

3,5,6,7,8,3',4'-Heptamethoxyflavone reduces interleukin-4 production in the spleen cells of mice

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ABSTRACT

In our previous studies, we reported anti-inflammatory functions of 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF), which is a polymethoxyflavone rich in various citrus fruits. Here, we investigated the immunomodulatory function of HMF in mice. HMF administration (50 mg/kg, i.p., 2 times/week) tended to reduce the production of antigen-specific IgE induced by ovalbumin in combination with aluminum hydroxide gel. Fluorescence-activated cell sorting analysis revealed the reduction of interleukin-4⁺CD4⁺ spleen cells and sustained presence of interferon- γ ⁺CD4⁺ spleen cells in mice administered HMF, whereas the ratio of CD4⁺CD8⁻ versus CD4⁺CD8⁺ spleen cells was not affected. Interleukin-4 release from CD3/CD28-stimulated spleen cells of mice administered HMF was reduced, whereas interferon- γ release was not affected. These results suggest that HMF has an immunomodulatory function via reduced interleukin-4 expression.

Gel and Coombs classification classifies hypersensitivity states into four types. Of these, the IgE-mediated hypersensitivity state is called allergy, which is involved in several conditions, such as allergic rhinitis, atopic dermatitis, asthma, and food allergy. Because IgE production is critical for the pathogenesis of these diseases, a treatment to reduce IgE is anticipated. IgE production is promoted by interleukin (IL)-4 and suppressed by interferon (IFN)- γ stimulation. Targeting production of these cytokines might contribute to alleviate the symptoms of diseases mediated by IgE modulation (16).

Polymethoxyflavones (PMFs) have a broad spectrum of biologic activities (5). Nobiletin (3',4',5,6,

7,8-hexamethoxyflavone) is a PMF present in the peel of citrus fruits, and various biologic activities have been demonstrated in *in vitro* and *in vivo* experiments. Nobiletin stimulates the cAMP-dependent protein kinase and extracellular signal-regulated kinase signaling pathways in PC12 cells and cultured hippocampus neurons (7, 8). In *in vivo* experiments, nobiletin has been shown to improve memory deficits in an animal model of Alzheimer's disease (12) as well as ischemia-induced memory deficits (18), and to improve motor and cognitive deficits in an animal model of MPTP-induced Parkinson disease (17). 3,5,6,7,8,3',4'-Heptamethoxyflavone (HMF) is a PMF structurally related to nobiletin (4, 9). HMF is reported to have anti-inflammatory functions in the brain (10, 11), and to suppress inflammatory osteoclastogenesis and alveolar bone resorption (6). However, immunomodulatory functions of HMF have not been addressed.

In the present study, we demonstrate that IL-4 production in mice is reduced by HMF administration and there is a tendency for HMF to reduce antigen-

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specific IgE production, suggesting an immunomodulatory function of HMF.

MATERIALS AND METHODS

Animals. Mice were maintained under a controlled temperature and photoperiod (23°C, 12-h light and 12-h dark) with *ad libitum* food and water. All experimental procedures followed the Guideline for Animal Experimentation prepared by the Animal Care and Use Committee of Matsuyama University. C57BL/6J and 129 hybrid mice were used.

HMF treatment. HMF was generously provided by USHIO Chemix (lot no. H13166 and H13020, Omaezaki, Japan). HMF treatment of mice was started at 6 weeks of age. HMF was suspended in corn oil (Sigma-Aldrich #C8267, St. Louis, MO, USA) as a vehicle and intraperitoneally injected at a dose of 17 or 50 mg/kg, twice a week for 2 to 5 weeks.

Induction of OVA-specific IgE. Mice were sensitized and challenged subcutaneously at the base of tail with aluminum hydroxide gel (Wako #012-24241, Osaka, Japan) and chicken ovalbumin (OVA; albumin from chicken egg white, Sigma-Aldrich #A2512) at a dose of 50 µg/0.2 mL/mouse following the manufacturer's protocol for the gel. The first immunization was performed 2 weeks after the initiation of HMF treatment and boosted immunity was carried out 2 weeks after the first immunization. After 1 week of boosted immunity, whole blood was collected.

Measurement of total IgE and OVA-specific IgE levels. Whole blood was obtained via cardiac puncture using a 25-gauge needle on the indicated day. Whole blood was mixed with heparin (Wako #081-00136). The final concentration of heparin was approximately 150 µg/mL and plasma was isolated by centrifugation from whole blood. Total IgE in the plasma was determined using Mouse IgE ELISA MAX Deluxe Set (BioLegend #432404, San Diego, CA, USA). OVA-specific IgE was determined using LEGEND MAX Mouse OVA Specific IgE ELISA Kit (BioLegend #439807).

