ORIGINAL

Effects of polymethoxyflavonoids on T helper 17 cell differentiation *in vitro* and *in vivo*

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Abstract : We examined the effects of polymethoxyflavonoids (PMFs) on T helper (Th) 17 cell differentiation *in vitro* and *in vivo*. Five different PMFs including nobiletin (NOB), sudachitin (SUD), demethoxysudachitin, heptamethoxyflavone and natsudaidain were used for the *in vitro* study, and effects of those flavonoids on Th17 responses were investigated. NOB and heptamethoxyflavone significantly suppressed the proliferation response, but SUD, demethoxysudachitin and natsudaidain did not suppress the proliferation response. All of the five flavonoids decreased IL-17A production. Mice with experimentally induced autoimmune encephalomyelitis were used as an *in vivo* Th17 differentiation model. We focused on two flavonoids, NOB and SUD, and examined the effects of those flavonoids. NOB significantly suppressed Th17 cell proliferation and cytokine responses, but SUD only decreased proliferation responses. The results suggest that the suppressive effect of NOB on Th17 response *in vivo* is stronger than that of SUD. J. Med. Invest. 70 : 166-170, February, 2023

Keywords **:** *polymethoxyflavonoids, nobiletin, sudachitin, T helper 17 cell, IL-17A*

INTRODUCTION

Naïve $CD4^+$ T cells differentiated into T helper type 1 (Th1), Th2 , Th17 or regulatory T cells depending on exogenous cytokine conditions when an antigen (Ag) is presented by professional Ag-presenting cells (1). Among the Th subsets, interleukin-17 (IL-17)-producing Th17 cells are known to be an inflammatory Th subset that contributes to chronic tissue inflammation (2, 3). Indeed, some biologic agents targeting the effector cytokines of Th17 cells have been approved for treatment of immune response-mediated diseases (4).

We have been examining the effects of polymethoxyflavonoids (PMFs) on immune functions including Th1/Th2 responses. Sudachitin (SUD), 5,8,4'-trimethoxyflavone, is a PMF found in *Citrus sudachi*. Treatment of ovalbumin (OVA)-immunized mice with sudachitin enhanced OVA-specific IL-4 and IL-10 production, resulting in enhancement of humoral immunity. These responses have been shown to contribute to the enhancement of Ag-presenting function (5). We have also examined the effect of nobiletin (NOB), 5,6,7,8,3',4'-hexamethoxyflavone, in OVA-immunized mice. The effect of nobiletin on OVA-specific T cell and B cell response was similar to that of SUD (6). The biological function of a PMF is known to be dependent on the number and binding position of methoxy groups. In this study, we examined the effects of five different types of PMFs on Th17 response *in vitro*. Th17 cells and their cytokines are associated with several autoimmune and inflammatory diseases (7, 8). It is well known that Th17 cells are critical for the induction of experimental autoimmune encephalomyelitis (EAE) in mice (9). We also examined the effects of NOB and SUD, which showed different actions on *in vitro* Th17 cell differentiation, on Th17 induction *in vivo*.

MATERIALS AND METHODS

Reagents

NOB, heptamethoxyflavone (HMF) and natsudaidain (NAT) were provided by Ushio-Chemix Co. (Shizuoka, Japan). SUD was provided by Ikeda-Yakusou Co. (Tokushima, Japan). Demethoxysudatitine (DSU) was provided by Hitachi Chemical Co. (Tsukuba, Japan).

Mice

Eight-wk-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 maintained on a control diet (No. D10012G ; Research Diets Inc., NJ, USA). All studies were performed in accordance with the ethical guidelines for animal experimentation by the Graduate School of Biomedical Sciences, Tokushima University, Japan and were approved by the institution review board of the animal ethics committee (T2019-95).

