JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Dietary Flavonoid Naringenin Induces Regulatory T Cells via an Aryl Hydrocarbon Receptor Mediated Pathway

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ABSTRACT: The aryl hydrocarbon receptor (AhR), a transcription factor mediating xenobiotic detoxification, plays a considerable role in regulatory T cell (Treg) induction. Tregs regulate the immune system, thus suppressing allergies and autoimmune diseases. This study aims to identify new types of antiallergic dietary factors, with focus on the flavonoids with potential AhR agonistic activity. Among 25 dietary flavonoid samples tested using a reporter assay, 8 showed marked induction of AhR-dependent transcriptional activity. The subsequent T cell proliferation suppression assay identified naringenin as the only sample capable of stimulating Treg induction; notably, this induction was eliminated by cotreatment with AhR antagonists. Indeed, naringenin induced $CD4^+Foxp3^+$ Tregs, irrespective of the presence of the transforming growth factor-β (TGF-β), indicating that the conventional TGF-β-dependent signaling pathway might not be involved.

KEYWORDS: *antiallergic food, naringenin, phytochemicals, Tregs*

INTRODUCTION

Allergy is defined as an inappropriate, adverse immune reaction to harmless antigens, including those derived from food. These abnormal immunologic responses against allergens are classified into several types depending on the pathophysiological mechanism of the reaction.[1] The common therapy to prevent allergy is suppression of degranulation of activated mast cells or antagonistic drugs to block the action of allergic mediators. Injection of anti-IgE antibodies is also used as immunotherapy.[2] Although various therapies have been developed, preventing allergies using antiallergic dietary factors may be a promising strategy.

Among numerous plant-derived nutrients and phytochemicals in the human diet, flavonoids are the most common polyphenolic compounds and are ubiquitous in fruits, vegetables, and beverages, including tea, juice, wine, and coffee. According to their chemical structure, flavonoids can be categorized into several major subclasses, which include flavonols, flavones, flavanones, flavanols, isoflavones, anthocyanins, and chalcones.[3,4] Many flavonoids have so far been identified, some of which are reported to have diverse health-promoting functions, including antiallergic and anti-inflammatory activities.[5,6] Green tea polyphenol epigallocatechin-3-gallate and grape seed proanthocyanidin have been reported to affect the induction of Tregs.[7,8] Several mechanisms for antiallergic functions of dietary flavonoids have been also proposed, and the existence of a key molecule that regulates the functions of flavonoids has been reported.[9]

Recently, the aryl hydrocarbon receptor (AhR) has received increased attention as another receptor of flavonoids.[10] AhR is a transcription factor that is expressed constitutively in a variety of tissues and immune cells. AhR plays an important physiological role in protecting the human body from environmental pollutants by controlling the expression of detoxification-mediating genes in a ligand-dependent manner.[11] Ligand-free AhR localizes to the cytosol, but ligand binding triggers the association of AhR with the aryl hydrocarbon receptor nuclear translocator (Arnt), followed by nuclear translocation of the complex.[12] The AhR/Arnt heterodimer is capable of binding to the xenobiotic-responsive element (XRE) which is located in the regulatory region of the well-known AhR target genes.[13]

In addition to toxicological system-mediated regulation, AhR has been reported to participate in many other biological signal pathways.[14] AhR-knockout (KO) mice were resistant to the toxicological effects of the most potent dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), but were defective in T cell differentiation and more sensitive to bacterial infection. This suggests that AhR plays a role as an important factor in the differentiation of immune cells.[15,16]

Regulatory T cells (Tregs), which are a subpopulation of $CD4^+$ T lymphocytes, play an important role in immunological tolerance, which is associated with food allergy and oral tolerance.[17] Previous research has demonstrated that the functional XRE sequence exists on the gene promoter of Foxp3, which is a Treg-specific transcription factor considered a master gene of the Tregs. Quintana et al.,[18] using a chromatin immunoprecipitation (ChIP) assay, reported that AhR was recruited to the Foxp3 promoter. Recent evidence also suggests that regulation of Treg differentiation is possibly mediated by the interaction between AhR and its ligands.[19,20]

Considering that some phytochemicals are ligands to AhR,[21−23] AhR-agonistic phytochemicals must be potent enough to mediate the immunological function via regulating Foxp3 expression and, therefore, regulate different types of allergies, including foodborne allergies. The purpose of this

Received: November 11, 2011
Revised: February 1, 2012
Accepted: February 13, 2012
Published: February 13, 2012

