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The mast cell stabilizing activity of Chaga mushroom critical for its therapeutic effect on food allergy is derived from inotodiol

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ABSTRACT

While an anti-allergic effect of Chaga mushroom (*Inonotus obliquus*) has been indicated, its therapeutic effect on allergy and immunoregulatory mechanisms and chemical constituents directly responsible for that are hardly known. We examined the effect of 70% ethanol extract of Chaga mushroom (EE) and its dichloromethane (DF) and aqueous (AF) fractions using a mouse model of chicken ovalbumin (cOVA)-induced food allergy, and found that only EE and DF ameliorated allergy symptoms to a significant extent. The in vivo mast cell-stabilizing activity was also found only in EE and DF whereas the activities to suppress Th2 and Th17 immune responses and cOVA-specific IgE production in the small intestine were observed in all three treatment regimens, implying that inhibition of the mast cell function by lipophilic compounds was vital for the therapeutic effect. Results also indicated that inotodiol, a triterpenoid predominantly present in DF, played an active role as a mast cell stabilizer.

1. Introduction

Food allergy is defined as a series of unfavorable immune reactions against an ingested food protein. While innocuous food proteins taken up through the intestinal mucosa normally induce immune tolerance called oral tolerance, such an immune tolerance mechanism does not operate properly in certain circumstances to cause food allergy [1,2]. Symptoms of food allergy vary from mild itching, skin rash, breathing problem, vomiting and diarrhea to life-threatening anaphylaxis which claims thousands of death each year worldwide [3]. The population to suffer from food allergy has been increasing in an alarming rate during the last decades particularly in developed countries [4].

Food allergy is an IgE-mediated (type-1) hypersensitivity reaction. When a food allergen absorbed through the digestive track interacts with IgE decorating the surface of mast cells via the interaction with FcεRI, mast cells undergo a rapid activation process to release granules containing a variety of effector molecules such as histamine, leukotriene and mast cell proteases [5–7]. In general, mast cell activation and degranulation lead to dilation of blood vessels to cause redness and edema and the recruitment of eosinophil to the gut to exacerbate the allergic inflammation [8]. Besides mast cell and eosinophil, $CD4^+$ T lymphocytes also play a role. Production of Th2 cytokines by $CD4^+$ T cells is critical for class switching of IgM or IgG to IgE in B lymphocytes [9–11]. In addition, Th2 cytokines produced by $CD4^+$ T cells activated in the draining lymph node (dLN) promote recruitment of mast cell and eosinophil to the inflicted tissue as well as their development [12].

Epinephrine, a 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-agonist [13,14]. Still, as dangerous anaphylactic response tends to arise acutely and intensely within a few minutes to an hour after contact with a food allergen, development of preventative therapeutics for food allergy is highly desirable. Special traditional herbal formulas (THFs), hot water extract of assorted plant species, are known to have reliable therapeutic effects on food allergy [15]. While THFs have been used for treatments of various diseases and medical conditions in oriental medicine for generations, their preparation is often cumbersome as a group of selected medicinal herbs have to be collected and decocted in right ratio. In addition, reports that the use of THFs may cause renal impairment and other health complications raise concerns over long-term uses [16–18]. Single herbal preparations, pure substances or mixtures from aqueous or organic solvent extracts of special medicinal

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herbs, are also used for improving various medical conditions [39,40]. Single herbal preparations, which are often used as food (dietary) supplements, are considered as more cost-effective and safer alternatives to THFs.

Chaga mushroom (*Inonotus obliquus*) is a parasitic fungus to grow on birch and other trees belonging to the family *Hymenochaetaceae*. This fungus naturally inhabits the forests of Russia, Korea and Northern Europe. Chaga mushroom is well-known for its therapeutic effects on various diseases including mental, immunological and metabolic disorders and cancer, and has been used as a folk medicine in East Asian countries and Russia for centuries [19,20]. An anti-allergic effect of Chaga mushroom has been also inferred in a study by others [21]. Yet, its therapeutic effect has never been investigated in an animal allergy model. Studies on chemical components and their immunoregulatory activities directly responsible for the anti-allergic effect of the mushroom have not been conducted, either. In this study, we examined the therapeutic effect of 70% ethanol (EtOH) extract of Chaga mushroom and its dichloromethane (CH_2Cl_2) and aqueous fractions on food allergy using a mouse model of chicken ovalbumin (cOVA)-induced food allergy. We also analyzed immunoregulatory activities of the extract and the fractions to better understand the mechanism(s) directly linked to the therapeutic effect. Further, a role of inotodiol, a major tetracyclic triterpenoid in the organic fraction of the mushroom extract, with respect to the anti-allergic effect of the mushroom was investigated and characterized.

2. Materials and methods

2.1. Chemicals and other reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin-glutamine solution, and dinitrophenol (DNP)-bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (Carlsbad, USA). cOVA (grade VI), alum [$KAl(SO_4)_2$-$12H_2O$], eosin, hematoxylin and BSA were purchased from Sigma-Aldrich (St. Louis, USA). Toluidine blue was purchased from Samchun Chemical (Pyungtack, Korea). IL-4, IL-17 and IFN-γ ELISA kits were purchased from Biolegend (San Diego, USA). IL-5 and IL-10 ELISA kits were purchased from BD Biosciences (San Diego, USA). Biotinylated anti-mouse total IgG and IgG2a were purchased from Jackson ImmunoResearch (West Grove, 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 mast cell protease-1 (MCPT-1), and IL-13 ELISA kits were purchased from eBioscience (San Diego, USA). Anti-DNP IgE was purchased from Sigma-Aldrich (St. Louis, USA). IFN-γ intracellular staining kit was purchased from Biolegend (San Diego, USA).

2.2. Animals

Five week-old female BALB/C mice were purchased from SAMTAKO Co. (Osan, Korea) and housed in Core Animal Facility 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.

2.3. Preparation of the 70% EtOH Chaga extract and the fractions and purification of inotodiol

Chaga mushroom (*Inonotus obliquus*) used in this study was purchased from a herbal market in Kumsan, Korea, and identified by Professor Y. H. Kim, College of Pharmacy, Chungnam National University (Daejeon, Korea). A voucher specimen (CNU 15001) was deposited at the herbarium of the College of Pharmacy, Chungnam National University.

The Chaga mushroom (630.0 g) was ground into fine particles and extracted with 70% aqueous EtOH (3 × 3.0 L) under ultrasonic agitation at 90 Hz (40 °C). The ethanol solution was evaporated in vacuo to produce a dried brown extract (58.0 g). The EtOH extract was suspended in distilled water and successively partitioned with CH_2Cl_2 and EtOAc to prepare CH_2Cl_2 (6.0 g), EtOAc (3.2 g), and water fractions (48.5 g).

The CH_2Cl_2 fraction was separated via silica gel column chromatography and eluted repeatedly with *n*-hexane-EtOAc (20:1, 9:1, 4:1, 2:1 and 1:2) to yield thirteen smaller subfractions (C-1 to C-13). Subfractions C-6 and C-7 were combined and were subjected to a silica gel column with a solvent mixture of *n*-hexane-EtOAc (20:1) to afford inotodiol (210.0 mg) (Supp. Fig. 1).

For animal experiments, the dried powders obtained from the EtOH extract and the fractions were pulverized and mixed with the Tween 20-saline solution to the final concentration 10 mg/mL or 40 mg/mL [22].

2.4. High performance liquid chromatography (HPLC) analysis

The solid EtOH extract and the fractions were dissolved in methanol (MeOH) at concentrations of 0.2% (w/v). The standards were also dissolved in MeOH at concentrations of 0.2 mg/mL. All solutions were filtered through a 0.2 μm syringe filter before analysis. HPLC analysis was performed using a LC-10AD series HPLC system (Shimadzu, Kyoto, Japan) coupled with UV/VIS detector at ambient temperature. A HECTOR-M C_{18} column (250 × 4.6 mm, 5 μm, RStech, Korea) was used stationary phase for separation. The mobile phase consisted of water (A) and 100% acetonitrile (B) was operated under gradient program following conditions; 0 min at 60% B, 30 min at 95% B and 60 min at 95% B. The flow rate of 1 mL/min and the detection wavelength of 210 nm were used.

