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Original Article

Propolis suppresses cytokine production in activated basophils and basophil-mediated skin and intestinal allergic inflammation in mice

Jun-ichi Kashiwakura[*, 1], Mari Yoshihara [1], Kodai Saitoh, Kota Kagohashi, Yuto Sasaki, Fuki Kobayashi, Iori Inagaki, Yuichi Kitai, Ryuta Muromoto, Tadashi Matsuda[**]

Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

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Abbreviations:
BMBs, bone marrow-derived basophils; CAPE, caffeic acid phenethyl ester; DNP, 2,4-dinitrophenol; DSS, dextran sulfate sodium; FcεRI, high affinity IgE receptor; HSA, human serum albumin; IgE-CAI, IgE-dependent chronic allergic inflammation; mMCP-1, mouse mast cell protease-1; MMC9, IL-9-producing mucosal mast cell

ABSTRACT

Background: Propolis is a resinous mixture produced by honey bees that contains cinnamic acid derivatives and flavonoids. Although propolis has been reported to inhibit mast cell functions and mast cell-dependent allergic responses, the effect of propolis on basophil biology remains unknown. This study aimed to investigate the inhibitory effect of propolis on FcεRI-mediated basophil activation.
Methods: To determine the inhibitory effect of propolis on basophil activation *in vitro*, cytokine production and FcεRI signal transduction were analyzed by ELISA and western blotting, respectively. To investigate the inhibitory effect of propolis *in vivo*, IgE-CAI and a food allergy mouse model were employed.
Results: Propolis treatment resulted in the suppression of IgE/antigen-induced production of IL-4, IL-6 and IL-13 in basophils. Phosphorylation of FcεRI signaling molecules Lyn, Akt and ERK was inhibited in basophils treated with propolis. While propolis did not affect the basophil population in the treated mice, propolis did inhibit IgE-CAI. Finally, ovalbumin-induced intestinal anaphylaxis, which involves basophils and basophil-derived IL-4, was attenuated in mice prophylactically treated with propolis.
Conclusions: Taken together, these results demonstrate the ability of propolis to suppress IgE-dependent basophil activation and basophil-dependent allergic inflammation. Therefore, prophylactic treatment with propolis may be useful for protection against food allergic reactions in sensitive individuals.

Introduction

Propolis is a resinous mixture produced by honey bees that contains polyphenols, aromatic aldehydes, flavonoids and fatty acids. Brazilian propolis is characterized by its large content of cinnamic acid derivatives such as artepillin C.[1,2] There are several reports indicating that Brazilian propolis acts as an anti-inflammatory product in immune responses. Brazilian propolis ethanol extract has been shown to suppress colitis in the DSS-induced mouse model,[3] and artepillin C attenuates carrageenan-induced paw edema in mice.[4] Additionally, chrysin, a natural flavonoid found in propolis, reduces skin inflammation induced by the repeated topical application of 2,4-dinitrochlorobenzene.[5]

Propolis and its components also suppress allergic inflammatory reactions. Tani *et al.* reported that Brazilian propolis inhibits Cry j1-induced cytokine production and the release of cysteinyl-leukotrienes and histamine from peripheral blood mononuclear cells of patients with allergic rhinitis.[6] Furthermore, CAPE, a natural cinnamic acid derivative found in propolis, inhibits airway inflammation in asthmatic mice by repressing reactive oxygen species-mediated MAPK/Akt pathway activation,[7] and also modulates IL-4/TNF-α-induced eotaxin production and phosphorylation of STAT6 in human lung fibroblasts.[8] A major flavonoid component in propolis, pinocembrin, suppresses the NF-κB pathway and attenuates antigen-induced airway inflammation.[9]

* Corresponding author. Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 060-0812, Japan.
** Corresponding author. Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 060-0812, Japan.
E-mail addresses: junkashi@pharm.hokudai.ac.jp (J.-i. Kashiwakura), tmatsuda@pharm.hokudai.ac.jp (T. Matsuda).
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[1] These authors contributed equally to this work.

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The prevalence of food allergy has increased dramatically over the last two decades in the industrialized countries. Food allergy is characterized by itchy skin, hives, and diarrhea after eating the allergy-causing food, and in some cases, can cause severe life-threatening reactions, i.e., anaphylaxis.[10,11] There are currently no therapeutic strategies for the cure of food allergy, although oral immunotherapy is one attractive treatment with success in some patients. To develop new treatments for food allergy, we will need to understand the cellular and molecular mechanisms and identify potential therapeutic targets. Basophils are involved in the induction of food allergen sensitization in mice.[12] Using basophil-depleted mice, it has been previously demonstrated that basophils are required for induction of OVA-induced intestinal anaphylaxis in food allergy mice. It is also revealed that basophil-IL-4-mast cell axis plays a crucial role in effector phase of the OVA-induced food allergy,[13] indicating that basophils are a good therapeutic target.