Th cell differentiation

Naïve $CD4^+$ cells were purified by using a Naïve $CD4^+$ T Cell Isolation Kit (Milteny Biotec, CA. USA). For Th17 cell differentiation, $CD4^+$ cells (2×10^5 cells/well) from C57BL/6 mice were cultured in an anti-CD3 monoclonal (m) antibody (Ab) (1 μg/mL) and anti-CD28 mAb (1 μg/mL)-bound 24-well plate in the presence of IL-1β (10 ng/mL), IL-6 (20 ng/mL) , transforming growth

Abbreviations :
Ab, antibody ; Ag, antigen; DSU, demethoxysudachitin ; EAE, experimental autoimmune encephalomyelitis ; HMF, heptamethoxyflavone, IFN, interferon ; IL, interleukin ; m, monoclonal ; MOG, myelin oligodendrocyte glycolipid ; NAT, natsudaidain ; NOB, nobiletin ; OVA, ovalbumin ; SUD, sudachitin ; Th, T helper

Received for publication October 4, 2022 ; accepted December 16, 2022.

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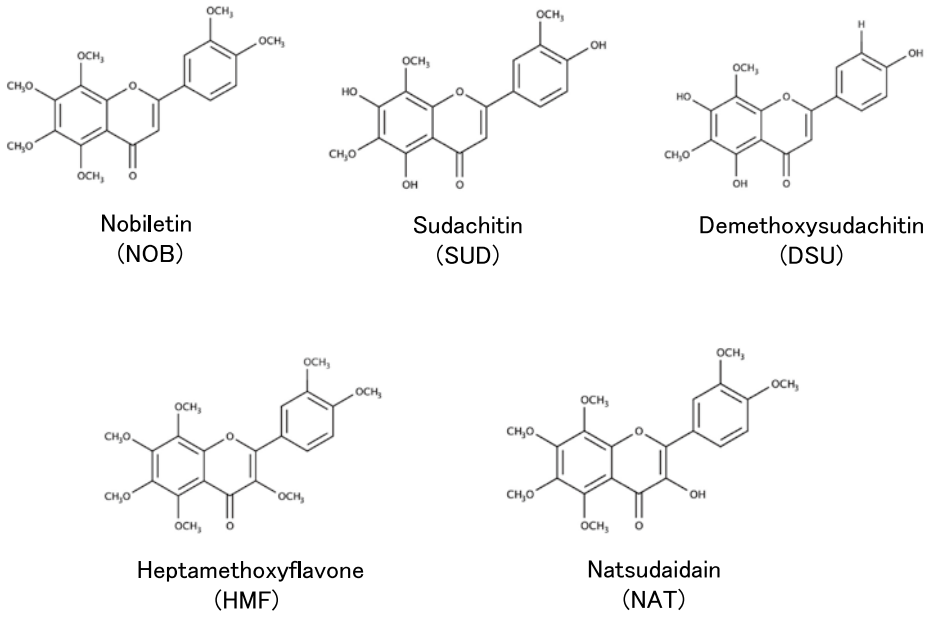

Figure 1. Chemical structures of NOB, SUD, DSU, HMF and NAT.

factor-β1 (2 ng/mL), anti-IFN-γ mAb (250 ng/mL) and anti-IL-4 mAb (250 ng/ml) for 18 h. For differentiation of Th1 and Th2 cells, $CD4^+$ cells were cultured with IL-12 (5 ng/mL), and IL-4 (30 ng/mL), IL-2 (10 ng/mL) and anti-IFN-γ (250 ng/mL) in the presence of anti-CD3 mAb and anti-CD28 mAb, respectively. Then 2 μM or 5 μM of a PMF was added and the cells were cultured in a 96-well flat-bottom plate at 37°C under 5% CO_2 for 48 hr. At the same time, an equal amount of dimethyl sulfoxide was added to the culture in the control group.

Proliferation response

$CD4^+$ cells were cultured in each Th differentiation condition. For the last 8 h of culture, 37 kBq of [^{3}H]thymidine deoxyribose was added to the wells, and the amount of [^{3}H]thymidine deoxyribose incorporated was measured by a scintillation counter (Aloka, Tokyo, Japan).

