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ARTICLE

Anti-allergic effects of *Rosae multiflorae fructus* via inhibition of T cell proliferation and the mast cell function

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Abstract Anti-allergic effects of the hot water extract of *Rosae multiflorae fructus* (Rosae extract), which has long been used in oriental medicine for treatment of various diseases, were explored with a chicken ovalbumin (cOVA)-induced mouse model of food allergy. Compared to the sham mice to show severe allergic symptoms (i.e., anaphylaxis, diarrhea and decrease in the body temperature) following oral cOVA challenge, the Rosae extract-treated mice showed a marked improvement in those symptoms. Histology data demonstrated that Rosae extract treatment resulted in a amelioration in the intestinal inflammatory lesion and a reduction in the numbers of mast cells and eosinophils in the small intestine. Studies using DO11.10 TCR transgenic T cells indicated that Rosae extract had an activity to subdue the antigen-specific T cell activation/proliferation in vivo and thereby to lower the level of Th2 cytokine production by T cells during the antigen-specific immune response. Moreover, passive systemic anaphylaxis study showed that the extract also had an activity to inhibit the mast cells function in vivo, i.e., release of granules triggered by specific IgE-antigen interaction. Altogether, the results from this study not only imply a potential clinical application of Rosae extract in prevention and treatment of food allergy but also clearly elucidate the immunoregulatory mechanisms of Rosae extract underlying its anti-allergic effect.

Keywords Eosinophil · Food allergy · Inflammation · Mast cells · Rosae multiflora fructus · Th2 cytokines

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Introduction

Food allergy is a series of adverse immune reactions against an innocuous protein in food [1]. The severities of food allergy symptoms vary widely, ranging from mild itching, skin rash, breathing problem, vomiting and diarrhea to life-threatening anaphylaxis [2]. While the prevalence rate of food allergy varies depending on the ages of subjects participating in the surveys and the regions where the surveys were conducted, according to those of World Allergy Organization, approximately 2–10% of the population worldwide suffer from a certain type of food allergy [3, 4]. Notably, the population suffering from food allergy has been increasing steadily last couple of decades as dietary patterns and lifestyle have changed [5].

Orally ingested food proteins generally induce immune tolerance rather than immunity in the body. The oral tolerance mechanism is, however, bleached occasionally to provoke food allergy. Most food allergies are attributed to IgE-mediated (type 1) immune reactions even though some exceptions have been reported [6–8]. Cross-linking of IgE tightly coupled to the Fc receptor (FcεRI) expressed on the surface of specific immune cells (e.g., mast cells and basophils) by a food allergen triggers release of intracellular granules to bring about the adversary allergic

symptoms [9, 10]. In light of IgE production and induction of subsequent allergic inflammation, the cytokine expression profile (i.e., Th1 and Th2 balance) of allergen-specific $CD4^+$ T cells is of critical importance. Th2 cytokines such as IL-4 and IL-5 play an importance role in immunoglobulin class switching to IgE. Other Th2 cytokines such as IL-10, IL-9 and IL-13 play a critical role in recruitment of mast cells and eosinophils to the small intestine [11, 12].

Epinephrine, an α-1 agonist to cause vasoconstriction, is routinely used for treating severe anaphylactic reactions in the clinic along with other medications such as anti-histamines, glucocorticoids and β-agonists [13, 14]. Nevertheless, as dangerous anaphylactic response tends to arise acutely and intensely within a few minutes to an hour after ingestion of a food allergen, development of preventive therapeutics for food allergy is highly desirable. Oral immunotherapy, which exploits the natural oral tolerance mechanism, has emerged as a preventive treatment option [15, 16]. Despite some success, however; their uses in the clinic have been limited for the risk that the administration of a food allergen during therapy may cause anaphylactic shock.

Special traditional herbal formulas (THFs), which are hot water extracts of assorted plant species, have been used as indigenous recipes for treatments of various inflammatory disorders and gastrointestinal disorders [17]. The therapeutic effects of THFs for food allergy have been also demonstrated by several studies by others [18, 19]. Among those, studies by Li and Sampson's group using a traditional Chinese medicinal formula called "food allergy herbal formula-2 (FAHF-2)" are of special interest. In the studies using a mouse peanut allergy model, they have shown that FAHF-2 ameliorates the allergic symptoms by modulating Th1 and Th2 cytokine production by T cells and also by subduing development of basophils, recruitment of eosinophils and mast cells to the gut and degranulation of mast cells [20].

Despite such reports, demands for more efficacious and economical allergy medications continue to increase. Dried fruit of *Rosa multiflora* Thunberg has long been used alone or as an ingredient of THFs in oriental medicine for treatment of various diseases as documented in both Korean and Chinese traditional medical encyclopedias [21, 22]. It is also a member of a group of food supplements and medicinal foods approved by Korean Food and Drug Administration [23]. The immunoregulatory effect of *Rosae multiflorae fructus* extract (shortened from now on to "Rosae extract") has been unveiled recently in a study using collagen-induced mouse rheumatoid arthritis model [24, 25]. Bearing that information in mind, we, in this study, examined therapeutic effects of Rosae extract on food allergy using chicken ovalbumin (cOVA)-induced mouse food allergy model [19] and also investigated the immunological mechanisms underlying its anti-allergic effects.

Materials and methods

Chemicals and other reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin–glutamine solution, carboxyfluorescein succinimidyl ester (CFSE) and DNP-bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). cOVA (grade VI), alum [$KAL(SO_4)_2$-$12H_2O$], eosin, hematoxylin and BSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Toluidine blue was purchased from Samchun Chemical (Pyeongtaek, Korea). IL-4 and IFN-γ ELISA kits were purchased from Biolegend (San Diego, CA, USA). IL-5 and IL-10 ELISA kits were purchased from BD Biosciences (San Diego, CA, USA). Biotinylated anti-mouse total IgG and IgG2a were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Biotinylated anti-IgG1 and IgE were purchased from Biolegend and BD Biosciences, respectively. Horse radish peroxidase (HRP)-conjugated streptavidin was purchased from Biolegend. Mouse MCPT-1 ELISA kit and APC-labeled anti-DO11.10 TCR (KJ1-26) and PE-labeled mouse CD4 (GK1.5) mAbs were purchased from eBioscience (San Diego, CA, USA). Anti-DNP IgE was purchased from Sigma-Aldrich (St. Louis, USA). DO11.10 peptide (ISQAVHAAHAEINEAGR) encompassing amino acid 323–339 of cOVA was purchased from Peptron (Daejeon, Korea).