Inotodiol and 3β-hydroxylanosta-8,24-dien-21-al purified from the CH_2Cl_2 fraction as described above were used as standards.

2.5. Protocol for the mouse model of cOVA-induced food allergy

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 7 groups (6 mice per group) (Fig. 1A). Two weeks after the second immunization, the mice were challenged p.o. with cOVA (50 mg) five times once every three days. During the challenge period, mice were either left untreated or treated daily with EE, DF, AF or Dexamethasone (p.o.). One hour after the fifth challenge, mice were examined for allergy symptoms (change in rectal temperature, diarrhea, and anaphylaxis) and the severities of allergy symptoms were rated and scored as previously reported [23]. The rectal temperature was measured using a rectal probe thermometer (Physitemp, Clifton, USA).

2.6. Histology

Histology experiments were performed as described by others [24]. 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 eosinophil in high power field (HPF) were enumerated as described [25,26].

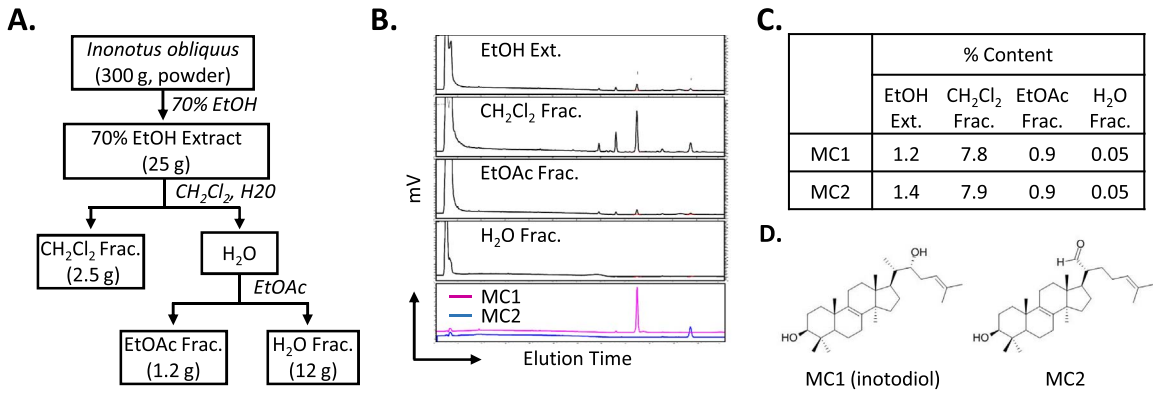


Fig. 1. Preparation of 70% EtOH extract of Chaga mushroom and its CH_2Cl_2 and H_2O fractions.
Experimental scheme for the preparation of 70% EtOH extract of Chaga mushroom and its CH_2Cl_2 and aqueous fractions is shown along with the amounts of the dry Chaga mushroom powder initially used in the EtOH extraction and the dry substances obtained after EtOH extraction and the fractionation (A). HPLC profiles of the ethanol extract and the CH_2Cl_2, EtOAc and H_2O fractions are shown along with the profiles of purified inotodiol (MC1) and 3β-hydroxylanosta-8,24-dien-21-al (MC2) used as the standards (B). The contents (% w/w) of inotodiol and 3β-hydroxylanosta-8,24-dien-21-al in the dry substances of the EtOH extract and the fractions are shown (C). The chemical structures of inotodiol and 3β-hydroxylanosta-8,24-dien-21-al are shown (D).

2.7. 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 with some modification [27]. 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 50–8000 folds, were then added to the plates and incubated for 2 h. After thorough wash, biotinylated anti-mouse IgE (1 μg/mL), IgG2a (1:100.000 dilution), IgG (1:100.000 dilution) or IgG1 (1 μg/mL) were 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.

2.8. Preparation of the tissue (small intestine) extract

The small intestine was cut into 7–10 cm pieces one day after the fifth challenge. The tissue fragment was washed with 1 × PBS on ice and snap-frozen using a dry-ice EtOH bath. The tissue sample was then transferred into a mortar containing liquid nitrogen (LN2) and ground with a pestle. The ground tissue sample was quickly transferred into a small centrifuge tube (1.5 mL) and mixed with 500 μL of the extraction buffer (10 mM of Tris-HCl pH 8.0, 150 mM of NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 1 mM of EDTA, 10% glycerol, 1 mM of PMSF, and a cocktail of freshly prepared protease inhibitors). After incubation (1.5 h at 4 °C), the tissue lysate was centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was then aliquoted and saved at − 70 °C.

2.9. 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 mesenteric lymph node (mLN) cells (1×10^6 cells) in of RPMI medium (10% FBS, 10 mM HEPES, 50 μM 2-mercaptoethanol, 1 × Penicillin-Streptomycin) were plated in a 96-well culture plate (200 μL) 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-4, IL-5, IL-10, IL-13, and IL-17 were quantified using ELISA kits in accordance with the manufacturer's manual.

2.10. 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 [28]. Thirty minutes after injection of DNP-BSA, the rectal temperature of the mice were 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 MCPT1. Mice were treated with EE, DF, AF or Dexamethasone daily for 5 days beginning 3 days prior to the injection of the mAb.

2.11. Intracellular cytokine staining

Cells were isolated from the mLNs of mice 1 day after the fifth challenge and stimulated with 200 μg/mL cOVA overnight at 37 °C. Then, the cells were restimulated with PMA (20 ng/mL) plus ionomycin (1 μg/mL) for 4 h at 37 °C in the presence of brefeldin-A (Biolegend, U.S.A). The cells were centrifuged at 1100 rpm for 10 min, washed with 200 μL of FACS buffer (1 × PBS, 1%BSA) and stained with mAb CD4-APC-Cy7 for 30 min on ice. Then, the cells were washed with FACS buffer and fixed and permeabilized with Fixation and Permeabilization Wash buffer purchased from Biolegends (CA, U.S.A), respectively, in accordance with manufacturer's manual. After permeabilization, the fixes cells were stained with anti-IFN-γ-FITC for 30 min on ice and analyzed with flow cytometry [29].

2.12. Statistical analysis

The significance of the difference between separate groups was calculated with nonparametric one-way ANOVA (Kruskal-Wallis test), and also confirmed with Bonferroni correction as post hoc analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of EE, DF and AF, and analysis for inotodiol and 3β-hydroxylanosta-8,24-dien-21-al as marker compounds

Molecules in the Chaga mushroom extract prepared by refluxing a dry mushroom power with 70% EtOH was fractionated based on the chemical property. The aqueous solution of the 70% EtOH extract was added to CH_2Cl_2, mixed and allowed to separate from the organic solvent. The CH_2Cl_2 layer was taken, and molecules in the fraction were

obtained in a solid form by evaporating the solvent. Lipophilic molecules still remaining in the aqueous layer were fractionated further with EtOAC. Then, molecules in those fractions were obtained in solid forms by evaporating water and EtOAc, respectively (Fig. 1A).

For checking the effectiveness of the fractionation process, the contents of inotodiol (MC1) and 3β-hydroxylanosta-8,24-dien-21-al (MC2) in each fractions were determined with HPLC; both inotodiol and 3β-hydroxylanosta-8,24-dien-21-al are tetracyclic triterpenoids unique in Chaga mushroom. Expectedly, both molecules were highly enriched in the CH_2Cl_2 fraction (Fig. 1B); the CH_2Cl_2 fraction contained almost 150 fold more inotodiol and 3β-Hydroxylanosta-8,24-dien-21-al than the aqueous fraction (Fig. 1C and D). The solid substances obtained from the 70% EtOH extract and the CH_2Cl_2 and the aqueous fractions, respectively, were reconstituted with Tween 20-saline solution to the final concentration of 1 mg/mL and used in the experiments described in the following sections. The reconstituted solutions were designated as EE for the 70% EtOH extract, DF for the CH_2Cl_2 fraction, and AF for the aqueous fraction.