Cytokine productions

After the culture, culture supernatants were collected and stored at -30°C until used. IFN-γ, IL-4 and IL-17A in the supernatants were quantified using mouse IFN-γ, IL-4 and IL-17A (Biolegend, San Diego, CA, USA) enzyme-linked immunosorbent assay kits according to the instructions of the manufacturer.

Induction of experimental autoimmune encephalomyelitis (EAE) in mice

Complete Freund's Adjuvant (Sigma Chemical Co., Mo, USA) containing 5 mg/mL *Mycobacterium tuberculosis* H37Ra (DIFCO Lab, NJ, USA) and 1 mg/mL myelin oligodendrocyte glycolipid (MOG) peptide (MEVGWYRSPFSRVVHLRNGK, Invitrogen, CA, USA) 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 injected with 300 ng of pertussis toxin again.

In vivo NOB and SUD treatment

For oral administration, NOB and SUD were dissolved in 0.5% sodium carboxyl methylcellulose. The mice were administered 500 μL of NOB or SUD solution containing 100 mg/kg body weight by gavage for 27 d. Control mice were treated with 500 μL of 0.5% sodium carboxyl methylcellulose solution.

MOG-specific proliferation and cytokine production

Spleens of EAE mice were collected and single cells were prepared. For determination of MOG-specific proliferation response, 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. The proliferation responses were determined by the incorporation of [^{3}H]thymidine deoxyribose. For determination of MOG-specific IL-17A and IL-6 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 contents of IL-17A and IL-6 were determined.

Statistics

Data were analyzed using ANOVA followed by post-hoc comparison tests. Data are expressed as means ± standard deviation. Differences were considered significant at $p < 0.05$.

RESULTS

Purified $CD4^+$ T cells were cultured in Th17-inducing conditions and the effects of five kinds of PMFs were investigated. Treatment with 2 μM and 5 μM NOB and 5 μM HMF significantly reduced the proliferation responses, but SUD, DSU and NAT did not reduce the proliferation responses (Fig. 2A). All of five PMFs significantly reduced the production of IL-17A (Fig. 2B). The results showed that the effects of PMFs on Th17-related markers are different *in vitro*.

In addition to Th17 cell differentiation, we examined the effects of the PMFs on Th1 and Th2 cell differentiation. Treatment with 2 and 5 μM NOB and 5 μM HMF significantly suppressed Th1 proliferation responses (Fig. 3A). Reduction of IFN-γ production was observed when cells were treated with 5 μM of

NOB and HMF (Fig. 3B). In contrast to IFN-γ production, IL-4 production was not reduced by treatment with any of the five PMFs (Fig. 4).

We next examined the effects of the PMFs on *in vivo* Th17 cell differentiation in EAE animal models. MOG-specific proliferation response and cytokine production were determined in EAE mice that had been treated with NOB or SUD. NOB suppressed both MOG-specific proliferation response and IL-17A production. In contrast, SUD significantly reduced MOG-specific proliferation response but did not reduce IL-17A production. Neither NOB nor SUD affected the production of IL-6, which is another Th17 marker cytokine (Fig. 5).