Animals

Five-week-old male BALB/C mice were purchased from Samtako Co. (Osan, Korea) and housed in Core Animal Facility in Chungnam National University. DO11.10 TCR Tg mice were purchased from Jackson Laboratory (Bar Harbor. ME, USA) and bred in Chungnam National University. The animal study protocol used in this study was approved by Animal Ethics Committee in Chungnam National University (Approval Number: CNU-00570), and animal experiments were carried out in accordance with the approved protocol.

Preparation of Rosae extract

Rosae multiflorae fructus used in this study was purchased from Kyeong-Dong Oriental Pharmacy Market (Seoul, Korea) and identified by Professor Y. H. Kim, College of Pharmacy, Chungnam National University (Daejeon, Korea). Ground-dried *Rosae multiflora fructus* powder was

infused in 10× volume of distilled water at 100 °C for 1 h. The infusion process was repeated twice. The infused water was centrifuged and filtered to remove solid ingredients. The resulting Rosae extract was then lyophilized and kept at 4 °C. The lyophilized materials were reconstituted with water before use [26]. Endotoxin level in Rosae extract was examined by using Pyrogent Plus assay kit (Lonza, Hopkinton, Massachusetts); the endotoxin level was determined undetectable. The dried Rosae extract powder was designated as KFRI-SL-2157-W and archived in the Functional Materials Research Group in Korea Food Research Institute.

cOVA-induced mouse food allergy model

Mice were immunized i.p. with cOVA (20 μg) plus alum (2 mg) twice with an interval of two weeks. One week after the second immunization, the blood was drawn from the immunized mice and the level of cOVA-specific IgG in the serum was measured with ELISA. The mice producing cOVA-specific IgG at a level in the range of mean ± SD were chosen and randomly divided into five groups (five mice per group). Two weeks after the second immunization, the mice were challenged p.o. with cOVA (50 mg) five times once every three days. During the entire challenge period, mice were either left untreated or treated daily with Rosae extract or Dexamethasone (p.o.). Immediately after the fifth challenge, mice were examined for allergic symptoms (change in rectal temperature, diarrhea and anaphylaxis) and the severities of allergic symptoms were rated and scored as previously reported [27]. The rectal temperature was measured using a rectal probe thermometer (Physitemp, Clifton, USA).

Histology

Histology experiments were performed as described by others [28, 29]. Briefly, the small intestine (duodenum) was collected 1 day after the fifth challenge and washed with PBS and fixed with 10% formalin. The tissue was embedded in paraffin, and the paraffin-embedded tissue sample was sliced into sections of 5 μm thickness with microtome. The thin sections were then stained either with toluidine blue or H&E staining solution. The stained samples were observed under the microscope, and the numbers of mast cells and eosinophils in high power field (HPF) were enumerated as described.

Measurement of the levels of cOVA-specific Igs and MCPT-1 in the serum

The blood was drawn from the mice one day after the fifth challenge, and the serum was prepared. ELISA was performed as previously described [19] with some modification. Briefly, the plates were coated overnight at 4 °C with 2 μg/mL of cOVA (100 μL). The cOVA-coated plates were blocked with 1% (w/v) BSA in 1× PBS. The serum samples, diluted 50- to 8.000-fold, were then added to the plates and incubated for 2 h. After thorough wash, biotinylated anti-mouse IgE (1 μg/mL), IgG2a (1:100 dilution), IgG (1:100 dilution) or IgG1 (1 μg/mL) was added to the plates and incubated for 1 h. After thorough wash, HRP-conjugated streptavidin (1:1500 dilution) was added and incubated for 30 min. After wash, tetramethylbenzidine (Sigma-Aldrich) was added and incubated at rt. The enzyme reaction was stopped by adding 100 μL of 2 N H_2SO_4. The optical density was read at 450 nm. MCPT-1 levels in the sera were quantified with MCPT-1 ELISA kit in accordance with the manufacturer's manual.

Measurement of the cytokine production by mesenteric lymph node cells

The mLNs were harvested from the mice 1 day after the fifth cOVA challenge, and single cell suspensions were prepared. The mLN cells (1×10^6 cells) in 200 μL of RPMI medium (10% FBS, 1× penicillin–streptomycin) were plated in a 96-well culture plate and cultured with cOVA (100 μg/mL) for three days in a 37 °C humidified CO_2 incubator. The culture supernatants were then collected, and the levels of IFN-γ, IL-5, IL-4 and IL-10 were quantified using ELISA kits in accordance with the manufacturer's manual.

Passive systemic anaphylaxis assay

BALB/C mice were injected i.v. with 3 μg of anti-DNP mAb (IgE isotype) one day before injection (i.v.) of 80 μg DNP-BSA for induction of allergic anaphylaxis reaction [30, 31]. Thirty minutes after injection of DNP-BSA, the rectal temperature of the mice was measured using a rectal probe thermometer. One hour after the injection, the blood was drawn from each mouse and the serum was prepared for analysis of the level of MCPT-1. Mice were treated with Rosae extract or Dexamethasone daily for 5 days beginning 3 days prior to the injection of the mAb.

Adoptive transfer of DO11.10 TCR Tg T cells and induction of the immune response with cOVA

Cells were isolated from the whole-body lymph nodes of DO11.10 TCR Tg mice and labeled with CFSE as described previously [32, 33]. Two million CFSE-labeled cells were injected i.v. into BALB/c mice. One day after the adoptive transfer, BALB/C mice were injected i.p. with cOVA (50 μg) plus alum (20 mg) for induction of

activation/proliferation of DO11.10 TCR Tg T cells. Eight days after the immunization, cells were isolated from the whole-body lymph nodes of the mice and stained with PE-labeled anti-mCD4 plus APC-labeled anti-KJ1-26 mAbs and analyzed with flow cytometry. The same cells were also cultured with DO11.10 peptide for 2 days, and the levels of IL-4, IL-5 and IFN-γ in the culture supernatants were measured using ELISA as described above. When the mice were treated, Rosae extract or Dexamethasone was orally administered daily for 8 days from the day when the mice were immunized with cOVA plus alum.

Statistical analysis

Statistical analyses were carried out with a program for independent-samples t test (Student's t test) in Microsoft Excel^{TM} software. p values less than 0.05 are considered statistically significant.