3.2. Amelioration of food allergy symptoms only by EE and DF but not by AF

When the mice pre-sensitized (immunized) with cOVA plus alum were orally challenged with cOVA, severe allergy symptoms, i.e., diarrhea, anaphylaxis and decrease in the body temperature, were clearly shown after the 5th challenge (Fig. 2). When the same mice were treated with EE during the challenge with cOVA, a significant improvement in the allergy symptoms were noticed in a dose-dependent manner; thus, their body temperature remained almost normal, and only mild, if any, anaphylaxis and diarrhea symptoms were shown. Similar effects were observed when the mice were treated with DF. Different from the treatments with EE and DF, however, treatment with AF caused no significant improvement in the allergy symptoms (Fig. 2).

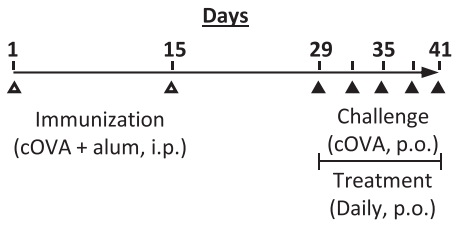
Expectedly, Dexamethasone (Dexa), a potent immunosuppressive agent, also ameliorated the symptoms (Fig. 2). Nevertheless, the Dexa treatment resulted in a significant weight loss and reduction in the size of the spleen and the cellularity of the lymph nodes (LNs), reflecting its well-known metabolic and immunologic side effects (Supp. Fig. 2). Of note, the treatment with either EE or DF had little effects on the body weight and the size and the cellularity of the spleen and the LNs, respectively.

3.3. Amelioration of the allergic inflammatory lesion in the small intestine by EE and DF

The oral challenge with cOVA caused a severe allergic inflammatory lesion in the small intestine. The pathological changes (mucosal edema), i.e., thickening of the intestinal wall particularly in the area of serosa and muscularis external, were easily observed in the small intestine of sham mice (Fig. 3A). The treatment with either EE or DF resulted in a marked improvement in the inflammatory lesion. Thus, the structure of the area of serosa and muscularis external of the treated mice appeared normal. The treatment of the mice with Dexa also ameliorated the lesion. Different from those treatments, however, no significant improvement of the lesion was observed after the treatment with AF.

Escalation in the numbers of mast cell and eosinophil in a tissue is a hallmark of allergic inflammation. Indeed, abnormally high numbers of mast cell and eosinophil (Fig. 3B and C and Supp. Fig. 3) were spotted in the small intestine of shame mice. The numbers of those cells were restored to the normal level when the mice were treated with either EE or DF. Similar results were also obtained after the Dexa treatment. Consistent with the results described above, AF treatment appeared to have no significant effect on the infiltration of mast cells and eosinophil to the tissue and/or their development; thus, the numbers of mast cell and eosinophil found in the small intestine of AF-treated mice were almost same as those of sham mice.

A.



B.

Diarrhea		Anaphylaxis	
Symptom	**Score**	**Symptom**	**Score**
Normal	0	Normal	0
Watery	1	Slow activity	1
Watery & Frequent	2	No response	2
Bloody	3	Shivering	3
Death	4	Death	4

C.

	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

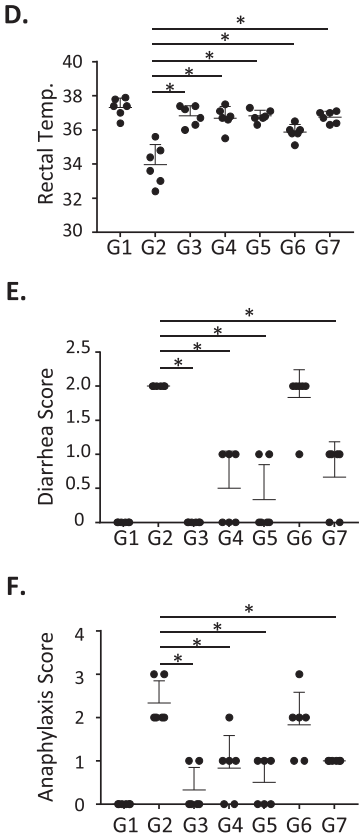


Fig. 2. EtOH ext. (EE) and CH_2Cl_2 frac. (DF) but not aqueous frac. (AF) can ameliorate food allergy symptoms.
The experimental scheme for the mouse model of food allergy used in this study is shown (A). Scoring systems used to rate the severities of diarrhea and anaphylaxis are shown in the table (B). The description of each experimental group is shown in the table; naïve group (G1), sham group (G2) and each treatment groups (G3–G7) (C). Dexa denotes dexamethasone. Thirty, sixty and ninety minutes after the 5th challenge, the rectal temperatures (D), severities of diarrhea (E) and anaphylaxis (F) were recorded according to the guidelines shown in the tables (B). The rectal temperatures measured 30 min after the last challenge, and the diarrhea and anaphylaxis scores recorded 60 min after the last challenge are plotted. Asterisk (*) denotes $p < 0.05$ by non-parametric one-way ANOVA (Kruskal-Wallis Test).