dx.doi.org/10.1021/jf204625y | J. Agric. Food Chem. 2012, 60, 2171−2178

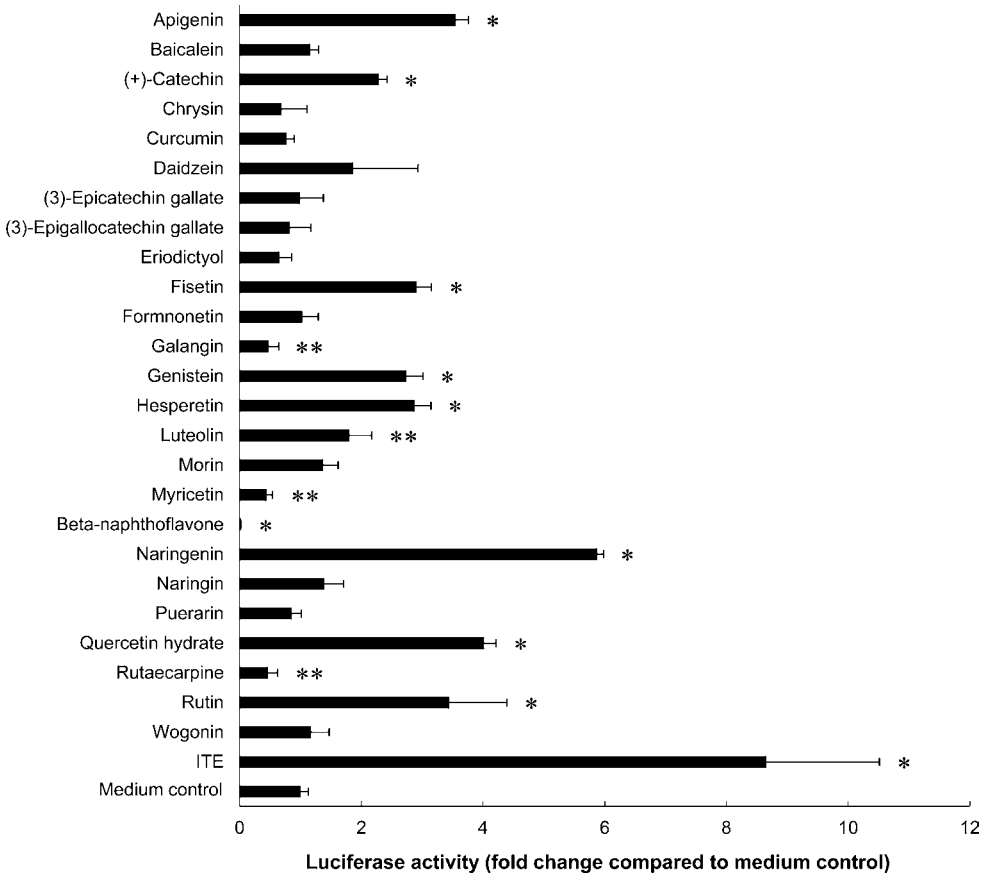

Figure 1. Agonistic effects of phytochemicals on the induction of AhR-dependent promoter activation. NIH 3T3 cells were transiently cotransfected with pGL3-XRE-Luciferase Reporter Vector together with pRL-CMV Vector for 5 h. The cells were treated with each phytochemical (10 μM) for 24 h, and then, the cell lysates were subjected to the luciferase assay. Each value is the mean ± SD (n = 3). Differences from the control were analyzed by Student's t test. Statistically significant differences are indicated by asterisks (*, $p < 0.01$; **, $0.01 < p < 0.05$).

study is to screen potential AhR-agonistic dietary phytochemicals for use as Treg-inducible dietary factors.

MATERIALS AND METHODS

Mice. Female BALB/c mice aged between 8 and 12 weeks old were obtained from Clea Japan (Tokyo, Japan) and used as experimental animals. All experiments were conducted in accordance with the institutional guidelines for animal welfare of the University of Tokyo.

Phytochemicals and Other Reagents. Apigenin and hesperetin were purchased from Sigma (St. Louis, MO, USA). Baicalein, chrysin, curcumin, (3)-epigallocatechin gallate, luteolin, myricetin, naringenin, and quercetrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Daidzein, eriodictyol, and galangin were purchased from Extrasynthese (Genay, France). Fisetin, genistein, and rutin were purchased from Wako Pure Chemicals (Tokyo, Japan). Wogonin was purchased from Calbiochem (Darmstadt, Germany). Morin was purchased from Nacalai Tesque (Kyoto, Japan). Naringin was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Puerarin was purchased from Funakoshi (Tokyo, Japan). (+)-Catechin and (3)-epicatechin gallate were purchased from Kurita Kogyo (Tokyo, Japan). Rutaecarpine, formononetin, and β-naphthoflavone were purchased from Santa Cruz (Santa Cruz, CA, USA). 2-(1′*H*-Indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and 1-methyl-*N*-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl]-1*H*-pyrazole-5-carboxamide (also known as 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide) (CH-223191) were purchased from Tocris Bioscience (Bristol, U.K.). 6,2′,4′-Trimethoxyflavone was purchased from Indofine Chemical (Hillsborough, NJ, USA).

Antibodies. The following antibodies were purchased from BD Pharmingen (San Diego, CA, USA): purified anti-mouse CD16/CD32 (Mouse BD Fc Block) (2.4G2), FITC-conjugated anti-mouse CD25 (7D4), PeCy7-conjugated anti-mouse CD4 (RM4−5), PE-conjugated anti-mouse IL10 (JES5-16E3). PE-conjugated anti-mouse Foxp3 (FJK-16s) was purchased from eBioscience (San Diego, CA, USA). Anti-mouse TGF-β1 Alexa Flour 488 (27232) was purchased from R&D systems (Minneapolis, MN, USA).

Cell Preparation and Culture. Mouse NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell cultures were performed in a 37 °C incubator with 5% CO_2. For preparing single-cell suspended primary splenocyte culture, spleens were removed from BALB/c mice and gently ground in 6 mL of ice-cold Roswell Park Memorial Institute (RPMI) medium. $CD4^+$ T cells were isolated from suspended splenocytes by magnetic-activated cell sorting (MACS) using anti-mouse CD4 beads (Miltenyi Biotec, Bergisch Gladash, Germany) with a typical purity of >95%. Isolated $CD4^+$ T cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) medium supplemented with 10% heat-treated FCS, 100 U/mL penicillin, 100 μg of streptomycin, and 3 mM L-glutamine.