Results

Amelioration of food allergy symptoms by Rosae extract treatment

BALB/c mice were immunized (sensitized) and challenged with cOVA as described in Fig. 1A to induce allergic reactions. The sensitized mice were treated with Rosae extract at two different doses during challenge with orally administered cOVA, and Dexamethasone (Dexa) was used as a positive control reagent. The severities of allergic symptoms (i.e., change in rectal temperature, diarrhea and anaphylaxis) were examined immediately after the fifth challenge. A group of mice (sham), sensitized and challenged but treated with no extract or drug, showed a significant drop in the rectal temperature, recurrent diarrhea and anaphylaxis. Stools in the colon of those mice appeared watery and amorphous as well (Fig. 1).

Meanwhile, when the sensitized mice were treated with Rosae extract during the challenge with cOVA, the allergy symptoms were ameliorated considerably (Fig. 1). Thus, the rectal temperature of Rosae extract-treated mice was almost normal, and the severities of diarrhea and anaphylaxis were lowered significantly compared to those of sham mice. In line, stools in the colon of Rosae extract-treated mice appeared less watery and held the normal shape and color. While the anti-allergic effects of Rosae extract were apparent in either doses (80 and 400 mg/kg), a dose–response relationship was also observed. Expectedly, Dexa treatment also resulted in a marked improvement in the allergic symptoms.

Lack of adverse effect of Rosae extract on the cellularities of the secondary lymphoid organs

Reflecting that active immune responses were underway during the challenge with cOVA, the average number of cells in the mLNs of sham mice was significantly higher than those of naïve mice (Fig. 2A). Still, the cellularities of the mLNs of Rosae-treated mice appeared normal. In agreement with the well-known side effect of Dexa (i.e., immunotoxicity), the cellularities of the mLNs of Dexa-treated mice decreased to a considerable extent [34]. Similar observations were made with the spleens as well (Fig. 2B).

The lack of immunotoxicity of Rosae extract was confirmed in a separate experiment in which normal (unimmunized) BALB/c mice were treated with either Dexa or Rosae extract. As shown in Fig. 2C and D, when the mice were treated with Dexa, marked decreases both in the cellularity of the LN and the size of the spleen were observed [35]. In contrast, both the cellularity and the size of the respective organs of Rosae extract-treated mice appeared normal. Together, these results suggested that Rosae extract modulates cOVA-induced allergic immune responses without causing a significant immunotoxicity.

Amelioration by Rosae extract of the intestinal inflammatory lesion and obstruction of mast cells and eosinophils infiltration

Conforming to the severe allergic symptoms, the structure of the small intestines of sham mice appeared highly deformed (Fig. 3A). Of note, thickening of the submucosa and circular muscle layer of the tissue was readily observed, indicating that strong allergic inflammation had been underway. In contrast, the structure of the small intestines of the Rosae extract-treated animals appeared less damaged. That is, thickening of the submucosa and circular muscle layer of the tissue in those animals was less palpable. Similar results were obtained after Dexa treatment.

Mast cells play a central role in triggering allergic reactions by releasing various effector molecules such as histamine and MCPT-1 upon interaction with a specific allergen. It is also known that the mast cell population increases in the afflicted tissue once the allergic immune response is initiated [27, 36, 37]. Expectedly, the numbers of mast cells found in the tissue samples of sham mice were markedly higher than those of naïve mice, an average of 1.3 cells/HPF for naïve mice versus 16 cells/HPF for sham mice (Fig. 3A and B). Of note, the treatment with Rosae extract resulted in a significant reduction in the numbers of mast cells located in the small intestines (average 6 cells/HPF).