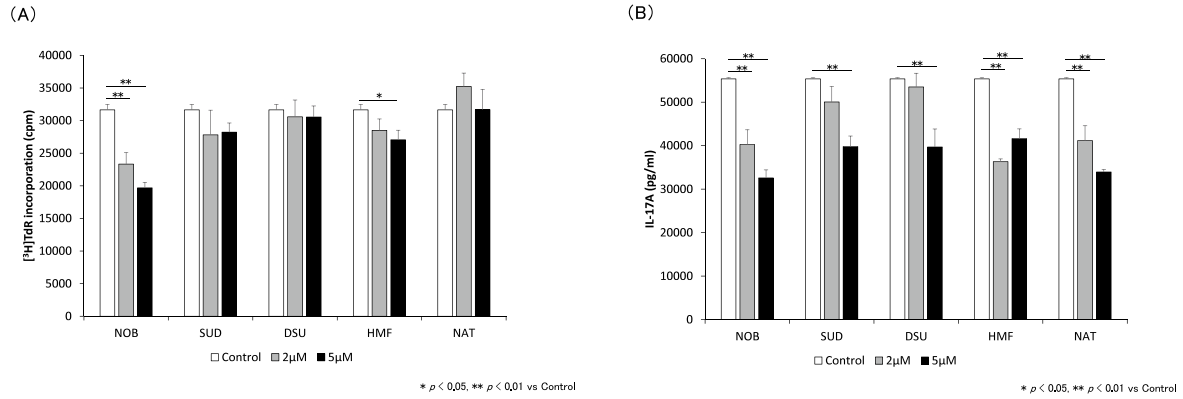

Figure 2. Effects of PMFs on Th17 cell differentiation. Mouse $CD4^+$ cells were cultured in Th17-inducing conditions. Proliferation response (A) and production of IL-17A (B) were determined as described in the Materials and Methods section. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

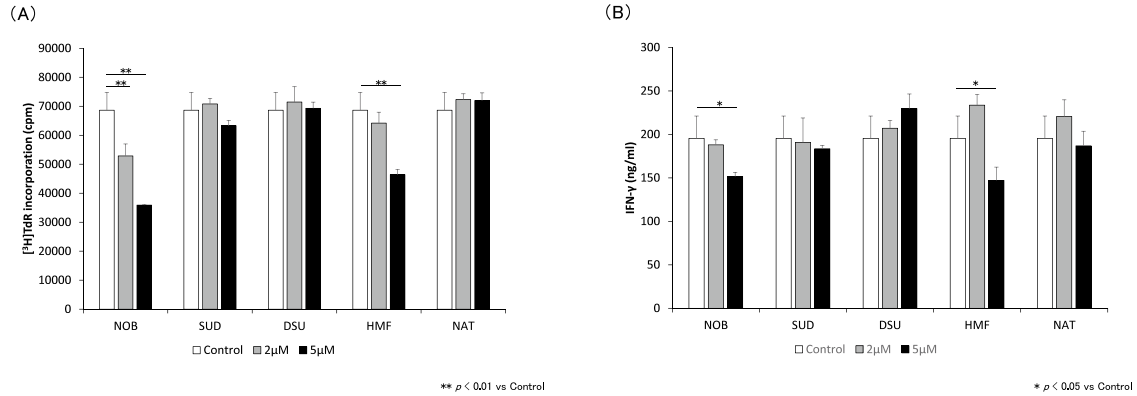

Figure 3. Effects of PMFs on Th1 cell differentiation. Mouse $CD4^+$ cells were cultured in Th1-inducing conditions. Proliferation response (A) and production of IFN-γ (B) were determined as described in the Materials and Methods section. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