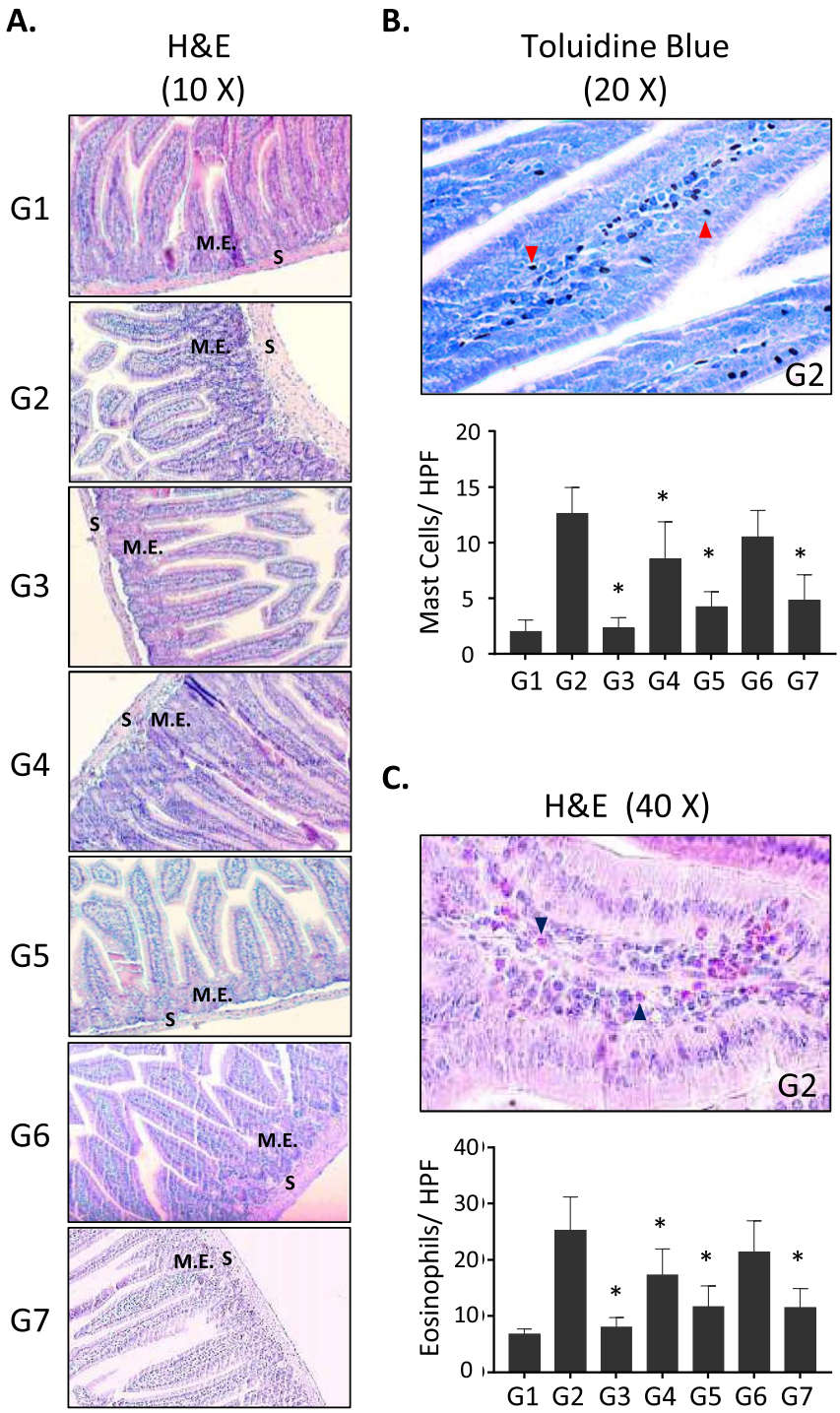
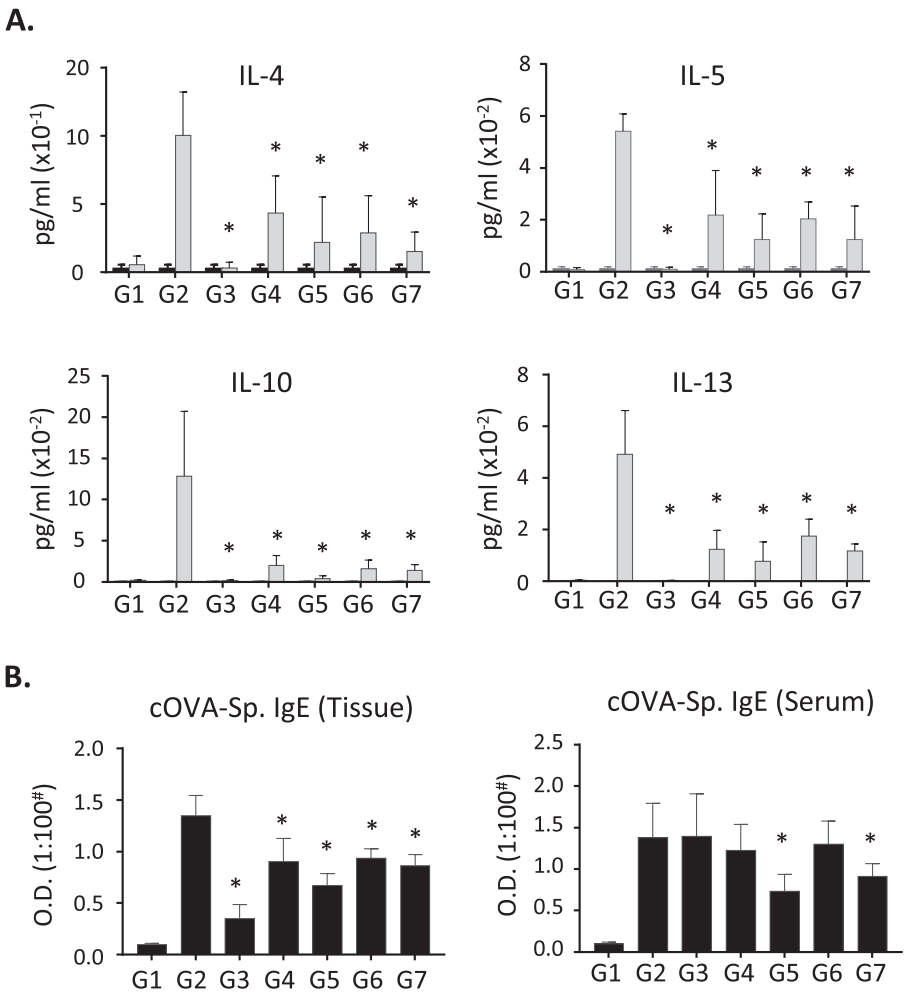


Fig. 3. EtOH ext. (EE) and CH_2Cl_2 frac. (DF) but not aqueous frac. (AF) can improve the allergic inflammatory lesion in the small intestine.

The small intestines (jejunum) were taken from the mice of each experimental group one day after the last challenge and fixed with formalin and sectioned with a microtome. The thin tissue sections were stained with hematoxylin plus eosin (H&E) and observed under the microscope for the inflammatory lesions (A). i.e., mucosal edema in the areas of serosa (S) and muscularis externa (M.E.), and eosinophil (stained deep red) (C). They were also stained with the toluidine solution and observed for mast cells (stained blue purple) (B). The representative pictures derived from the tissue sample of G2 are shown in (B, top) and (C, top), respectively. The numbers of mast cell (B, bottom) and eosinophil (C, bottom) per high power fields (HPFs) were counted and the average numbers were plotted. Cell numbers in at least six different HPFs (at least one HFP per mouse) were counted and the means and SDs were calculated. The definition of the groups (G1–G7) used in this figure are shown in Fig. 2C. Asterisk (*) denotes that *p* value by nonparametric one-way ANOVA (Kruskal-Wallis Test) between G2 and the indicated group is < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)



	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

Fig. 4. CD4$^+$ T cell immunity against cOVA and the level of cOVA-specific IgE in the small intestine are measurably affected not only by EtOH ext. (EE) and CH_2Cl_2 frac. (DF) but also by aqueous frac. (AF) treatment.
The cells prepared from the mLNs of the mice of each experimental group 15 h after the last challenge were cultured for 3 days in the presence (gray bar) or absence (black bar) of cOVA. The concentrations of a group of Th2 cytokines (A) in the culture supernatants were measured with ELISA. The definition of the groups (G1–G7) used in this figure are shown in Fig. 2C. The levels of cOVA-specific IgE in the extracts prepared from the small intestines (B, left) and the serums (B, right) of the mice of each experimental group were measured with ELISA, respectively. # denotes the dilution factor for the each samples used in ELISA. The levels of IgE in the tissue extracts and the serums are represented by O.D. obtained from ELISA. The data represent means ± SD Asterisk (*) denotes that p value by nonparametric one-way ANOVA (Kruskal-Wallis Test) between G2 and the indicated group is < 0.05.

3.4. Diminution of Th2 and Th17 CD4$^+$ effector T cell functions by EE, DF and AF treatments

Th2 cytokines produced by CD4$^+$ T cells play an important role in the recruitment of mast cell and eosinophil to the tissue as well as IgE production by B cells. In order to examine how EE, DF, and AF treatments had effect on CD4$^+$ T cell immune responses against cOVA during oral challenge with cOVA, the cells prepared from the mLN of the mice in each treatment group were cultured ex vivo with cOVA, and the levels of Th2 cytokines in the culture supernatants were compared. Expectedly, the cells from sham mice produced noticeably high levels of Th2 cytokines (i.e., IL-4, IL-5, IL-10, IL-13) (Fig. 4A). Meanwhile, the cells from EE- or DF-treated mice produced the same cytokines at markedly lower levels compared to those from sham mice, indicating that those treatments had attenuated the CD4$^+$ T cell response against cOVA. Of note, the cells prepared from AF-treated mice also produced the cytokines at a similarly reduced level, which was quite unexpected as the same treatment brought about no significant improvement in the allergy symptoms and the allergic inflammatory lesion in the gut. In order to know whether the levels of Th2 cytokines were also reduced in the small intestines of the mice treated with EE-, DF- and AF, respectively, we carried out ELISA assay with the tissue extracts. Indeed, it was found that the levels of IL-5, IL-10 and IL-13 in the treated mice were significantly lower compared to those in sham mice (Supp. Fig. 4). Note that the level of IL-4 in sham mice was too low to measure.

We also examined the IL-17 levels in the culture supernatants, and found that production of IL-17 was also reduced considerably when the mice were treated with each one of those regimens (Supp. Fig. 5A). In contrast to Th2 cytokines production, IFN-γ production was not significantly affected by any of those treatments except Dexa (Supp. Fig. 5B). Here, it is of note that a majority of IFN-γ-producing cells in the mLNs belonged to a CD4-negative population, indicating that cOVA-specific CD8$^+$ T cells made a major contribution to the production of IFN-γ during the culture (Supp. Fig. 5C). Reflecting the immunotoxicity of Dexa, the cells from the Dexa-treated mice produced a lower level of IFN-γ even than the cells from the naïve mice.