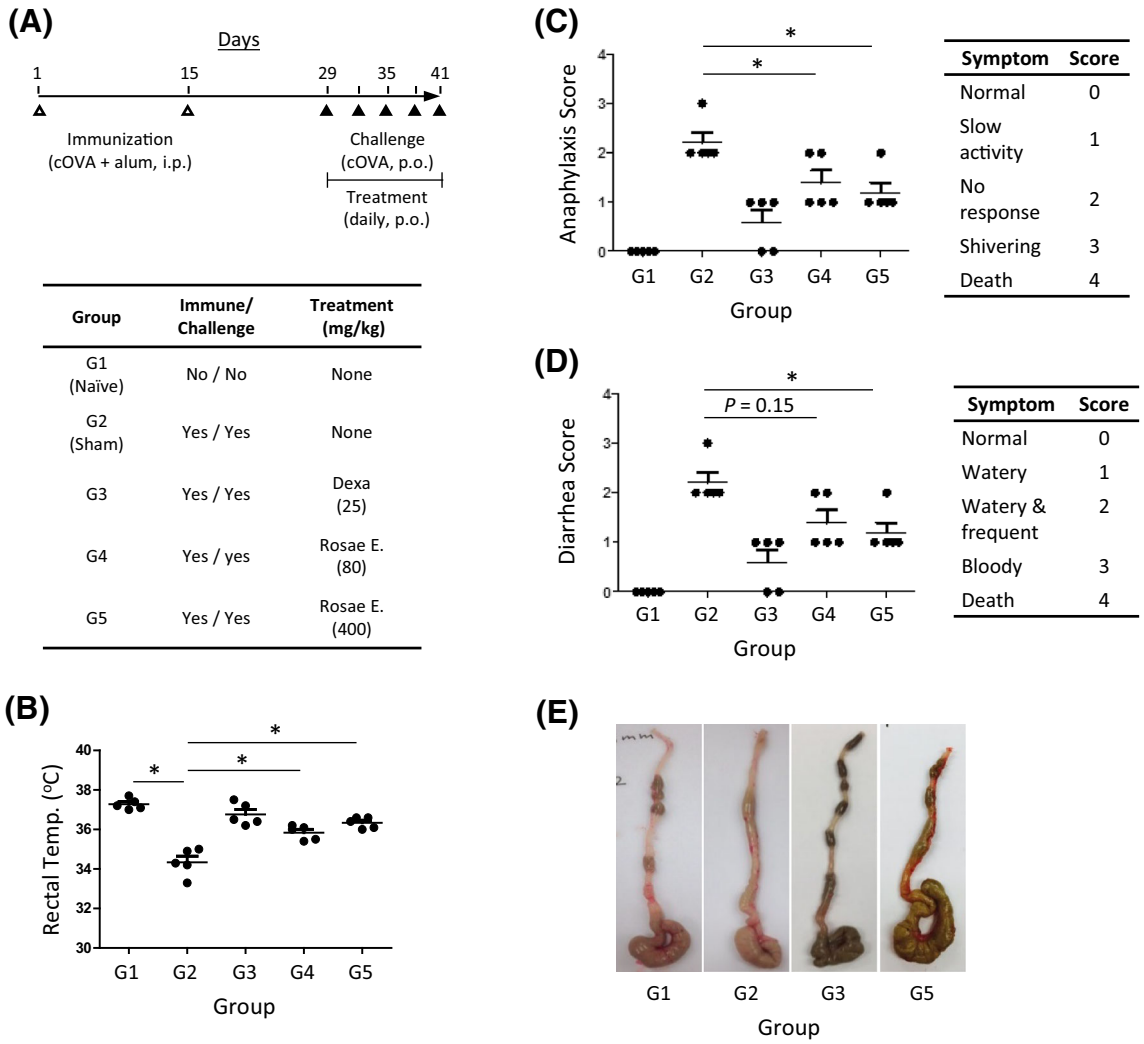

Fig. 1 Amelioration of food allergy symptoms by Rosae extract. The experimental scheme of the mouse model of cOVA-induced food allergy including the treatment schedule and the description of the experimental groups are shown (**A**). Thirty, sixty and ninety minutes after the fifth challenge, rectal temperature (**B**) and the severities of anaphylaxis (**C**) and diarrhea (**D**) were examined and scored according to the tables shown in the figure. Results obtained 30 min (**B**) and 60 min (**C** and **D**), respectively, after the challenge are shown. Stools in the colons taken from the respective group of mice 15 h after the fifth challenge are shown (**E**). Asterisk (*) denotes $p < 0.05$ by Student's t test

Recruitment of eosinophils to the small intestine is regarded as a critical event for the development of allergic inflammation [38]. Eosinophils recruited to the afflicted tissue secrete various toxic molecules to exacerbate the allergic inflammation [37]. As expected, a strong infiltration of eosinophils was observed in the small intestine of sham mice (Fig. 3A and B); the average number of eosinophils found in the tissue samples of sham mice (23.3 cells/HPF) was almost sixfold higher than that of naïve mice (4 cells/HPF). When the mice were treated with Rosae extract, however, the extent of eosinophil infiltration was significantly reduced; the average number of eosinophils found in the tissue samples of Rosae extract-treated mice was only about 2.5-fold higher than that of naïve mice. Similar results were obtained after Dexa treatment.

Control effect of Rosae extract on the levels of cOVA-specific antibodies and MCPT-1 in the serum

The presence of allergen-specific immunoglobulins (Igs) in the blood, particularly allergen-specific IgG1 and IgE, is one of the key factors for the occurrence of food allergy. Expectedly, a high titer of cOVA-specific IgG was detected in the sera of sham mice, while it was hardly detected in the sera of naïve mice (Fig. 4A). The average level of cOVA-specific IgG in the sera of Rosae-treated mice was lower than that of sham mice ($p < 0.05$), but the difference was only marginal (less than 20%). Concentrations of cOVA-specific IgG1 in the sera were also examined; IgG1 is a major subclass of IgG in the serum whose production is

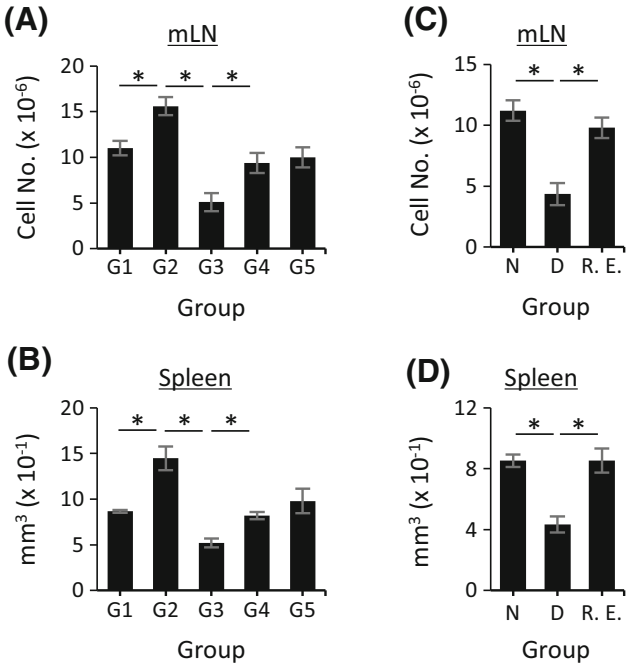

Fig. 2 The lack of immunotoxicity of Rosae extract; its effects on the cellularities of the secondary lymphoid organs. The mice were killed 15 h after the fifth challenge, and the mesenteric LNs (mLNs) and the spleens were taken from the respective group of mice. The numbers of cells in the mLNs (**A**) and the spleens (**B**) were examined. Normal BALB/C mice (neither immunized nor challenged) were left untreated or treated p.o. with either Dexamethasone (2.5 mg/kg) or Rosae extract (400 mg/kg) as indicated for 15 days, and the numbers of cells in the mLNs (**C**) and the spleens (**D**) from the respective group of mice were examined. Group description is shown as shown in Fig. 1A. Asterisk (*) denotes $p < 0.05$ by Student's t test. The data represent mean ± SD of five mice per group

known to be driven by Th2 cytokines (i.e., IL-4) (Fig. 4B). The levels of cOVA-specific IgG1 in the sera of sham mice, Dexa-treated and Rosae-treated mice were almost the same.

A high level of cOVA-specific IgG2a, another subclass of IgG whose production is known to be driven by Th1 cytokine (i.e., IFN-γ), was also detected in the sera of sham mice (Fig. 4C). The levels of cOVA-specific IgG2a in the sera of Rosae extract-treated mice appeared lower than those in the sera of sham mice, even though the difference turned out statistically not significant. The presence of cOVA-specific IgE was also clearly detected in the sera of sham mice (Fig. 4D). And, as in the case of IgG1, treatments with either Rosae extract or Dexa appeared to have little effect on the levels of cOVA-specific IgE in the sera.