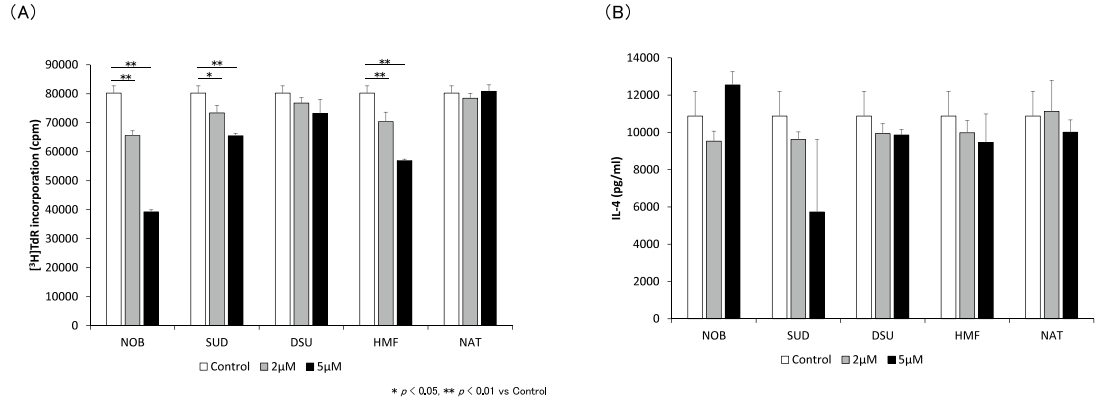

Figure 4. Effects of PMFs on Th2 cell differentiation. Mouse $CD4^+$ cells were cultured in Th2-inducing conditions. Proliferation response (A) and production of IL-4 (B) were determined as described in the Materials and Methods section. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

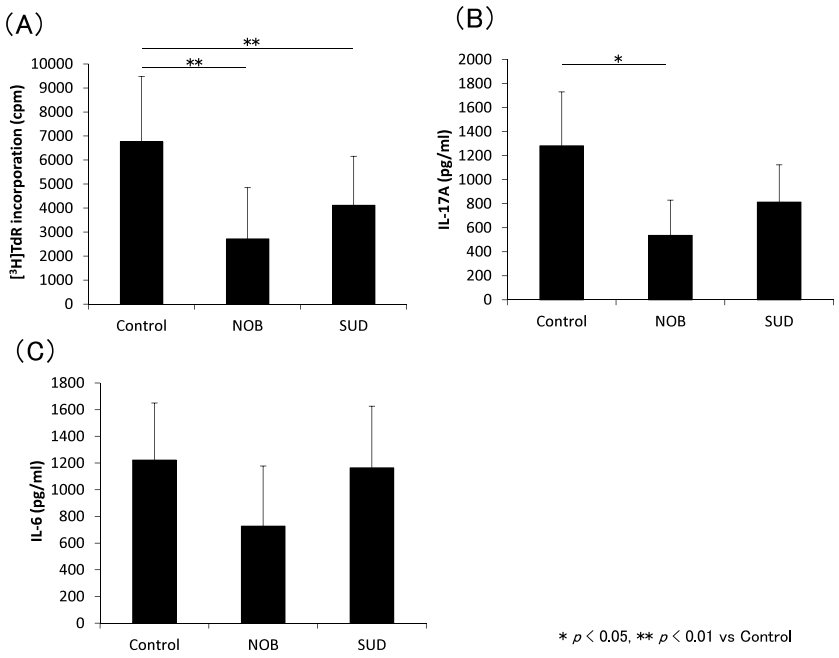

Figure 5. Effects of NOB and SUD on Th17 response in EAE mice. MOG-specific proliferation response (A), IL-17A production (B) and IL-6 production (C) were determined as described in the Materials and Methods section. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

DISCUSSION

There has been no previous study in which the effects of various types of PMFs on Th17 cell differentiation were examined. In this study, we found that PMFs inhibit Th17 cell differentiation *in vitro*, while the effects of PMFs in proliferation response are different. Differentiation of Th17 cells *in vivo* was suppressed by treatment with NOB but not by treatment with SUD (Fig. 5).

All of the five PMFs suppressed Th17 cell differentiation as evaluated by IL-17A production by $CD4^+$ cells. The proliferation response was suppressed by treatment with NOB and HMF but not by treatment with the other PMFs (Fig. 2). Since it is known that both cytokine production by T cells and proliferation of T cells are equivalent markers of cell activation (10), different effects on cytokine and proliferation responses are expected in the presence of different signal transduction pathways between proliferation and cytokine production.