Flow cytometry. To examine the lymphocyte population in the spleen, single cell suspensions were prepared by mincing and then passed through a 40-µm nylon mesh (FALCON #352340, Corning, NY, USA). The spleen cells were treated with ACK lysis buffer for 5 min. After washing with Hanks' Balanced Salt

Solution (HBSS, GIBCO #14170112), the pellet was used as spleen leukocytes.

For the analysis of CD4- and CD8-defined cell populations in spleen leukocytes, cells were incubated with a panel of antibodies, including purified anti-mouse CD16/32 antibody (BioLegend #101301), APC anti-mouse CD8a antibody (BioLegend #100711), and PE anti-mouse CD4 antibody (BioLegend #100511). After washing with PBS, the cells were fixed with 2% paraformaldehyde in PBS.

For intracellular staining of IL-4 and IFN-γ, spleen leukocytes were stimulated for 4 h with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich #79346) and 1 µg/mL ionomycin (Sigma-Aldrich #10634) in the presence of Golgi Plug Protein Transport Inhibitor (Becton Dickinson #555029, Franklin Lakes, NJ, USA). The cells were stained for CD4 (APC anti-mouse CD4 antibody: BioLegend #100515) as described above. After fixation with 2% paraformaldehyde in PBS, IL-4 and IFN-γ were stained in the saponin buffer containing 0.1% saponin, 2% fetal calf serum, 0.1% NaN₃, and Hanks balanced salt solution. The following antibodies were used: PE anti-mouse IL-4 antibody (BioLegend #504103), PE anti-IFN-γ antibody (BioLegend #505807), and PE Rat IgG1, κ isotype control antibody (BioLegend #400407).

Flow cytometry was performed using FACS Canto with Diva software (Becton Dickinson).

Cytokine assay. For the assay of cytokines produced by T lymphocytes in HMF-treated mice, spleen leukocytes were prepared from mice administered HMF for 2 weeks. The spleen leukocytes were stimulated with coated 1 µg/mL anti-CD3 (BioLegend #100313) and soluble 1 µg/mL anti-CD28 (BioLegend #102111) antibodies for 72 h. IL-4 and IFN-γ in the culture supernatant were assessed by enzyme-linked immunosorbent assay (ELISA) kits (BioLegend #431107 and #430807, respectively).

Statistical analysis. Student's *t*-test was used to analyze the intergroup differences. A *P* value of less than 0.05 was considered statistically significant. All data are presented as mean ± SEM.

RESULTS

HMF alleviates IgE production

To evaluate immunomodulatory functions of HMF, mice were treated with HMF for 5 consecutive weeks. Administration of HMF at a dose of 17 mg/kg (twice a week) did not affect the production of

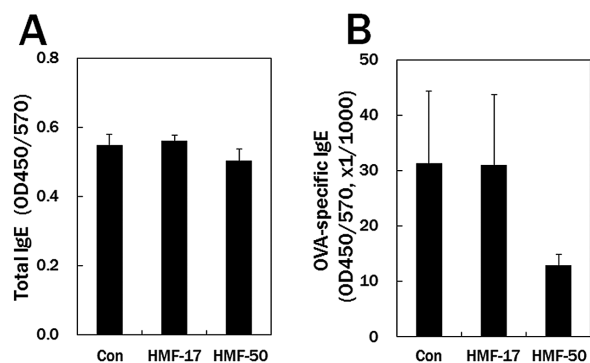


Fig. 1 Total IgE and OVA-specific IgE in mice administered HMF. HMF was intraperitoneally injected into mice at a dose of 17 or 50 mg/kg, twice a week for 5 weeks. Some groups of mice were immunized with OVA during the period of HMF administration, as described in Materials and Methods. Total IgE in the plasma of mice administered HMF (A) ($n = 4$ mice per group) and OVA-specific IgE in the plasma of mice administered HMF and OVA (B) ($n = 5-6$ mice per group) were measured.

total IgE in the plasma. At a dose of 50 mg/kg (twice a week), HMF treatment showed a tendency to reduce total IgE (Fig. 1A). Then, we challenged OVA with aluminum hydroxide gel during the period of HMF administration. The higher dose of HMF (50 mg/kg) tended to reduce OVA-specific IgE induced with adjuvant, although there was no significant difference (Fig. 1B).

HMF reduces a population of IL-4⁺CD4⁺ spleen leukocytes. To explore the immunomodulatory action of HMF, we first examined the change in T cell populations distinguished by a cluster of differentiation markers. Fluorescence-activated cell sorting (FACS) analysis revealed that the ratio of CD4⁺CD8⁻ cells/CD4⁻CD8⁺ cells was not affected by HMF administration (Fig. 2), suggesting that HMF does not affect the ratio of helper to cytotoxic T-cell population.