Mast cell protease-1 (MCPT-1) is released from mast cells when a specific allergen binds to the antigen-specific IgE to form complexes with Fcε receptors (FcεRs) on the surface [39]. MCPT-1 released from mast cells in the afflicted small intestine enters the bloodstream and stays in the blood for a period of time. As shown in Fig. 4E, significant levels of MCPT-1 were detected in the sera of sham mice, while no MCPT-1 was detected in the sera of naïve mice. Different from cOVA-specific IgGs and IgE, MCPT-1 levels in the sera of Rosae extract-treated mice were markedly lower than those in the sera of sham mice. MCPT-1 levels in the sera of Dexa-treated mice were also lower than those of sham mice.

Suppression by Rosae extract of the formation of Th2 cytokine-producing cOVA-specific $CD4^+$ T cells

Th2 cytokines (e.g., IL-4, IL-5, IL-10) produced by $CD4^+$ T cells are key mediators of allergic immune responses. They play central roles not only in antibody class switching toward IgE but also in the development and activation of mast cells and eosinophils. In an effort to understand a mechanism underlying the anti-allergic effects of Rosae extract, cells prepared from the mLNs were cultured with cOVA, and the amounts of Th2 (IL-4, IL-5, IL-10) and Th1 (IFN-γ) cytokines produced by T cells during the culture were examined (Fig. 5). Expectedly, high levels of IL-4 were detected in the culture supernatants obtained from the cells from sham mice, whereas it was hardly detected in the culture supernatants obtained from the cells from naïve mice (Fig. 5A). Levels of IL-4 in the culture supernatants obtained from the cells from Rosae extract-treated mice were considerably lower (more than fourfold) than those in the supernatants obtained from the cells from sham mice. The level of IL-4 production by the mLN cells from Dexa-treated mice was barely detectable. Similar results were obtained for other Th2 cytokines examined, i.e., IL-5 and IL-10 (Fig. 5B and C).

Different from the Th2 cytokines mentioned above, significant levels of IFN-γ were detected even in the culture supernatants obtained from the cells from naive mice (Fig. 5D). Overall concentrations of IFN-γ in the supernatants obtained from the cells from sham mice were higher than those in the supernatants obtained from the cells from naive mice, but the difference was less than twofold and turned out statistically not significant ($p > 0.05$). Meanwhile, concentrations of IFN-γ in the culture supernatants obtained from the cells from Rosae extract-treated mice were comparable to those in the supernatants obtained from the cells from naïve mice.

Inhibition by Rosae extract of the antigen-specific $CD4^+$ T cell proliferation in vivo

In order to better understand the effect of Rosae extract on antigen-specific $CD4^+$ T cell immune responses, we used DO11.10 TCR Tg T cells. The Tg T cells adoptively transferred into syngenic mice (BALB/C) were stimulated

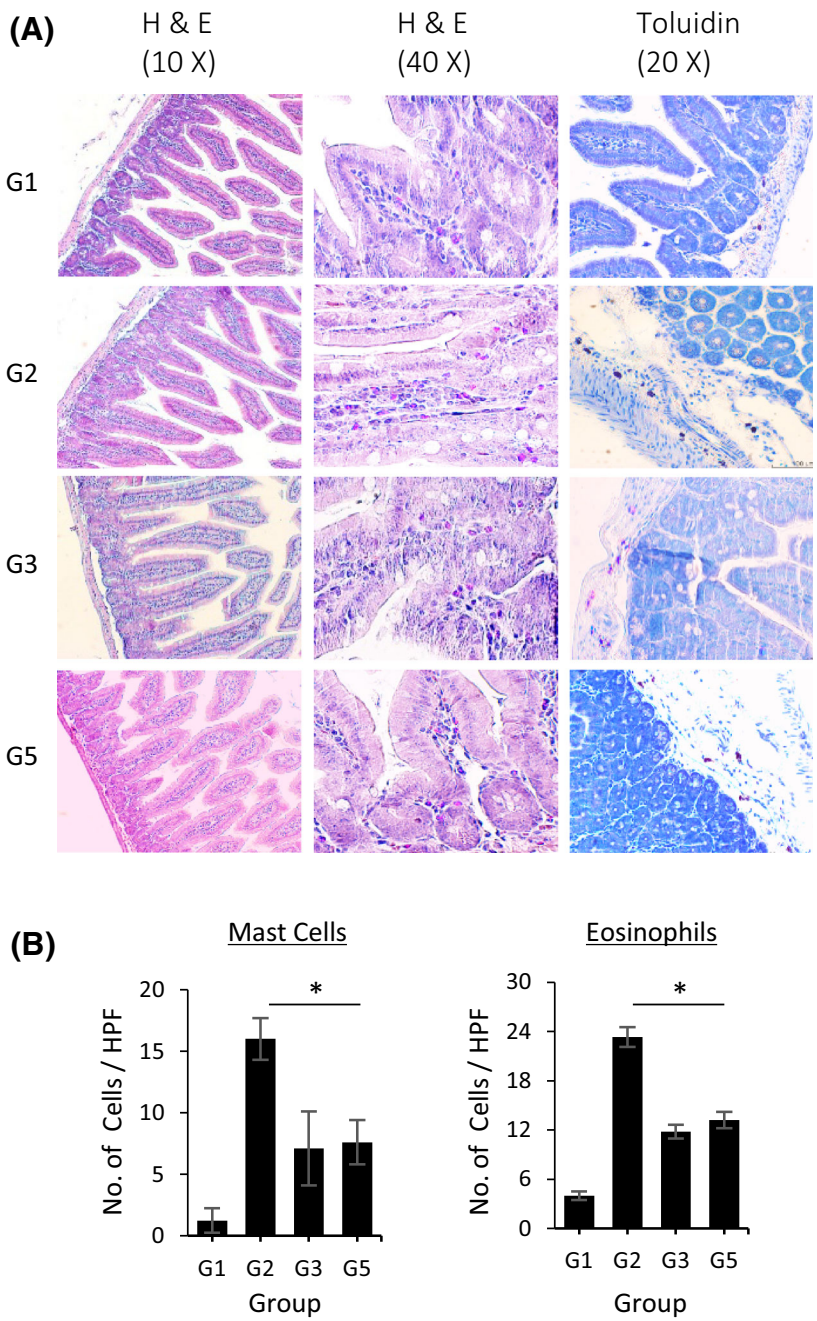

Fig. 3 Amelioration of the intestinal inflammatory lesion and obstruction of the infiltration of mast cells and eosinophils by Rosae extract. The small intestines (jejunum) were taken from the respective group of mice one day after the fifth cOVA challenge and fixed with formalin and sectioned with microtome. The thin tissue sections were stained with hematoxylin plus eosin (H&E) and observed under the microscope for inflammatory lesions (**A**, *left*) and for eosinophils (stained *dark red*) (**A**, *center*). They were also stained with toluidine solution and observed for mast cells (stained *deep blue*) (**A** *right*). The numbers of mast cells (**B**, *left*) and eosinophils (**B**, *right*) in high power fields were counted, and the average numbers were plotted. Cell numbers in at least five different HPFs were counted, and the means and SDs were calculated. Group description is shown as shown in Fig. 1A. Asterisk (*) denotes $p < 0.05$ by Student's *t* test