In Vitro Induction of Tregs. For in vitro induction and differentiation of Tregs, 5×10^6 $CD4^+$ T cells isolated from murine splenocytes were cocultured with plate-bound anti-CD3 antibody (1 μg/mL) (BD Pharmingen, San Diego, CA, USA), soluble anti-CD28 antibody (0.5 μg/mL) (BD Pharmingen), and recombinant murine IL-2 (0.2 ng/mL) (PeproTech, Rocky Hill, NJ, USA) in the presence or absence of recombinant murine TGF-β (0.125 ng/mL) (R & D Systems, Minneapolis, MN, USA) together with the phytochemicals for 72 h.

T-Cell Proliferation Suppression Assay. The $CD4^+$ T cells cultured with phytochemicals for 72 h were recovered and assayed for

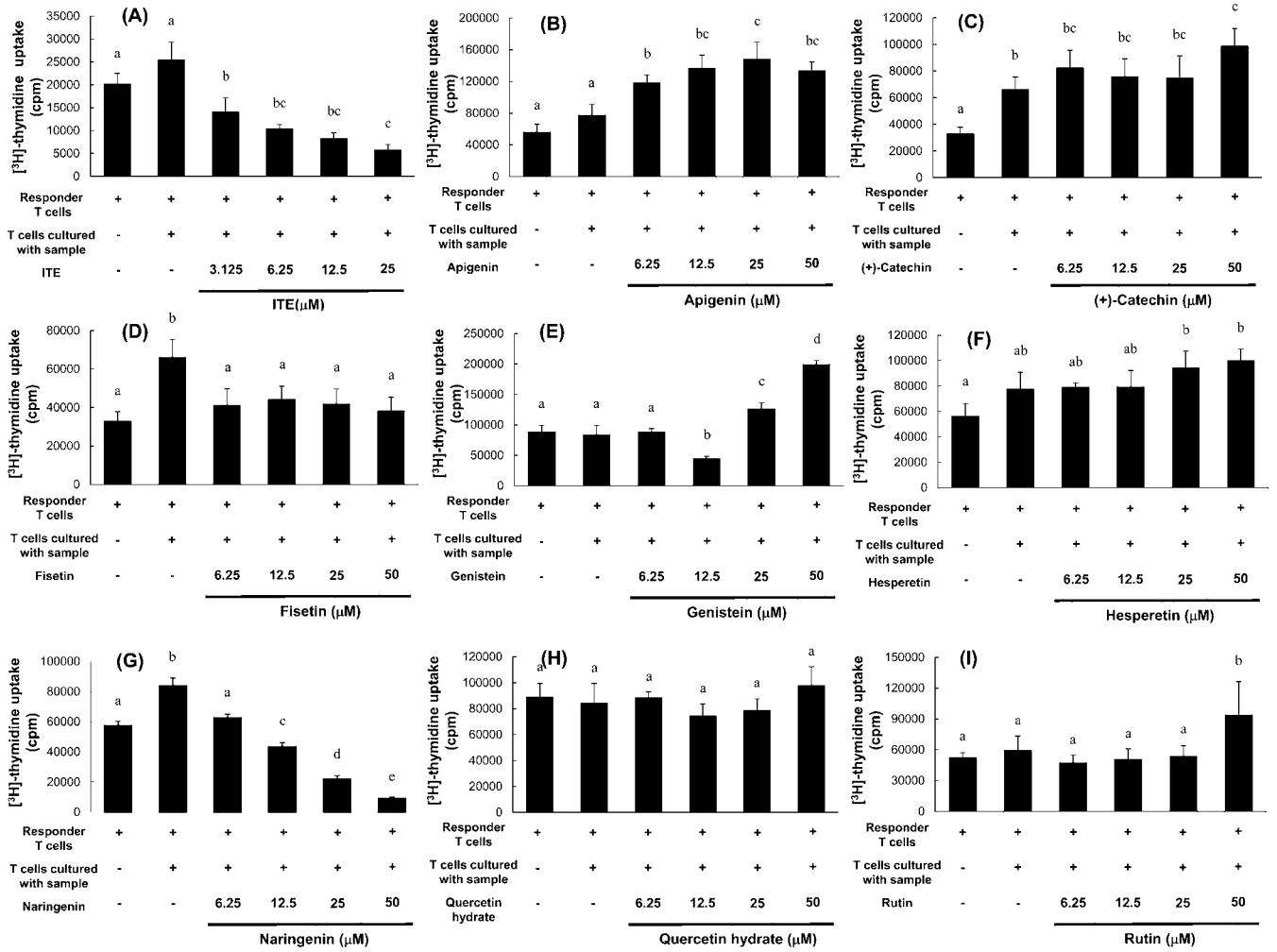

Figure 2. Effect of phytochemicals on the induction of immunosuppressive activity of $CD4^+$ T cells in the presence of TGF-β in vitro. Murine splenic $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of selected phytochemicals (6.25, 12.5, 25, and 50 μM) for 72 h and recombinant mouse TGF-β (0.125 ng/mL), and then, the cells were recovered. The recovered $CD4^+$ T cells (2.5×10^4 per well) were coincubated with responder $CD4^+$ T cells (5×10^4 per well) for 96 h in the presence of anti-CD3 (2.5 μg/mL) and anti-CD28 (2.5 μg/mL) antibodies. The immunosuppressive activities of the $CD4^+$ T cells cultured with phytochemicals were assessed by measuring ^{3}H-thymidine intake. Endogenous AhR ligand ITE (A) and other selected phytochemicals with AhR-agonistic activity, namely, apigenin (B), (+)-catechin (C), fisetin (D), genistein (E), hesperetin (F), naringenin (G), quercetin hydrate (H), and rutin (I), were examined. Each value is the mean ± SD (n = 3). Statistically significant differences (*, $p < 0.05$) are indicated by different letters (Tukey's test).