To examine the cytokine expression in the helper T cells, we performed the second FACS analysis. The population of IL-4⁺CD4⁺ cells was significantly reduced by HMF administration, whereas the population of IFN- γ ⁺CD4⁺ cells was not affected (Fig. 3).

HMF reduces IL-4 secretion from spleen leukocytes To confirm the reduction of IL-4⁺CD4⁺ Th2 cells, spleen leukocytes were isolated from mice administered HMF for 2 weeks and stimulated with anti-CD3/anti-CD28 antibodies for 72 h. Culture supernatants were examined for IL-4 and IFN- γ secretion using ELISA analysis. IL-4 and IFN- γ secretion was synergistically induced by the anti-CD3 and anti-CD28

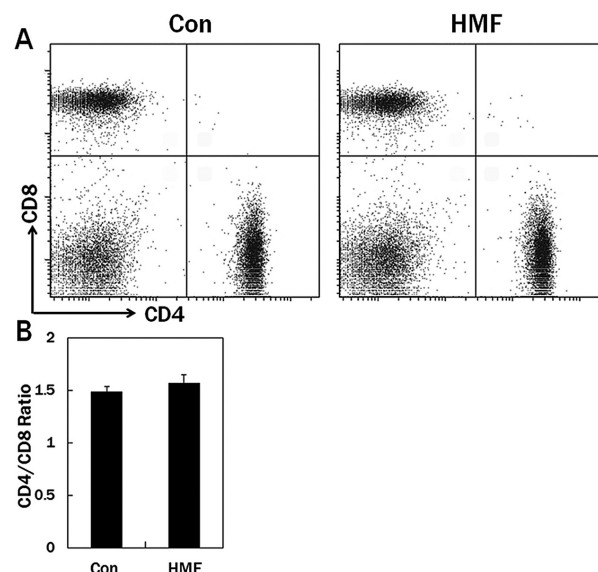


Fig. 2 CD4/CD8 T cell ratio in the spleen of mice administered HMF. HMF was intraperitoneally injected into mice at a dose of 50 mg/kg, twice a week for 3 weeks. (A) The ratio of CD4⁺CD8⁻ cells/CD4⁻CD8⁺ cells in the spleen cells of mice administered HMF was determined. (B) HMF administration did not affect the CD4/CD8 ratio. Data are from 6 mice per group.

antibodies. IL-4 secretion in the presence of two types of antibodies was significantly reduced by HMF, whereas IFN- γ secretion was not affected (Fig. 4).

DISCUSSION

In the present study, we used two different assay systems of FACS and ELISA analyses, to demonstrate that HMF reduces IL-4 expression in helper T cells. IL-4 is known to promote IgE production, and targeting IL-4 signaling has been tried in a number of clinical studies for the treatment of allergic diseases (2, 3, 14-16). As HMF treatment showed a tendency to reduce antigen-specific IgE production in mice, HMF administration might contribute to modulate immunological conditions via reduced IL-4 production in humans. In future studies, we will modify the protocols of immunization and HMF treatment in mice, and try to confirm a statistically significant effect of HMF on antigen-specific IgE production.

What kinds of T cell population are affected by HMF? In the Fig. 2, HMF has been shown not to affect the ratio of helper to cytotoxic T-cell population. Moreover, it was demonstrated that HMF reduced IL-4 production, but did not affect IFN- γ production in the Figs. 3 and 4. These evidences

suggest that HMF affects mainly T helper type 2 cells, but not type 1 cells.