with cOVA plus alum. And, the levels of DO11.10 Tg T cell proliferation in the presence or absence of Rosae extract administered into the mice were compared with the extents of CFSE dilution (Fig. 6A). Expectedly, when the mice were immunized with cOVA plus alum, a robust proliferation of DO11.10 TCR Tg T cells determined by a marked increase in the population of $CFSE^{low}$ and $Kj1\text{-}26^{+}$ cells was observed (Fig. 6B). Of note, however, when the mice immunized with cOVA plus alum were also treated with Rosae extract at the same time, it was found that the population of $CFSE^{low}.Kj1\text{-}26^{+}$ cells decreased considerably, while the population of $CFSE^{high}.Kji\text{-}26^{+}$ cells remained relatively high, indicating that Rosae extract suppressed the proliferation of T cells induced by cOVA. Meanwhile, Dexa treatment resulted in a dramatic reduction in the population of both $CFSE^{low}.Kj1\text{-}26^{+}$ and $CFSE^{high}.Kji\text{-}26^{+}$ cells.

Levels of cytokine production by DO11.10 TCR Tg T cells in the LNs of the mice were also examined (Fig. 6C). When the cells from the host mice (BLAB/C mice) were cultured with the peptide specifically recognized by DO11.10 TCR Tg T cells, production of Th2 cytokines (i.e., IL-4 and IL-5) was hardly detected. Likewise, when the cells from the LNs of the mice which had adoptively

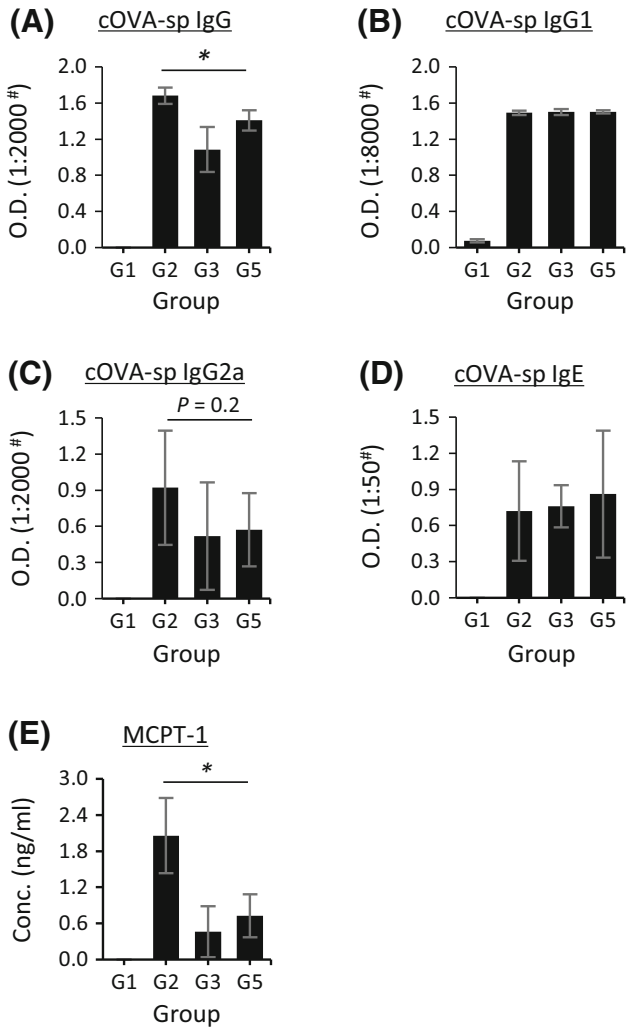

Fig. 4 Control effects of Rosae extract on the levels of cOVA-specific antibodies and MCPT-1 in the serum. The serums were prepared from the blood drawn from the respective group of mice 15 h after the fifth challenge. Levels of cOVA-specific total IgG (**A**), IgG1 (**B**), IgG2a (**C**), IgE (**D**) and mouse mast cell protease-1 (MCPT-1) (**E**) in the serums were measured with ELISA. Group description is shown as shown in Fig. 1A. The data represent mean ± SD. Asterisk (*) denotes $p < 0.05$ by Student's t test

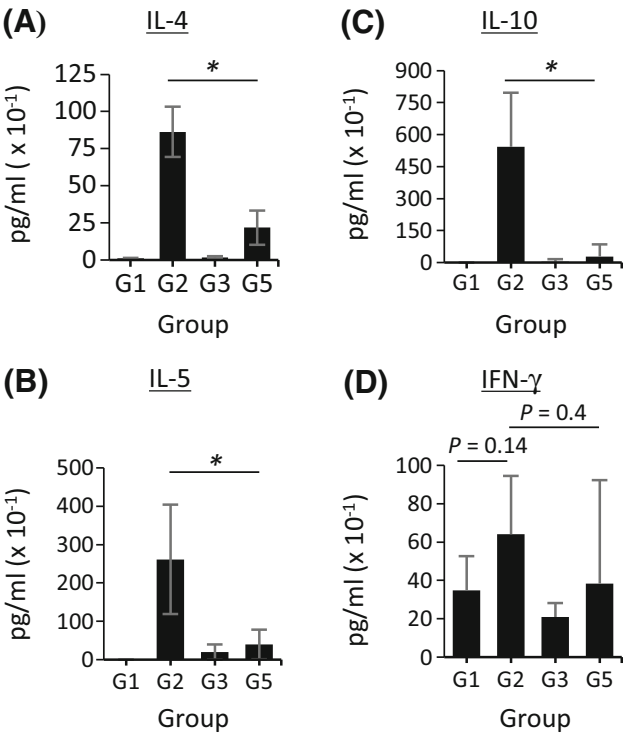

Fig. 5 Suppressive effects of Rosae extract on the differentiation of cOVA-specific T cells. The cells prepared from the mLNs of the respective group of mice (five mice per group) 15 h after the fifth challenge were cultured for 3 days with cOVA. The concentrations of IL-4 (**A**), IL-5 (**B**), IL-10 (**C**) and IFN-γ (**D**) in the culture supernatants were measured with ELISA and plotted. Group description is shown as shown in Fig. 1A. The data represent mean ± SD. Asterisk (*) denotes $p < 0.05$ by Student's t test