Focusing on the number of methoxy groups binding flavonoid structures, PMFs with many methoxy groups tended to show higher activity as evaluated by suppression of proliferation response and cytokine production (Fig. 2). We compared the suppressive effects of NOB and SUD on Th17 cell differentiation in the EAE model, and we found that the effect of NOB, which has 6 methoxy groups, was superior to the effect of SUD, which has 3 methoxy groups (Fig. 5). *In vitro* Th17 differentiation condition, all PMFs used in the present study significantly decrease IL-17 production (Fig.2B). In contrast, only NOB and HMF decrease the proliferation response (Fig.2A). We cannot explain the mechanism. The number of methoxy groups might contribute to these differences because NOB and HMF have more methoxy groups than SUD and DSUD. But further study is needed because the number of methoxy groups in NOB is the same as that in NAT (Fig.1). There are studies that show the number of methoxy groups and metabolites from PMF contributes to the functions of PMFs. Miyata *et al*. examined the effects of NOB (5,6,7,8,3',4'-hexamethoxyflavone) and tangeretin (5,6,7,8,4'-pentamethoxyflavone) on differentiation of 3T3-L1 preadipocytes into adipocytes (11). They found that both NOB and tangeretin increased secretion of adiponectin and decreased MCP-1 in 3T3-L1 cells. NOB had a much greater effect than tangeretin on the accumulation of intracellular triglycerides in 3T3-L1 cells, suggesting that the presence of one methoxy group influences lipid synthesis pathways. Naturally occurring flavonoids have been shown to be metabolized *in vivo* by P450 CYP1-enzyme, which leads to the activation of metabolites showing greater biological effects than their parent compounds. Li *et al*. examined the effects of NOB and NOB-derived metabolites on LPS-induced nitrite production by macrophage-like cell lines and found that the suppressive effects NOB-derived metabolites on the production were stronger than the suppressive effect of NOB (12). A superior inhibitory effect of NOB-derived metabolites on tumor growth has also been shown (13, 14). It is possible that the types of metabolites derived from NOB and SUD contribute to the differentiation of Th17 cells in EAE models.

There have been some studies in which the effects of flavonoids in an EAE mouse model were investigated. The flavonoids kurarinone and glucoside icariin have been shown to ameliorate EAE diseases (15, 16). Icariin has been shown to decrease the production of both IFN-γ and IL-17 *in vitro*, but its effect on proliferation response has not been investigated in the study. Kurarinone has been shown to suppress IFN-γ and IL-4 production *in vitro*. Our study showed that PMFs do not affect IL-4 production *in vitro* (Fig. 4). In our previous study, PMF suppressed Ag-specific proliferation response and IFN-γ, IL-2 and IL-10 production but enhanced IL-4 production (17). The effect of a PMF on IL-4 production might be different from other cytokine production pathways.

Our previous studies show that NOD suppresses Th17 differentiation in EAE models (18) and enhances Th2 differentiation in ovalbumin-immunized mice (6). The exposure of cytokines and the type of antigen-presenting cells have been shown to be crucial for the determination of T helper cell differentiation (1).

Since the pathway for Th17 and Th2 differentiation is complex and independent, a direct comparison of the effect of PMFs in these two models is difficult.

In this study, we used five different types of PMFs and compared the effects of those PMFs on Th cell differentiation *in vitro*. Although we compared the effects of NOB and SUD in an EAE model, further *in vivo* study using various types of PMFs is needed.

Authorship

A.N., Y.H. and T.S. conceived and designed the study. A.N., Y.H., C.A. and E.S. performed the experiments. A.N., Y.H. and M.N. analyzed the data. A.N., Y.H. and T.S. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

ACKNOWLEDGMENTS

Nobiletin, heptamethoxyflavone and natsudaidain, and sudachitin and demethoxysudachitin were kindly provided by Ushio-Chemix Co., Ikeda Yakuso Co., and Hitachi Chemical. Co., respectively. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science (KAKENHI 22K11701), and Technology and Hitachi Chemical Co., Ltd.

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