their suppressive activity on T-cell proliferation. The $CD4^+$ T cells (2.5×10^4 per well) were coincubated with responder $CD4^+$ T cells (5×10^4 per well) isolated from BALB/c mouse splenocytes in a U-bottomed 96-well plate for 96 h in the presence of anti-CD3 (2.5 μg/mL) and anti-CD28 (2.5 μg/mL). Proliferation of the $CD4^+$ T cells was evaluated by measuring incorporation of ^{3}H-thymidine (37 kBq/well) (ICN Pharmaceuticals, Costa Mesa, CA, USA) added into the culture during the final 24 h of incubation.

Immunofluorescence Staining. After incubating T cells with naringenin, the cells were recovered and then subjected to immunofluorescence staining. For intracellular staining, the cells were restimulated with PMA/ionomycin (Sigma-Aldrich, St. Louis, MO, USA) and Golgistop (BD Pharmingen, San Diego, CA, USA). After 5 h restimulation, the cells were recovered and incubated with Fc Block antibody and stained with anti-CD25-FITC and anti-CD4-PeCy7 according to each experimental design. Then cells were fixed and permeabilized followed by labeling with anti-Foxp3-PE, anti-IL-10-PE, and anti-TGF-β1 Alexa Flour 488. For intracellular staining of Foxp3, cells were fixed in Foxp3 fixation/permeabilization concentrate and diluent (eBioscience, San Diego, USA), and permeabilized with permeabilization buffer (eBioscience). For intracellular staining of IL-10 and TGF-β, cells were fixed in IC fixation buffer (eBioscience), and permeabilized with permeabilization buffer.

Luciferase Reporter Assay for Murine AhR Activity. Mouse NIH 3T3 cells were seeded on 24-well plates and transiently cotransfected with pGL3-XRE-Luciferase Reporter Vector together with pRL-CMV Vector using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The pRL-CMV vector, constitutive expression of *Renilla* luciferase, was used as an internal control value. After transfection, the cells were treated with each phytochemical (10 μM) and cultured for 24 h, and then they were subjected to the luciferase assay. In this experiment, we chose ITE, a nontoxic tryptophan-derived endogenous AhR ligand, as a positive control, because 2,3,7,8-tetrachlorodibenzo-*p*-dioxins (TCDD) and 3-methylcholanthrene (3MC), which are frequently used as ligands in AhR-dependent reporter analysis, showed toxicity to the cells. Luciferase reporter activities were tested after 24 h of incubation according to the instruction manual for the Dual-Luciferase reporter assay (Promega, Madison, WI, USA).

Statistical Analyses. All data presented are the mean ± standard deviation (SD) values of 3 or more independent experiments performed in triplicate. Statistical comparisons were performed by

dx.doi.org/10.1021/jf204625y | J. Agric. Food Chem. 2012, 60, 2171–2178

analysis of Student's t test or Tukey's tests. Results were considered significant when p values were <0.05.

RESULTS

Agonistic Effects of Phytochemicals on the Induction of AhR-Dependent Transcriptional Activity. The XRE-luciferase reporter assay was carried out to examine whether the test compounds could increase or decrease the AhR-dependent transcriptional activity. NIH3T3 cell lines transfected with an XRE-luciferase reporter plasmid were used for this purpose. ITE, used as a positive control, exhibited high activity in the XRE-luciferase reporter assay (Figure 1). Among the 25 samples tested, apigenin, (+)-catechin, fisetin, genistein, hesperetin, naringenin, quercetin hydrate, and rutin showed markedly high luciferase activities, whereas galangin, myricetin, β-naphthoflavone, resveratrol, and rutaecarpine showed significantly low luciferase activities (Figure 1). The 8 flavonoids with AhR ligand activity, which induced luciferase expression as shown above, were subsequently analyzed with respect to the induction of immunosuppressive $CD4^+$ T cells.

Naringenin Affects Immunosuppressive Activity of Tregs in the Presence of TGF-β. To investigate whether phytochemicals affect the induction of Tregs and their suppressive function, we cultured mouse splenic $CD4^+$ T cells with the phytochemicals in the presence of anti-CD3 and anti-CD28 antibody stimulation, and then we performed the T cell proliferation suppression assay in vitro. $CD4^+$ T cells isolated from splenocytes were cultured with the 8 potent AhR-agonistic flavonoids in the presence of a suboptimal dose of TGF-β, which is a crucial cytokine in the in vitro induction of Tregs.[24] Immunosuppressive activity of the cultured $CD4^+$ T cells was evaluated by measuring their effect on the proliferation of responder $CD4^+$ T cells.