3.5. Decrease in the level of cOVA-specific IgE in the small intestine after EE, DF and AF treatments

IgE which plays a leading role in the allergic immune response is localized mainly in the mucosal tissues and acts there. Thus, we next examined levels of cOVA-specific IgE in the small intestines. Expectedly, a considerable level of cOVA-specific IgE was detected in the tissues of shame mice (Fig. 4B, left). Similar to the results obtained from the experiments for the production of Th2 cytokines by the mLN cells (Fig. 4A), the treatment of the mice with each one of EE, DF and AF resulted in a significant reduction in the levels of cOVA-specific IgE. Despite the fact, it has to be noted that the levels of IgE in the tissues of the treated mice, except the Dexa-treated mice, were still considerably higher than those of naïve mice.

Levels of cOVA-specific IgE in the blood were also examined. As in the small intestines, a measurable level of cOVA-specific IgE was detected in the serums of sham mice (Fig. 4B, right). Different from the results obtained from the tissue samples, however, the treatment only with the high dose of EE or DF caused reduction in its level to a significant extent; other treatments including Dexa treatment brought about almost no change.

Levels of cOVA-specific IgG in the blood were examined as well (Supp. Fig. 6). Effects of the treatments on the levels of cOVA-specific IgG seemed only marginal and also varied depending on the isotype of IgG. Thus, Dexa, the high dose of EE and DF treatments caused a slight but statistically significant reduction in the levels of cOVA-specific total IgG in the serum, while the same treatments had little effect on the level of cOVA-specific IgG1 whose production is known to be strongly influenced by Th2 cytokines.

3.6. Inhibition of mast cell function only by EE and DF

It is known that MCPT-1 which is released by mast cell following interaction with an allergen in the local tissue quickly appears in the blood and stays for a relatively long period of time [30]. Indeed, a noticeably high level of MCPT-1 was detected in the serums of sham mice prepared one day after the 5th oral challenge (Fig. 5A). And, as shown in the figure, all the treatments including AF resulted in a significant reduction in the levels of MCPT-1 in the blood. Nevertheless, the extents of the reduction were markedly different; that is, the level of MCPT-1 found in the serums of AF-treated mice was considerably higher than those found in the serums of EE- and DF-treated mice. Note that almost no MCPT-1 was detected in the blood of Dexa-treated mice.

In order to know whether EE and DF exerted a direct effect on the mast cell function, we carried out the passive anaphylaxis experiment, in which mast cells were systemically activated via sequential administration of anti-DNP IgE and DNP-conjugated BSA. Expectedly, when the mice were given i.v. anti-DNP IgE plus DNP-BSA without any pretreatment, they showed strong anaphylactic responses revealed by sharp decrease in the body temperature and the appearance of a high level of MCPT-1 in the blood (Fig. 5B). Meanwhile, treatment of the mice with either EE or DF resulted in a significant reduction in the extent of the body temperature change and the level of MCPT-1; Dexa treatment also showed a similar effect. Expectedly, however, AF treatment exerted no significant effect. Note that the dose of Dexa was reduced to 10 mg/kg to minimize the immune cells death by Dexa. Together, these results indicated that EE and DF directly acted on mast cells to compromise their immune function (i.e., release of granules).

3.7. Inhibition of the mast cell function by inotodiol

Inotodiol is a tetracyclic triterpenoid uniquely present in Chaga mushroom (*Inonotus obliquus*). Pharmacological activities of inotodiol have been investigated previously by others, but its immunoregulatory activities have not been explored [31,32]. It was present almost exclusively in DF after the fractionation of 70% EtOH extract of Chaga mushroom (Fig. 1). In order to know whether the anti-mast cell function of EE and DF was attributed to inotodiol at least in part, we used inotodiol in the passive anaphylaxis experiment. Strikingly, treatment of mice with inotodiol prior to injection of anti-DNP IgE and DNP-BSA resulted in a significant reduction in the extent of the body temperature change and the level of MCPT-1 in the blood (Fig. 5C). Of note, treatment of the mice with cholesterol, a structural analog of inotodiol (Supp. Fig. 7), had no effect. These results suggested that inotodiol have a specific anti-mast cell activity, and make contribution to the anti-allergic effect of EE and DF.

3.8. Prevention of recurrence of food allergy symptoms by EE

Next, we examined whether EE could prevent recurrence of the allergy symptoms in allergic mice. For addressing the question, the mice challenged with cOVA five times (sham mice) were treated with EE for 12 days before re-challenge with the allergen, and the severities of the allergy symptoms were compared with those of the mice treated only with the vehicle (Fig. 6A). Expectedly, when shame mice were fed cOVA two weeks after the last (5th) challenge, all of them (G2') showed severe allergy symptoms as depicted in Fig. 6B. When the same mice were treated with EE before feeding cOVA (G3'), it was noticed that the allergy symptoms were markedly ameliorated (Fig. 6B). We also found that the level of MCPT-1 in the serums of EE-treated mice were significantly lower than those of the vehicle-treated mice (Fig. 6C).

4. Discussion

Many studies using Chaga mushroom have focused on its anti-cancer activity [33–36]. Several studies also have shown that specific β-glucans in the mushroom are responsible for the anti-cancer activity. Our results that only EE and DF held the therapeutic effect on food allergy, however, elucidated that the anti-allergic effect of Chaga mushroom were mainly attributed to lipophilic molecules partitioned into the organic layer (Figs. 2 and 3). Taken together, it seems evident that differential health effects of Chaga mushroom are exerted by distinct groups of substances in the mushroom. Given that the use of Dexa is limited due to severe side effects [37], the results that EE and DF treatments brought about a strong improvement in the allergy symptoms without noticeable side effects (Supp. Fig. 2) are highly encouraging of using them as an therapeutics or a food supplement for food allergy.

As it is well-known that Th2 cytokines play an important role in isotype class switching of IgM (or IgG) to IgE and also in the recruitment and/or development of mast cell and eosinophil, it was somewhat predicted that EE and DF treatments had a negative effect on cOVA-specific $CD4^+$ T cell immune response (Fig. 4A and Supp. Fig. 4 and 5A). Still, the results that AF also had the similar activity are quite puzzling as AF treatment had little effect on the allergy symptoms. It is not clear at this point whether a group of related molecules present both in DF and AF were responsible, or whether distinct molecules present separately in DF and AF exerted the same effect. No matter what is the case, these results appear to indicate that the activity to compromise the Th2 immune reaction is not directly correlated with the activity to ameliorate the allergy symptoms.

As in the case of cOVA-specific Th2 immune responses discussed above, the reduction in the level of IgE by EE and DF was not surprising (Fig. 4B). But, the reduction in the level of IgE by AF was totally unexpected. The exact mechanisms underlying the reduction of cOVA-specific IgE were not fully understood at this point. Still, it can be speculated that suppression of cOVA-specific Th2 immune response by those treatments limited the $CD4^+$ T cell help for the germinal center formation in the dLN for antigen-specific B cell proliferation and isotype class switching, curtailing the number of cOVA-specific IgE producing memory B cells or plasma cells. In addition, the results that AF treatment failed to ameliorate the allergy symptoms even though it