transferred DO11.10 TCR Tg T cells but had not been immunized with cOVA plus alum were cultured with the peptide, the production of those cytokines was hardly detected. In contrast, when the cells from the mice immunized with the antigen after adoptive transfer of DO11.10 TCR Tg T cells were cultured with the peptide, high levels of IL-4 and IL-5 were detected in the culture supernatants. In parallel with the decrease in the population of $CFSE^{low}.K1\text{-}26^{+}$ cells after Rosae extract treatment, the production of Th2 cytokines by the cells from the mice treated with Rosae extract was significantly lower than the production by the cells from the mice which were immunized without Rosae extract treatment. Expectedly, the level of the cytokine production by the cells from Dexa-treated mice was also reduced; the extent of reduction observed after Dexa treatment was even more profound than that observed after Rosae treatment.

Suppression by Rosae extract of the mast cell activity in vivo

The result that the level of MCPT-1 in the serum decreased after Rosae extract treatment (Fig. 4E) prompted us to examine the effect of Rosae extract on the function of mast cells. For the experiment, mice were injected with IgE specific for a happen, dinitrophenol (DNP) in this case, followed by administration of a protein antigen conjugated with the same hapten (DNP-BSA) to induce systemic activation of mast cells to cause degranulation and anaphylaxis (Fig. 7A).

Expectedly, when DNP-BSA was administered to the mice injected with anti-DNP-IgE before, a conspicuous decrease in the body temperature and a high level of MCPT-1 in the serum were detected within 60 min of DNP-BSA injection (Fig. 7B). Of note, treatment of the mice with Rosae extract

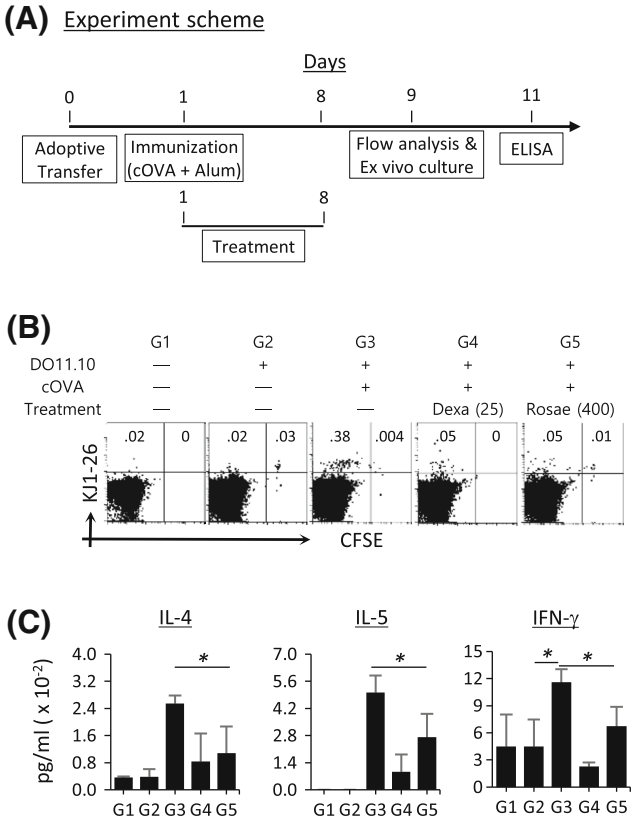

Fig. 6 Inhibition of activation/proliferation of DO11.10 T cells induced by cOVA in vivo. DO11.10 TCR Tg T cells labeled with CFSE were adoptively transferred and systemically stimulated with cOVA plus alum with or without treatment with either Rosae extract or Dexa as depicted in (**A**). The cells prepared from the whole-body LNs were stained with anti-CD4 plus KJ1-26 mAbs and analyzed with flow cytometry. Dot plots show the population of KJ1-26$^+$ (DO11.10) T cells among the gated total CD4$^+$ T cells and the extent of CFSE dilution in those cells (**B**). The cells from the LNs were cultured ex vivo with the DO11.10-specific peptide for 3 days, and the levels of IL-4, IL-5 and IFN-γ were measured with ELISA (**C**). The experiments were carried out with three mice per group

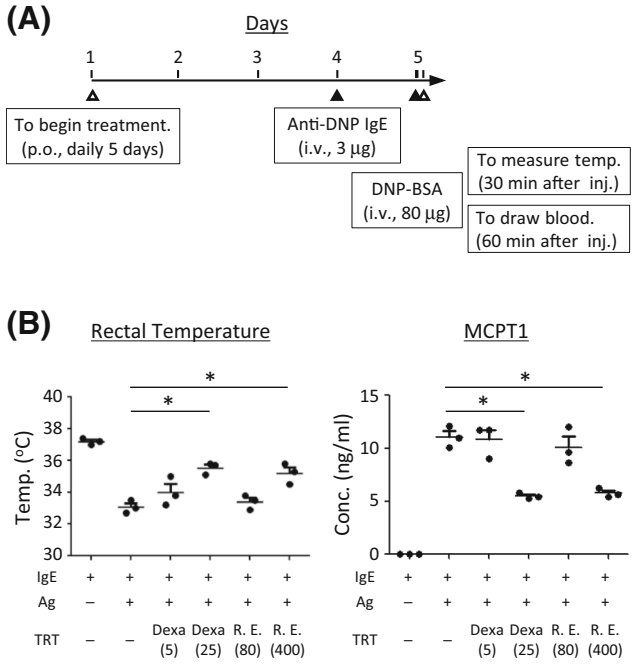

Fig. 7 Inhibition by Rosae extract of the function of mast cells in vivo. DNP-BSA was administered into BALB/C mice injected with anti-DNP mouse IgE beforehand to cause passive systemic anaphylaxis. The mice were treated with either Rosae extract or Dexa in a couple of different doses as described in (**A**). The changes in the body temperature caused by the allergic reaction were examined 30 min after the administration of DNP-BSA (**B**). Then, the blood was drawn from the mice to prepare the serums. The levels of MCPT-1 in the serums were measured with ELISA (**C**). The experiments were carried out with three mice per group

significantly reduced the extents of those changes in a dose-dependent manner. Thus, when the mice were treated with the extract at 400 mg/kg, the extent of the body temperature change and the level of MCPT-1 in the serum were reduced to less than half of those observed in the mice to which DNP-BSA was administered without any treatment. Dexa treatment also showed similar results; treatment of the mice with 25 mg/kg of Dexa provided a comparable result to the treatment with 400 mg/kg of Rosae extract.