The $CD4^+$ T cells, prepared by culturing with the endogenous AhR ligand ITE, in the presence of TGF-β, exhibited dose-dependent immunosuppressive activity (Figure 2A). Immunosuppressive effects of the $CD4^+$ T cells incubated with each of the 8 flavonoids were also investigated, and the results are shown in Figure 2B–I. Unexpectedly, apigenin, (+)-catechin, fisetin, genistein, hesperetin, quercetin hydrate, and rutin did not show any immunosuppressive activity. In marked contrast, the $CD4^+$ T cells cultured with naringenin showed dose-dependent suppression of the proliferation of responder $CD4^+$ T cells. This suggests that naringenin enhances the immunosuppressive activity of $CD4^+$ T cells in the presence of TGF-β.

Naringenin Affects Treg Induction, Thereby Inhibiting the Proliferation of Responder $CD4^+$ T Cells in the Absence of TGF-β. Since TGF-β modulates the differentiation of T cells to Tregs, the increased naringenin-induced immunosuppressive activity (Figure 2G) might be due to the modulation of TGF-β-mediated pathways. We, therefore, investigated whether naringenin promotes the induction of Tregs or affects the activity of Tregs in the absence of TGF-β. $CD4^+$ T cells incubated with naringenin in the absence of TGF-β also showed immunosuppressive activity on responder $CD4^+$ T cells (Figure 3). This suggests that naringenin directly affects the induction of Tregs, irrespective of the involvement of the TGF-β signaling pathway.

To further confirm whether the immunosuppressive effect was induced by naringenin via the Treg induction, we characterized the immunological phenotype expression of $CD4^+$ T cells cultured with naringenin. Our data showed that

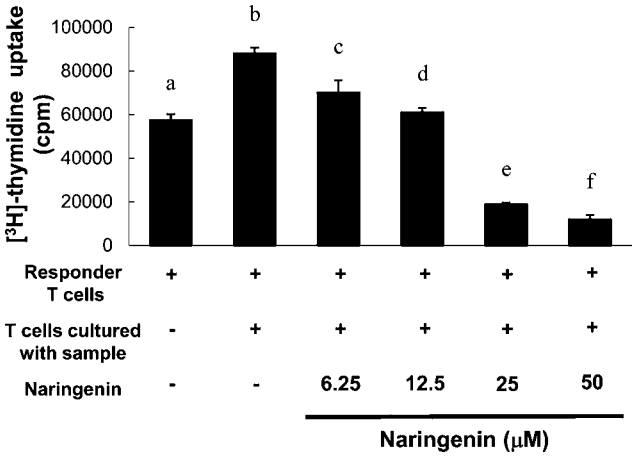

Figure 3. Naringenin upregulates the induction of immunosuppressive $CD4^+$ T cells in the absence of TGF-β in vitro. Murine splenic $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of naringenin (6.25, 12.5, 25, and 50 μM) and in the absence of TGF-β for 72 h, and then, the cells were recovered. The recovered $CD4^+$ T cells were assessed for their suppressive activity on T cell proliferation, as described in Figure 2. Each value is the mean ± SD (n = 3). Statistically significant differences (*, $p < 0.05$) are indicated by different letters (Tukey's test).

the expression of $CD4^+Foxp3^+$ Tregs and $CD4^+CD25^+Foxp3^+$ Tregs was upregulated by incubating cells with naringenin (Figure 4). We also found that the population of $CD4^+TGF\text{-}\beta^+$ cells was significantly increased by naringenin (Figure 5B). The increased tendency of population of $CD4^+IL\text{-}10^+$ cells was also observed (Figure 5A). These results suggest that naringenin induces functional $CD4^+$ Tregs, which possess immunosuppressive activities in the absence of TGF-β.

Naringenin Affects Treg Induction through AhR-Ligand Specific Interaction in Vitro. To further confirm whether naringenin affects the induction of Tregs via AhR–ligand interaction, we investigated the effect of AhR antagonists on induction of Tregs. Three different AhR antagonists, namely, resveratrol, 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-o-tolyazo-phenyl)-amide (CH-223191), and 6,2′,4′-trimethoxyflavone (TMF), were tested to verify whether the immunosuppressive effect of naringenin was blocked by the AhR antagonists. Antagonists were added to the culture medium when $CD4^+$ T cells were treated with naringenin, and then, the T cell proliferation suppression assay was performed.

Our results suggest that naringenin affects the immunosuppressive activity of Tregs in an AhR-dependent manner. Immunosuppressive activities of the $CD4^+$ T cells cultured with naringenin were attenuated by adding AhR antagonists to the culture, and this effect was AhR antagonist dose-dependent in nature (Figure 6). These results suggest that a specific interaction between AhR and naringenin is involved in the naringenin-mediated immunosuppressive activity of $CD4^+$ T cells. These data confirm that naringenin can affect the induction of Tregs, thereby enhancing immunosuppressive activities.