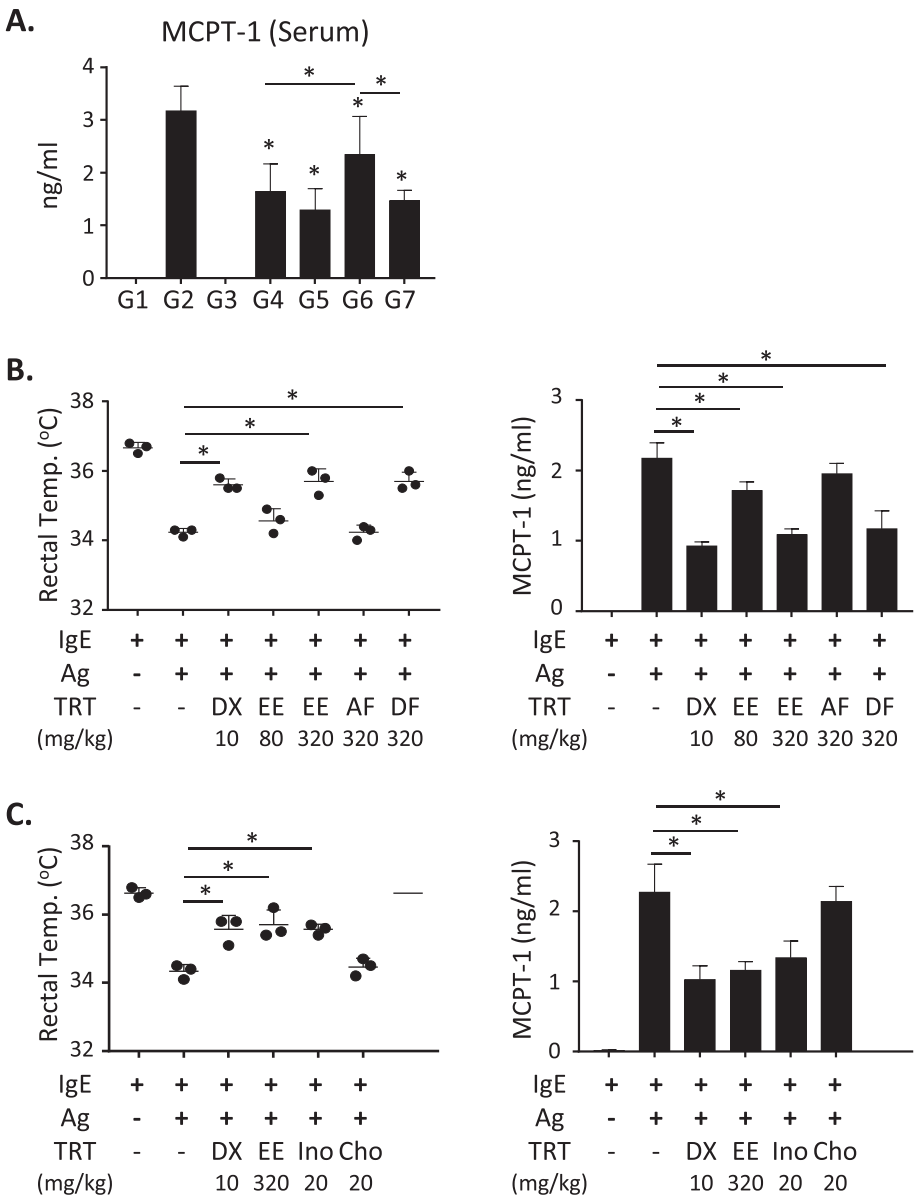


Fig. 5. EtOH ext. (EE), CH_2Cl_2 frac. (DF) and inotodiol but not aqueous frac. (AF) and cholesterol can inhibit the mast cell function.

The concentrations of MCPT-1 in the serums prepared from the mice of each experimental group were measured with ELISA (A). The definition of the groups used in A (G1–G7) is shown in Fig. 2C. A set of passive anaphylaxis experiments were carried out for comparing the efficacies of Dexa, EE, AF and DF for the inhibition of the mast cell function (degranulation) (B). The changes in the rectal temperature that reflect the extents of the anaphylactic reaction were examined 30 min after DNP-BSA injection (B, left). And, the levels of MCPT-1 in the serums that reflect the extents of mast cell degranulation were measured with ELISA (B, right). The serums were prepared 1 h after DNP-BSA injection. Another set of the passive anaphylaxis experiments were carried out for comparing the efficacies of Dexa, EE, inotodiol and cholesterol (C). The rectal temperature changes (C, left) and the levels of MCPT-1 (C, right) in the mice of the each experimental group are shown. Experiments shown in (B) and (C) were performed with 3 mice per group. Asterisk (*) denotes $p < 0.05$ by nonparametric one-way ANOVA (Kruskal-Wallis Test).

brought about a significant reduction in the level of cOVA-specific IgE in the small intestine imply that the level of allergen-specific IgE is not always 'proportionally' related with the severities of allergy symptoms. More works are warranted to better understand the effect of the level of allergen-specific IgE on the occurrence of allergy and the severities of its symptoms.

The data presented in Fig. 5A clearly indicate that the effects of EE and DF on the release of the granules by mast cells are different from that of AF (Fig. 5A). Given that the level of MCPT-1 in the blood reflects the extent of granule release by mast cells in the local tissue, it seems clear that EE and DF are much more effective in preventing the release of the granules by mast cell than AF (Fig. 5A). Moreover, the levels of MCPT-1 in the serums of the mice from respective treatment groups appear to mirror their effects on the allergy symptoms. That is, AF treatment that resulted in little improvement in the allergy symptoms also had little effect on the level of MCPT-1 in the blood. On the other hand, EE (both the high dose and the low dose) and DF treatments that resulted in marked improvement in the symptoms also caused a significant reduction in the levels of MCPT-1 in the blood. One may argue that the low levels of MCPT-1 in the blood of EE and DF-treated mice were due to the low number of mast cells dwelling in the tissue (Fig. 3 and Supp. Fig. 3). Even though such a possibility cannot be ruled out, it is of note that the results from the passive anaphylaxis experiments are conforming to the idea that only EE and DF have an activity to attenuate the mast cell function (Fig. 5B). Thus, it is also conceived that the low numbers of mast cell and eosinophil in the small intestine resulted from the inhibition of mast cell function by EE and DF, respectively.

It is not known whether a single compound in DF can exert the same effects as DF itself. However, given the importance of the mast cell degranulation in the occurrence of the allergy symptoms and the lesions in the small intestine, the result that highly purified inotodiol (> 95% purity) (Fig. 1B) exerted a comparable effect on the mast cell function as DF in the passive anaphylaxis experiment clearly hints such a possibility (Fig. 5C). A few studies by others have shown the anti-cancer activity of inotodiol [38]. Even though exact mechanisms underlying the anti-cancer activity have not been understood, those results indicate that inotodiol may alter the cellular mechanism(s) critical for the survival and proliferation of cancer cells [31]. More works are warranted to better understand the anti-allergic effects of inotodiol.

For validating the therapeutic (preventive) effect of Chaga mushroom on food allergy, it seemed important to provide evidences that EE

