown to have many physiological actions, and one of its actions is a neuroprotective effect on the CNS.[24] The anatomical structure of the CNS includes a blood-brain barrier and a blood-spinal cord barrier. Nobiletin has been shown to be able to cross the blood-brain barrier and suppress the activation of extracellular signal-regulated kinase, c-Jun N-terminal kinase and nuclear factor-kappa B signaling pathway of mitogen-activated protein kinase family. It has been shown that nobiletin suppressed the overproduction of nitric oxide in microglia and the release of inflammatory cytokines, resulting in a neuroprotective effect.[25] In EAE model mice, dendritic cells and T cells infiltrating into the brain and spinal cord produce inflammatory cytokines such as TNF-α, IL-6, IL-17, and IFN-γ and then cause neurodegeneration in the model mice.[26] In addition, IL-10 has been shown to suppress Th17 activity and exhibit an anti-inflammatory effect.[27] In rheumatoid arthritis that overexpresses IL-17, it has been reported that IL-10 reduces the number of Th17 cells and induces Foxp3-positive regulatory T cells.[28] Since an anti-inflammatory action is one of the most extensively studied functions of a polymethoxy flavonoid, especially nobiletin, we speculated that suppression of inflammation in the CNS by nobiletin is one of mechanisms for improvement of EAE disease in addition to suppression of Th17 cell differentiation.

In this study, we found that PMF nobiletin suppresses Th17 differentiation. In the case of Th1/Th2 differentiation, nobiletin has been shown to enhance Th2-mediated immune responses.[13] We focused on immune regulatory action of PMFs and found that sudachitin (5,7,4'-trihydroxy-6,8,3'-trimethoxyflavone) which contain three methoxy groups also enhance Ag-specific Th2 response in ovalbumin-immunized mice.[29] It is interesting to determine how the number and/or position of methoxy group that bind to the flavonoid structure affect Th differentiation both *in vitro* and *in vivo*.

In summary, we have provided the first evidence that nobiletin can inhibit Th17 cell differentiation and expression of RORγτ, which is a master regulator of Th17 cells, resulting in alleviation of EAE. In view of our data, we demonstrated that nobiletin may became a potential novel therapeutic drug for MS, and nobiletin has numerous pharmacological actions to further explore.

Acknowledgments

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Abbreviations

Ab	antibody
Ag	antigen
AUC	area under the curve
CNS	central nervous system
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
MOG	myelin oligo-dendrocyto glycoprotein
MS	multiple sclerosis
PMF	polymethoxyflavone
RT-qPCR	reverse transcription-quantitative PCR
TdR	thymidine deoxyribose
TGF	transforming growth factor
Th	helper T

Conflict of Interest

No potential conflicts of interest were disclosed.

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