DISCUSSION

This study was undertaken to find new antiallergic dietary factors by screening dietary phytochemicals with AhR-agonistic activity in order to identify the compound inducing

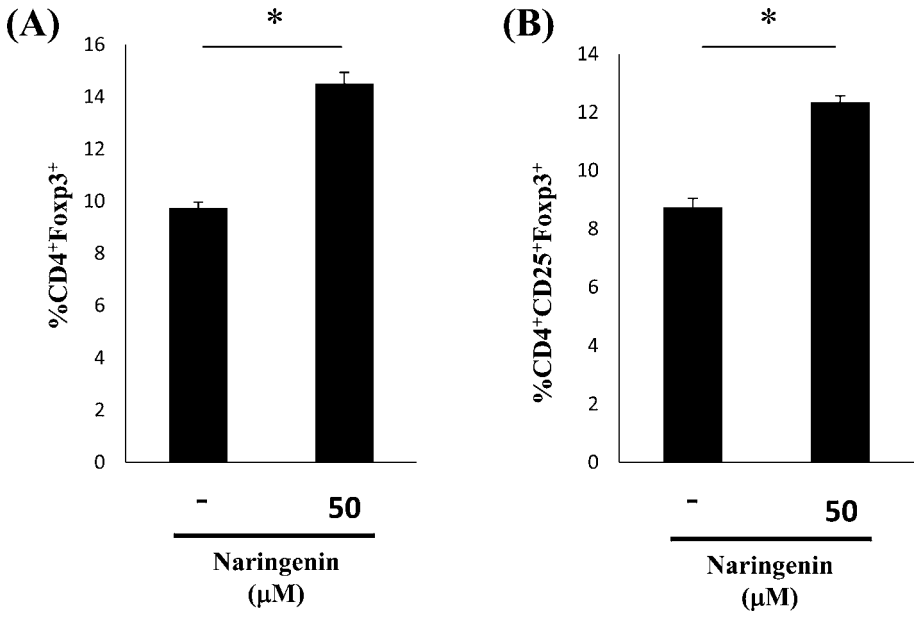

Figure 4. Phenotypic characterization of T cell populations induced by naringenin in the absence of TGF-β. Frequencies of CD4 T cell populations were analyzed by flow cytometry. CD4 cells isolated from spleens of BALB/c mice were stained with anti-CD4, anti-CD25, and anti-Foxp3 antibodies. $CD4^+Foxp3^+$ T cell population (A) and $CD4^+CD25^+Foxp3^+$ T cell population (B) were analyzed by FACS. Each value is the mean ± SD (n = 3). Statistically significant differences are indicated by asterisks (*, $p < 0.01$).

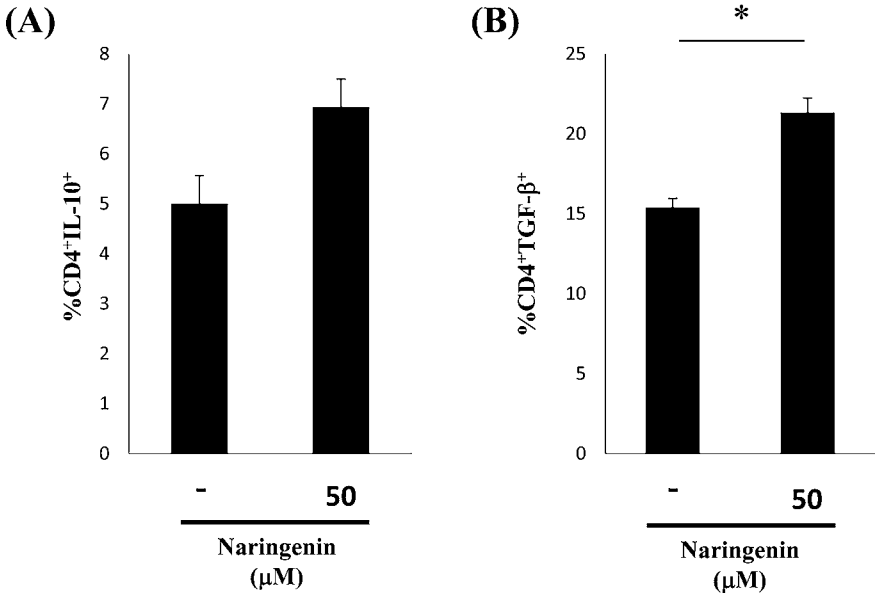

Figure 5. Flow cytometric analysis of cytokine producing cells among $CD4^+$ T cells induced by naringenin in the absence of TGF-β. Frequencies of CD4 T cell populations were analyzed by flow cytometry. CD4 cells isolated from spleens of BALB/c mice were stained with anti-CD4, anti-IL-10, and anti-TGF-β antibodies. $CD4^+IL\text{-}10^+$ T cell population (A) and $CD4^+TGF\text{-}\beta^+$ T cell population (B) were analyzed by FACS. Each value is the mean ± SD (n = 3). Statistically significant differences are indicated by asterisks (*, $p < 0.01$).

immunosuppressive activity of $CD4^+$ T cells. Among the 25 samples tested, 8 were identified as AhR ligands, and from these, only naringenin was found to enhance the induction of immunosuppressive $CD4^+$ T cells in an AhR-dependent manner. These results indicate that not all AhR ligands can induce Tregs and suggest that the specific activity of naringenin is a promising immunosuppressive dietary factor.

Naringenin is a phytochemical, classified as a flavanone, ubiquitously present in citrus fruits. Previous studies have shown that naringenin has several biological activities, including potential immunomodulatory functions.[25−28] Remarkably high naringenin-induced luciferase reporter activity as observed in our experiment (Figure 1) suggests that naringenin acts as a potent AhR ligand inducing the expression of AhR target genes.