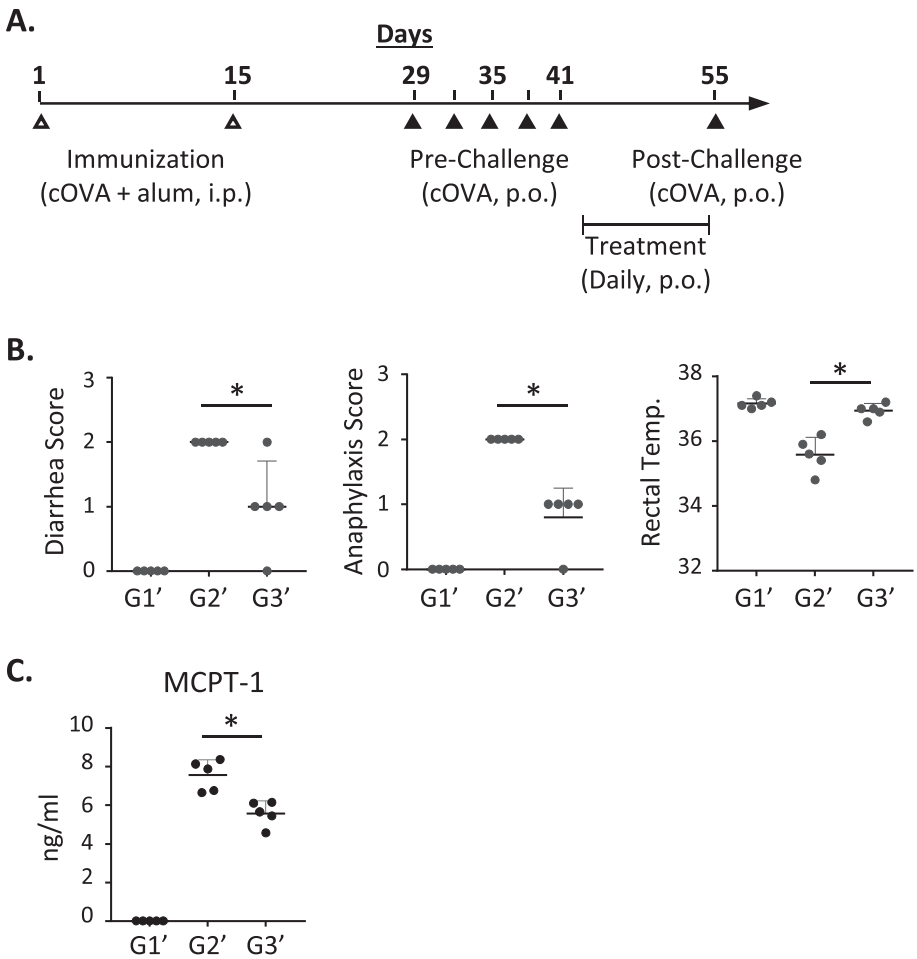


Fig. 6. EtOH ext. (EE) can prevent the recurrence of allergy symptoms in the mice that have already developed the symptoms.
The time frame of the experiment to examine the efficacy of EE for the prevention of the recurrence of allergy symptoms is shown; the mice immunized and challenged with cOVA (pre-challenge) as in Fig. 1A were left untreated or treated with EE (320 mg/kg) for 12 days beginning 1 day after the 5th challenge, and challenged with cOVA one more time (post-challenge) 1 day after the end of the treatment (A). The levels of allergy symptoms shown in the mice of each experimental group, i.e., the extents of diarrhea, anaphylaxis and the rectal temperature change, are shown (B). G1' denotes the group of mice (naïve) with no immunization, no challenge and no EE treatment. G2' denotes the group of mice with immunization and pre- and post-challenges but no EE treatment. G3' denote the group of mice with immunization, pre- and post-challenges and EE treatment. The serums were prepared 2 h after the post-challenge and the levels of MCPT-1 in the serums were measured with ELISA (C) as described in Fig. 2. Asterisk (*) denotes $p < 0.05$ by nonparametric one-way ANOVA (Kruskal-Wallis Test).

treatment could ameliorate the allergy symptoms in the mice that had already developed the symptoms. Thus, we carried out the experiment described in Fig. 6, and the results proved that EE could also prevent recurrence of the allergy symptoms in animals that were already highly allergic to cOVA. Since this experiment mimics the situation that food allergy patients are contacted with a cognate food allergen, the results shown in Fig. 6 also seems to demonstrate the value of EE (and DF) as a therapeutics or a dietary supplement for food allergy patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2017.11.025.

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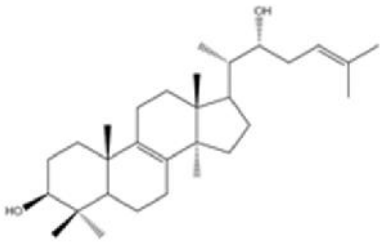
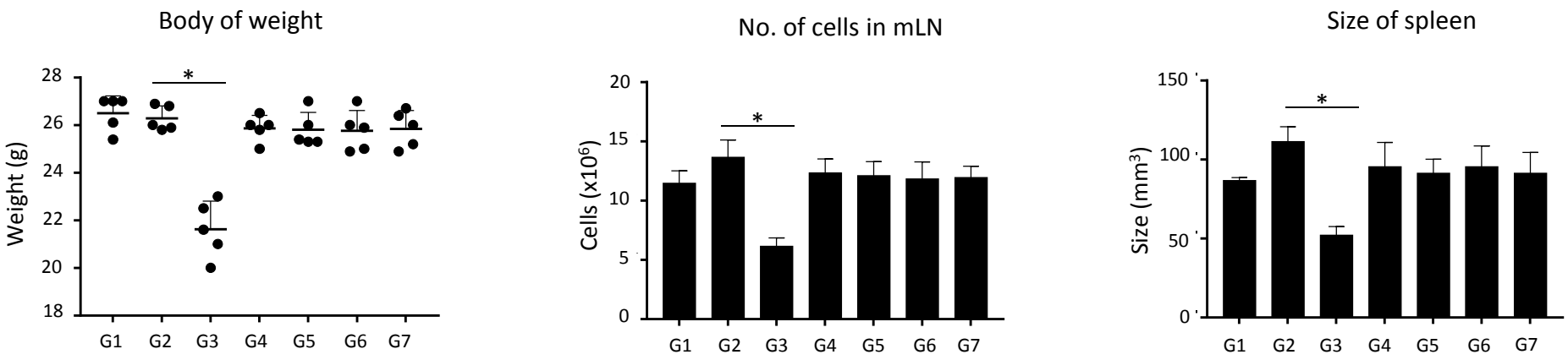


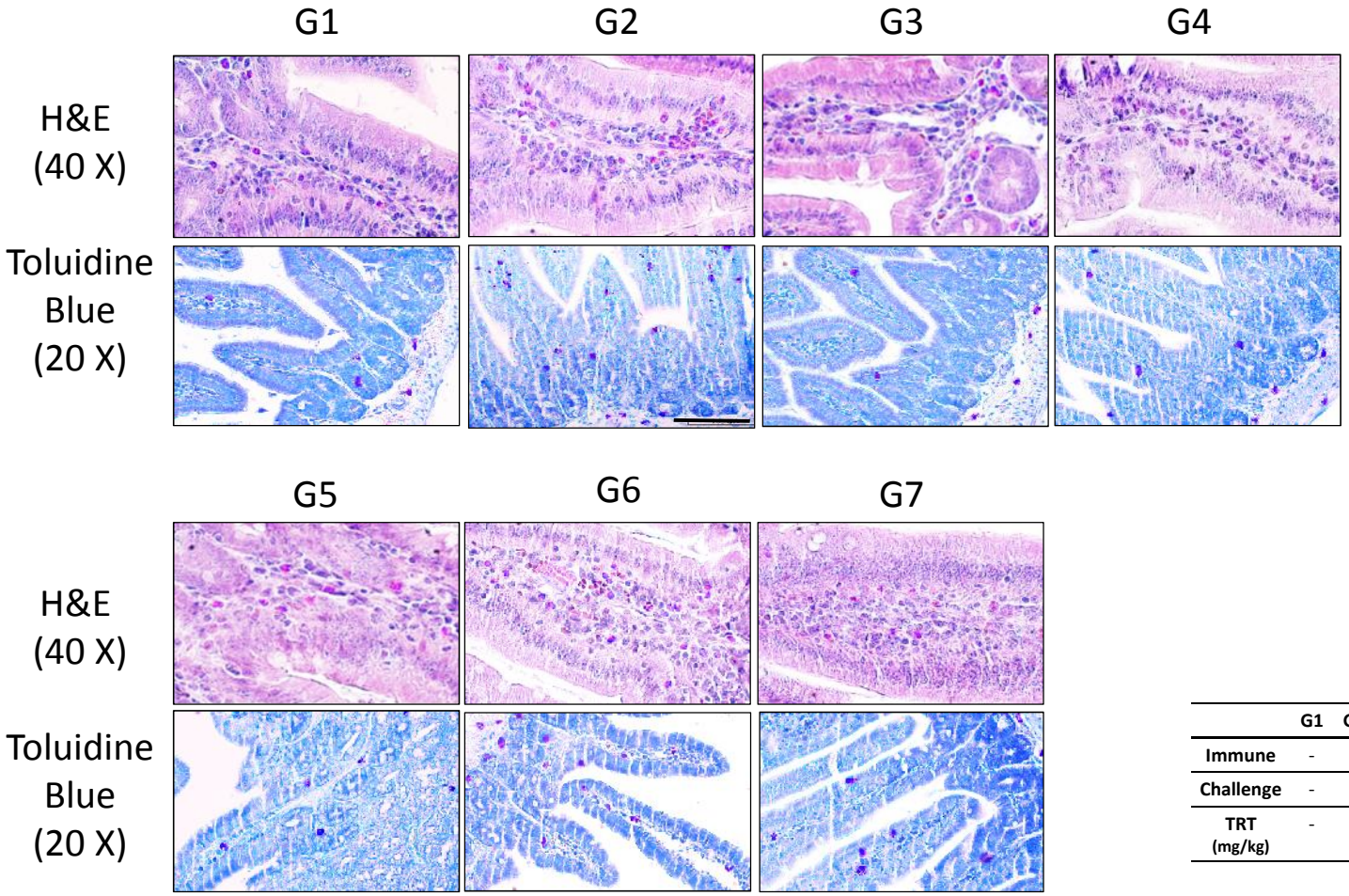
	δ_C (100 MHz)	δ_H (400 MHz)
1	35.4	
2	27.6	
3	78.7	3.18 (*dd*, *J* = 4.43,1H)
4	38.7	
5	50.2	0.94 (m, 1H)
6	18.9	
7	26.3	
8	134.4	
9	134.0	
10	36.8	
11	20.8	
12	28.9	
13	44.5	
14	49.2	
15	30.7	
16	30.7	
17	47.0	1.55 (m, 1H)
18	15.5	0.67 (s, 3H)
19	18.0	0.93 (s, 3H)
20	41.5	1.97 (m, 1H)
21	12.4	0.88 (s, 3H)
22	73.2	3.65 (m, 1H)
23	27.0	
24	121.2	5.16 (t, 1H)
25	134.8	
26	25.7	1.60 (s, 3H)
27	17.9	1.69 (s, 3H)
28	27.7	0.90 (s, 3H)
29	15.2	0.76 (s, 3H)
30	24.1	0.82 (s, 3H)