Target cells of HMF for the immunomodulatory

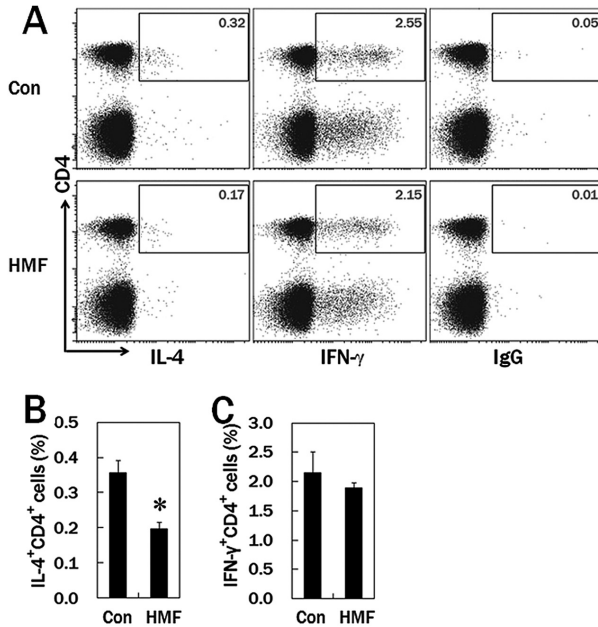


Fig. 3 Population analysis of IL-4⁺CD4⁺ and IFN- γ ⁺CD4⁺ spleen cells in mice administered HMF. HMF was intraperitoneally injected into mice at a dose of 50 mg/kg, twice a week for 3 weeks. (A) IL-4 and IFN- γ expression in the CD4-positive spleen cells from mice administered HMF was determined by FACS analysis using anti-IL-4 (IL-4), anti-IFN- γ (IFN- γ), and isotype-matched control (IgG) antibodies. (B) The IL-4⁺CD4⁺ spleen cell population was reduced by HMF administration. (C) The IFN- γ ⁺CD4⁺ spleen cell population was unchanged by HMF administration. Data are from 4 mice per group. *: $P < 0.05$.

functions are not clear. When the spleen T cells from non-treated mice were activated using anti-CD3/CD28 antibodies under the presence of HMF *in vitro*, we failed to detect the reduced IL-4 production by HMF (data not shown). This result is contrast to the result in the spleen T cells from HMF-treated mice *in vivo* (Fig. 4), suggesting a possibility that HMF does not affect T cells directly, but affect indirectly via other cells such as monocytes/macrophages or dendritic cells.

In the present stage, we speculate the involvement of monocytes/macrophages in the mechanisms of immunomodulatory functions of HMF. Recently, Okuyama *et al.* reported anti-inflammatory effect of HMF in the central nervous system (11), in which ischemia-induced microglial activation in the brain was suppressed by HMF. Onozuka *et al.* reported reduced A β pathology in an Alzheimer's disease model mice by nobiletin (3',4',5,6,7,8-hexamethoxyflavone), a structurally related compound to HMF (3,5,6,7,8,3',4'-heptamethoxyflavone). On the reduction of A β burden in the mouse brain, an involvement of microglial function has been reported (13). We are interested in the point that both PMFs (HMF and nobiletin) were administered peripherally in the experiments of these reports. Because monocyte/macrophage can enter into the central nervous system from the peripheral tissues and mediate inflammatory responses, peripheral modification of monocyte/macrophage by PMFs might affect the brain inflammations partially. Overall, including the description above mentioned, it could be possible that HMF might affect monocyte/macrophage functions, resulting in the modulation of T-cell functions.

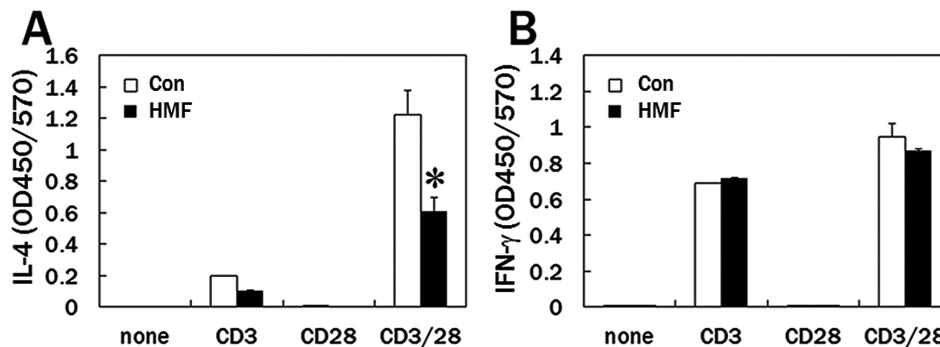


Fig. 4 IL-4 and IFN- γ secretion from CD3 and/or CD28-stimulated spleen cells of mice administered HMF. HMF was intraperitoneally injected into mice at a dose of 50 mg/kg, twice a week for 2 weeks. Spleen leukocytes were prepared and stimulated *in vitro* with anti-CD3 and/or anti-CD28 antibodies for 72 h. IL-4 (A) and IFN- γ (B) in the culture supernatant were determined by ELISA. IL-4 secretion from CD3/CD28-stimulated spleen leukocytes was reduced by HMF administration. Data from cells stimulated with anti-CD3/anti-CD28 antibodies are shown as mean $\pm$ SEM of quadruplicate samples from two mice in each group. Data from cells stimulated with anti-CD3 antibody or anti-CD28 antibody or none are of single sample. *: $P < 0.05$.

Although HMF is included in various citrus fruits, the HMF content in the peel of *Citrus kawachiensis* (Japanese name, kawachibankan) is markedly higher (over twice) than that in other citrus fruits such as *Citrus unshiu* Marcov (Japanese name, unshiu mikan), *Citrus hassaku* Hort. Ex Y. Tanaka (Japanese name, hassaku), and grapefruit (1). Therefore, administration of the peel of kawachibankan might have some immunomodulatory effects. Drinking an extract or eating a powder or paste of the peel of kawachibankan could be possible as dosing methods for HMF.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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