Tregs, which have suppressive activity on other immune cells, play a central role in the regulation of immune responses, controlling immunological self-tolerance, and balancing the immune system. TGF-β, which modulates $Foxp3^+$ Treg expression and its activity, has been reported to play an important role in the differentiation and maintenance of $Foxp3^+$ Tregs.[24] Our present results demonstrate that naringenin enhances the immunosuppressive activities of

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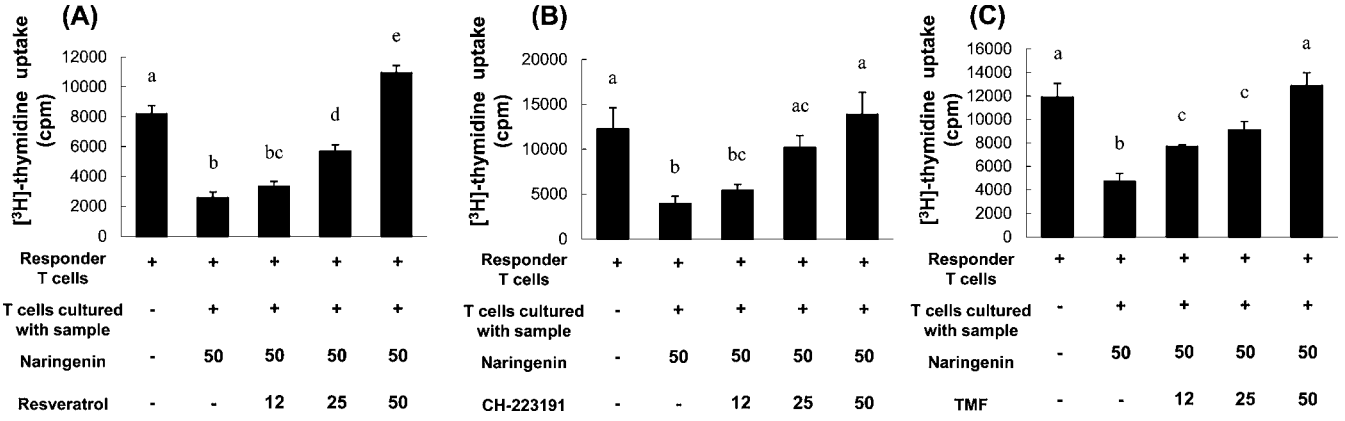

Figure 6. Naringenin directly affects the induction of immunosuppressive $CD4^+$ T cells in an AhR-dependent manner in vitro. Murine splenic $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of naringenin (50 μM) together with each AhR antagonist (12.5, 25, and 50 μM) for 72 h, and then, the cells were recovered. The recovered $CD4^+$ T cells were assessed for their suppressive activity on T cell proliferation, as described in Figure 2. The effect of the 3 AhR antagonists, namely, resveratrol (A), CH-223191 (B), and TMF (C), was examined. Each value is the mean ± SD (n = 3). Statistically significant differences (*, $p < 0.05$) are indicated by different letters (Tukey's test).

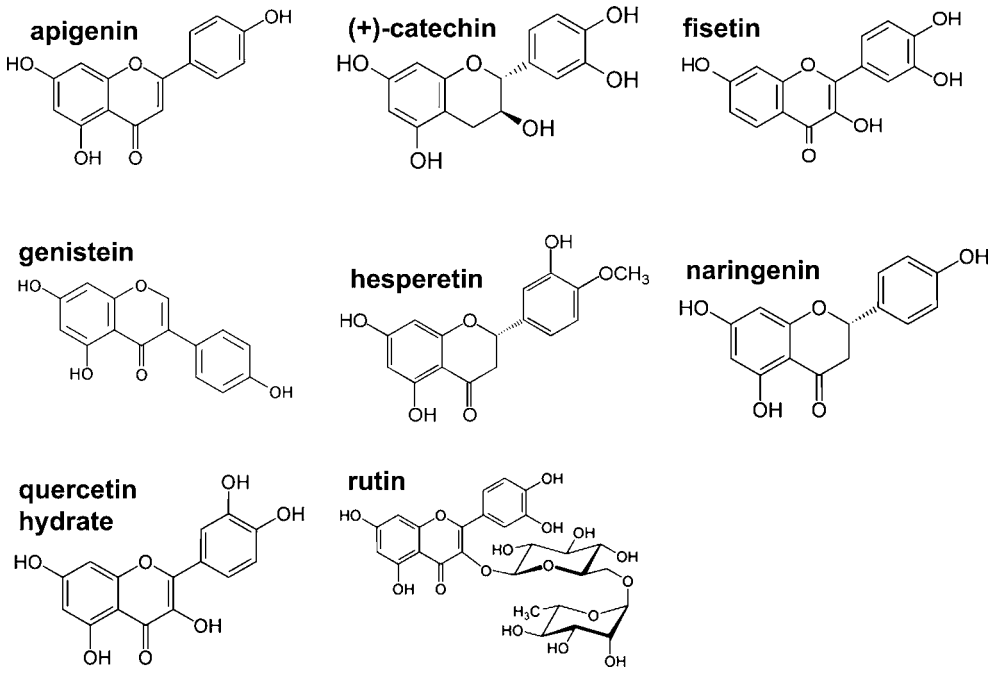

Figure 7. Structures of agonistic AhR compounds screened in this study.

$CD4^+$ T cells activated in the presence of TGF-β in vitro. Interestingly, in the absence of TGF-β also, naringenin induces typical $CD4^+CD25^+Foxp3^+$ Tregs (Figure 4B). This indicates that a TGF-β-independent signaling pathway might be involved in the induction of naringenin-mediated Treg differentiation. Recently, Gandhi et al.[29] have reported that the in vitro activation of AhR by its ligand TCDD induced different patterns of T cell differentiation depending on the cytokines added. The presence of TGF-β promoted differentiation to $Foxp3^+$-inducible Tregs (iTregs), whereas Tr1-type cells producing IL-10 were induced in the absence of TGF-β. We then investigated which types of Tregs were induced by naringenin. Our data suggested that both types of Tregs were induced by naringenin, although the increase in the population of $CD4^+IL\text{-}10^+$ cells was not significant (Figure 5B).