Supp. Fig. 1. NMR analysis data of purified inotodiol.



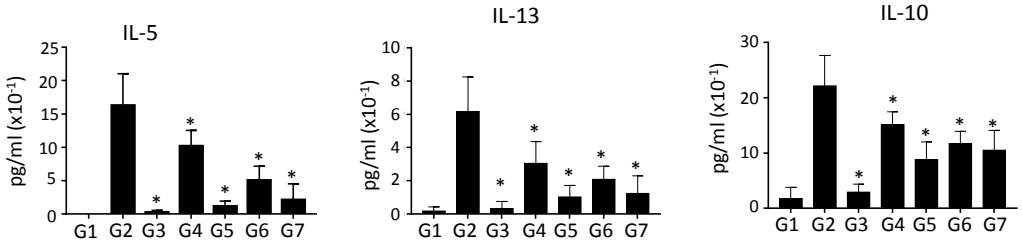
	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

Supp. Fig. 2. The Effects of each treatment regimens on the body weight, the cellularity of mLN and the size of the spleen.



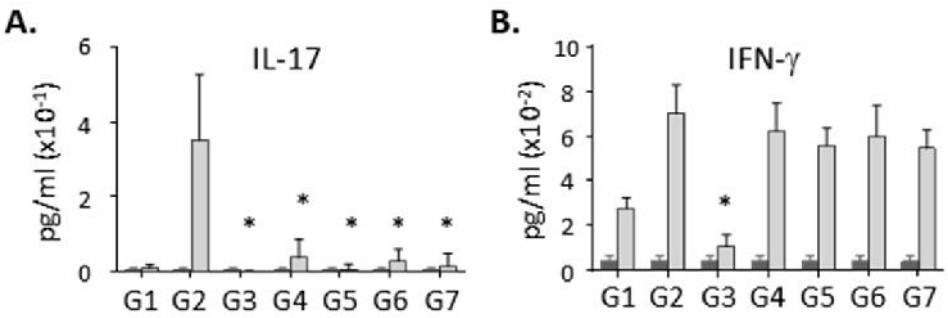
	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

Supp. Fig. 3. Effects of each treatment regimens on the inflammatory lesion, i.e., the numbers of eosinophil and mast cell in the gut

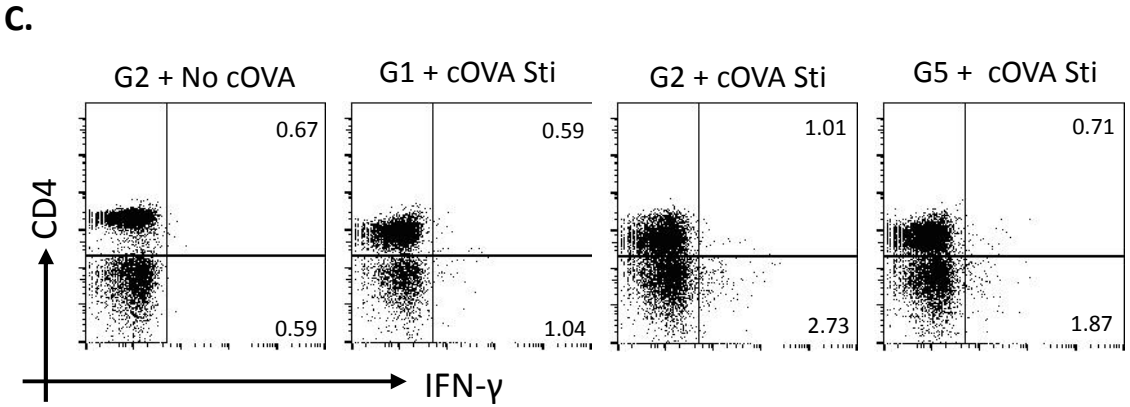


	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

Supp. Fig. 4. The levels of Th2 cytokines in the small intestine

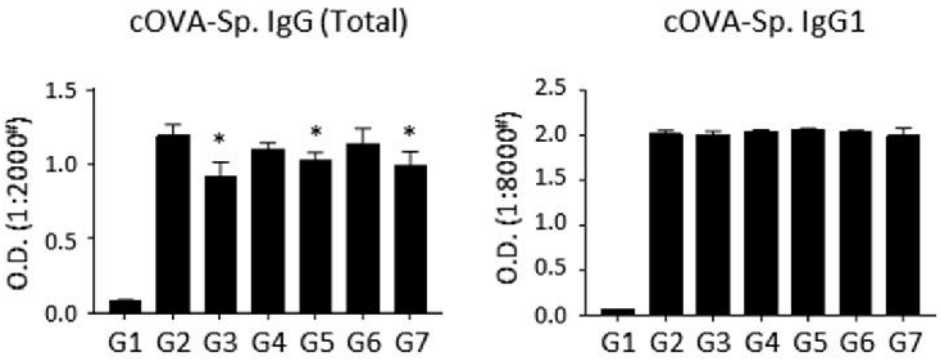


	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)



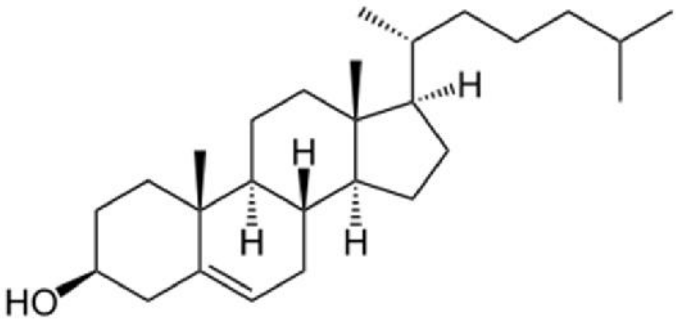
Mesenteric lymph node cells cultured with or without cOVA were fixed and permeabilized as described in **Materials and Methods** and stained for CD4 and IFN-γ. The percentages of the cells belonging to each quadrants are shown inside the plot.

Supp. Fig. 5. Effects of each treatment regimens on development of cOVA-specific T cell immune function



	G1	G2	G3	G4	G5	G6	G7
Immune	-	+	+	+	+	+	+
Challenge	-	+	+	+	+	+	+
TRT (mg/kg)	-	-	Dexa (25)	EE (80)	EE (320)	AF (320)	DF (320)

Supp. Fig. 6. Effects of each treatment regimens on the levels of cOVA-specific total IgG and IgG1 in the blood



Supp. Fig. 7. Chemical structure of cholesterol