Discussion

Hot water extracts of special herbs or THFs have been mainstream treatment regimens for a variety of pathologic symptoms and syndromes in oriental medicine. The use of such extracts is no longer confined to Asian countries, becoming increasingly popular in Western countries as well. Reflecting such a trend, National Institute of Health in USA has been promoting studies on herbal extract medications as a part of alternative medicines. Nevertheless, the fact that exact molecular and cellular mechanisms underlying the therapeutic effects of many herbal extracts and herbal formula are largely illusive is a one of the issues to be settled for broader use of herbal extract medications [18, 40].

The *Rosae multiflorae fructus*, dried fruit of *Rosa multiflora* Thunberg, has long been used as an ingredient of THFs for various pathologic symptoms [17]. Its immunoregulatory effects have been also reported in recent studies on its anti-arthritic effect [25]; Yan [41]. Our study also verifies such an immunoregulatory effect of Rosae extract. As various types of immune cells are actively involved in food allergy [6, 37], the results that oral administration of Rosae extract during the challenge with cOVA clearly diminished the severities of allergic symptoms (i.e., decrease in body temperature, diarrhea, anaphylaxis) (Fig. 1) provide a clear evidence for the immune regulation by Rosae extract. In addition, since the enlargement of the mLN and the spleen observed in sham

mice is thought to occur as a result of active host immune responses against orally administered cOVA, the finding that the cellularity and size of the mLN and the spleen of Rosae-treated animals were maintained at a steady-state condition during the challenge also supports the immunoregulatory activity of Rosae extract (Fig. 2). The immunoregulatory effect of Rosae extract is further delineated by the histology data that Rosae extract treatment resulted in amelioration of the intestinal inflammatory lesion induced by orally administered cOVA and reduction in the numbers of mast cells and eosinophils residing in the small intestine (Fig. 3).

It has to be noted here that the anti-allergic effect of Rosae extract had been explored in a mouse model of asthma [42]. According to that study, treatment of the mice with the hot water extract of Rosae multiflora fructus ameliorates the inflammatory lesion in the lung induced by nasally administered cOVA-containing aerosol into the mice sensitized with cOVA beforehand. The same study also reported that the treatment resulted in a considerable reduction in the numbers of mast cells and eosinophils recruited to the lung lesion. Despite such a report, the mechanisms underlying the anti-allergic effects of Rosae extract have remained largely obscure. In light of the mechanisms underlying the anti-allergic effects of Rosae extract, we may speculate several possibilities. Firstly, it is possible that Rosae extract may suppress effector functions of mast cells and/or basophils such as release of the intracellular granules and production of the pro-allergic cytokines (e.g., IL-9 and IL-13), both of which are critical for triggering a cascade of immune reactions to invoke allergic symptoms and inflammation [6, 37]. Secondly, it is also possible that Rosae extract may have effect on T cell activation and development of effector functions induced by a specific antigen. As a result, the expression of pro-allergic cytokines (e.g., IL-4 and IL-5 and IL-10) by allergen-specific $CD4^+$ T cells is compromised and the recruitment of mast cells and eosinophils to the small intestine is also subdued. Lastly, it may have effect on eosinophil function to prevent progression of the inflammatory lesion [43]. These possibilities are not mutually exclusive, and therefore, it is also possible that Rosae extract has effect on all those cell types directly or indirectly to a certain degree to reduce the levels of allergic symptoms and inflammatory lesion.

The results from the passive anaphylaxis experiment indicate that Rosae extract could have a direct effect on mast cells to inhibit release of the granules containing MCPT-1 and to modulate the allergic responses for the systemic anaphylaxis (Fig. 7). Given that, it seems likely that the amelioration of the allergic symptoms (Fig. 1) and the reduction in the level of MCPT-1 in the serum (Fig. 4E) observed in the allergic mice treated with Rosae extract result at least in part from the direct suppression of mast cell functions by the extract.

In addition, it seems also true that the anti-allergic effect of Rosae extract is at least in part attributed to the activity to modulate T cell immune functions [42]. The results that LN cells from Rosae extract-treated mice produced considerably lower levels of IL-4, IL-5 and IL-10 than LN cells from sham mice (Fig. 5) indicate that Rosae extract may directly or indirectly have effect on cOVA-specific $CD4^+$ T cell activation and/or development of their effector functions during cOVA challenge (Fig. 5). In line, the results from the experiment using DO11.10 TCR Tg T cells (Fig. 6B) that Rosae extract administered orally can have effect on T cell activation and proliferation by a specific antigen to suppress clonal expansion and the generation of effector and/or memory T cells also support the idea. Thus, it seems likely that the reduction in the levels of cytokine production by the cells from the mLNs of Rosae extract-treated mice (Figs. 5 and 6C) resulted from the decrease in the number of the antigen-specific T cells by the treatment. We speculate that the attenuation of T cell activation/proliferation by Rosae extract is closely related to the lower numbers of mast cells and eosinophils populated in the small intestines of the Rosae extract-treated mice (Fig. 3).

Levels of cOVA-specific IgG and IgE in the sera of different groups of mice were also examined to know whether the effect of Rosae extract on T cells brought about changes in Ab production and Ab class switching, but the results indicate that Rosae extract treatment had little effect on those processes. Together, it seems unlikely that the anti-allergic effects of Rosae extract are attributed to the reduction in the levels of cOVA-specific IgE and IgG1. These results also imply that while Rosae extract treatment can be an effective prevention and treatment option for food allergy, it may not be a cure for the disease. In other words, it is possible that the allergen-specific IgE persistent in the body may cause food allergy symptoms when the patient re-encounters a food allergen after termination of Rosae extract treatment. Yet, if the extract exerts a long-term effect on allergen-specific T cells, it is still possible that the severity of food allergy invoked after termination of Rosae extract treatment can be reduced. Together, further studies are warranted for better understanding of molecular and cellular mechanisms for the anti-allergic effects of Rosae extract and for improving its therapeutic efficacies.

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