To determine whether the immunosuppressive effects of naringenin were mediated by AhR, we investigated the effect of AhR antagonists on the induction of Tregs. Three different AhR antagonists, namely, resveratrol, TMF, and CH-223191, were used in this study. Resveratrol is a natural compound present in a variety of plants. Carlson et al.[30] reported that resveratrol has several bioactivities, including chemopreventive activity that arises from its AhR antagonistic activity. Resveratrol has been reported to prevent TCDD-induced transformation of the cytosolic AhR to its nuclear DNA-binding form.[31] It also inhibits TCDD-induced CYP1A1 enzyme activity by preventing binding of AhR to the promoter sequences that regulate CYP1A1 transcription.[32,33] Hence, resveratrol is known to inhibit AhR–ligand interaction as well as AhR binding to the promoter region of the target genes. Comparatively, TMF is known as an AhR antagonist that competes with AhR agonists, displaying no partial agonistic activity and exhibiting no cell line/promoter dependence in vitro.[34] Kim et al.[35] reported that CH-223191 blocks the

binding of TCDD to AhR, inhibits TCDD-induced nuclear translocation, and suppresses DNA binding of AhR both in vitro and in vivo. CH-223191 is, therefore, recognized as a specific AhR antagonist.

Our investigation showed that the immunosuppressive effect of naringenin was attenuated by cotreatment with AhR antagonists, suggesting that AhR activation is indispensable for the immunosuppressive activity of naringenin. Considering that the antagonists used in this experiment have different characteristics and action mechanisms, this result might indicate that the immunosuppressive activity of naringenin is regulated by its direct interaction with the ligand-binding site of the AhR molecule.

In our screening system, apigenin, (+)-catechin, fisetin, genistein, hesperetin, naringenin, quercetin, and rutin exhibited markedly high luciferase reporter activities, suggesting that they are potent AhR agonists. However, only naringenin demonstrated immunosuppressive activity in the T cell proliferation suppression assay. It is possible that simply being an AhR agonist is not sufficient to be an immunosuppressive factor.

The relationship between the structure and bioactivity of flavonoid has been investigated in depth.[36,37] Peng et al.[38] reported, for example, that flavonoid structure affects the lipid antioxidant activity. To investigate the relationship between the structure and immunoregulatory activity of the flavonoid, we compared the structure of the 8 flavonoids that induced luciferase expression in this study (Figure 7). The common structural components of flavonoids are 2 benzene rings on either side of a 3-carbon ring. In addition, flavonoids are classified into various classes according to the combinations of oxygen atoms, hydroxyl groups, methyl groups, and sugars attached to the component structures.[39] Among the 8 potent agonistic AhR flavonoids we found, apigenin is categorized as a flavone. Hesperetin and naringenin are classified as flavanones. Genistein, quercetin, and (+)-catechins are categorized as isoflavone, flavonol, and flavanol, respectively. Thus, these agonistic AhR phytochemicals belong to a range of different groups of flavonoids. Although the structures of the 8 flavonoids are diverse, we found that the structure of apigenin resembled that of naringenin. Nevertheless, apigenin did not show any functional immunosuppressive activity. In contrast to the suppressive effect, apigenin as well as (+)-catechin and hesperetin promoted the proliferation of responder $CD4^+$ T cells.

Quintana et al.[18] have demonstrated that the activated AhR, indeed, plays an important role in the promotion and differentiation of T cells in an AhR ligand-dependent manner. Our data suggests that different modes of interaction between each flavonoid with AhR induce disparate downstream signaling pathways, thereby leading to variant immunomodulatory activities. The specific structure of naringenin, including lack of double bond in the C-ring, which is different from that of apigenin, might contribute to its functional immunosuppressive activity.

Considering the specificity of naringenin in the induction of Tregs, it might be possible that another receptor which is specific to naringenin exists and participates in the Treg induction. Looking for such a receptor may be an interesting approach to clarify the mechanism for this Treg induction by naringenin.

In conclusion, we investigated the immunoregulatory effect of several potent AhR-agonistic phytochemicals on the induction of $CD4^+$ Tregs. Although activated AhR is reported to participate in the regulation of Tregs, our research reveals that not all AhR agonistic flavonoids are able to regulate the immunosuppressive effect of Tregs. Induction of $CD4^+Foxp3^+$ iTreg cells by naringenin via an AhR-mediated pathway observed in this study may be because of the unique structure or conformation of naringenin. This study showing the immunoregulatory activity of naringenin via the AhR signaling pathway may provide new information on the antiallergic function of dietary flavonoids.

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Funding

This work was partly supported by a Grant-in Aid from the Japan Society for the Promotion of Science (No. 22658040).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; CH-223191, 1-methyl-*N*-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl]-1*H*-pyrazole-5-carboxamide, 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide; ITE, 2-(1′*H*-indole-3′-carbonyl)-thiazone-4-carboxylic acid methyl ester; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF-β, transforming growth factor beta; TMF, 6,2′,4′-trimethoxyflavone; Tregs, regulatory T cells

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