Basophils are the least abundant granulocyte in the blood and share several features with mast cells such as the expression of FcεRI, a high affinity IgE receptor, on their surface. Basophils and mast cells are key effector cells in IgE-dependent allergic inflammatory reactions.[14] Recent studies have indicated that certain immunological reactions depend on basophils but not mast cells.[15] Basophils are essential for the induction of IgE-CAI[16,17] and play a pivotal role in IgG-dependent anaphylaxis by secreting platelet activating factor.[18] Kubo and colleagues have reported that basophil-derived IL-4 is required for ILC2 activation and papain-induced asthmatic reaction.[19] Basophils are also essential for secondary immune responses against helminth infection.[20]

Although propolis has been reported to inhibit mast cell activation,[21] it is still unknown whether propolis directly inhibits FcεRI signaling. It is also unclear whether propolis affects basophil activation and basophil-related allergic inflammatory reactions. Therefore, in this study, we sought to analyze the ability of propolis to inhibit IgE-mediated basophil activation and the basophil-related food allergy response.

Methods

Propolis

Propolis powder (LY-009), standardized to contain 8.0% artepillin C was obtained from Yamada Bee (Okayama, Japan).

Mice

Balb/c mice were purchased from Sankyo Labo Service (Hokkaido, Japan). All animal studies were approved by the Hokkaido University animal ethics committee. All mice were housed in the Pharmaceutical Sciences Animal Center of Hokkaido University under specific pathogen-free conditions.

Antibodies

Anti-DNP IgE mAb (clone: DNP H1-ε-206) was described previously.[22] FITC anti-mouse IgE (clone: RME-1), PE anti-mouse CD200R3 (clone: Ba13) and APC anti-mouse CD49b mAb (clone: HMα2) mAbs were purchased from BioLegend (San Diego, CA, USA). Anti-phospho Src Y416, anti-phospho Erk, anti-phospho Akt, anti-Lyn and anti-Akt Abs were purchased from Cell Signaling Technology (Beverly, MA, USA). Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Flowcytometric analysis

Flowcytometric analysis was performed as previously described.[23]

BMBs

Bone marrow cells from Balb/c mice were cultured in RPMI1640 medium supplemented with 10% fetal calf serum and recombinant mouse IL-3 (1 ng/ml, Tonbo Biosciences, San Diego, CA, USA) for 9 days. $CD49^+$ cells were purified by magnetic cell sorting using biotin anti-mouse CD49b mAb and Streptavidin Particle Plus − DM (BD Biosciences, San Jose, CA, USA) for obtaining BMBs.

Cytokine production

IgE-sensitized BMBs were stimulated with indicated concentrations of DNP_{23}-HSA (Biosearch Technologies, Novato, CA, USA) for 24 h. Cytokine levels in supernatants were measured using ELISA kits (IL-4 and IL-6; BioLegend, IL-9 and IL-13; Affymetrix, San Diego, CA, USA).

Western blot

Western blot analysis was performed as previously described.[23]

IgE-CAI

IgE-CAI was performed as previously described.[23] Briefly, Balb/c mice were intragastrically administered with 0.3 mg propolis in PBS at day 1. At day 4, the mice were intravenously injected with 100 μg anti-DNP IgE mAb. At day 5, the mice were intradermally injected with 10 μg DNP_{11}-OVA (Biosearch Technologies) and ear thickness was monitored once daily for 6 days. Expression of *Mcpt8* in the ear tissues of IgE-CAI was measured by qPCR using the primer set as previously described.[23]

OVA-induced intestinal inflammation

OVA-induced intestinal inflammation was generated as described previously.[24] Diarrhea occurrence were monitored. In some experiments, mice were orally administrated with 0.3 mg propolis twice a week from day 27. Twenty-four h after 9th challenge, sera and jejunum tissues were collected. Serum levels of total and OVA specific antibodies were measured by ELISA as described previously.[13,25] Serum mMCP-1 concentrations were measured by ELISA kit (Affymetrix). Expression of *Il4* and *Enpp3* in jejuna of food allergy mice was measured by qPCR using the primer set as previously described.[23,26]

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.02. Mann–Whitney test, Bonferroni's multiple comparisons test and Tukey's multiple comparisons test were employed. Data were considered significant at $p < 0.05$. Data were shown mean + SEM.

Results

Propolis suppresses IgE/Ag-induced basophil activation in vitro

To investigate the effect of propolis on basophil biology, we generated BMBs. After culturing bone marrow cells with IL-3 and

purification by a magnetic sorting technique, we obtained high purity CD200R3$^+$CD49b$^+$ basophils (>95%, Fig. 1A). We first tested whether propolis affected FcεRI expression on these cells. The BMBs were incubated with 100 μg/ml propolis for 6 h in the presence of 0.5 μg/ml anti-DNP IgE mAb, and FcεRI expression was detected using FITC anti-mouse IgE as previously described.[27] FcεRI expression levels on propolis-treated basophils were comparable to the levels on untreated basophils (Fig. 1B, C), suggesting propolis does not suppress FcεRI expression in these cells.

We next investigated the effect of propolis on antigen-induced basophil cytokine production. The BMBs were sensitized with 0.5 μg/ml anti-DNP IgE mAb overnight and then stimulated with various concentrations of Ag in the presence or absence of propolis (1–100 μg/ml) for 24 h. After stimulation, cytokine levels in the supernatants of the activated basophils, with or without propolis

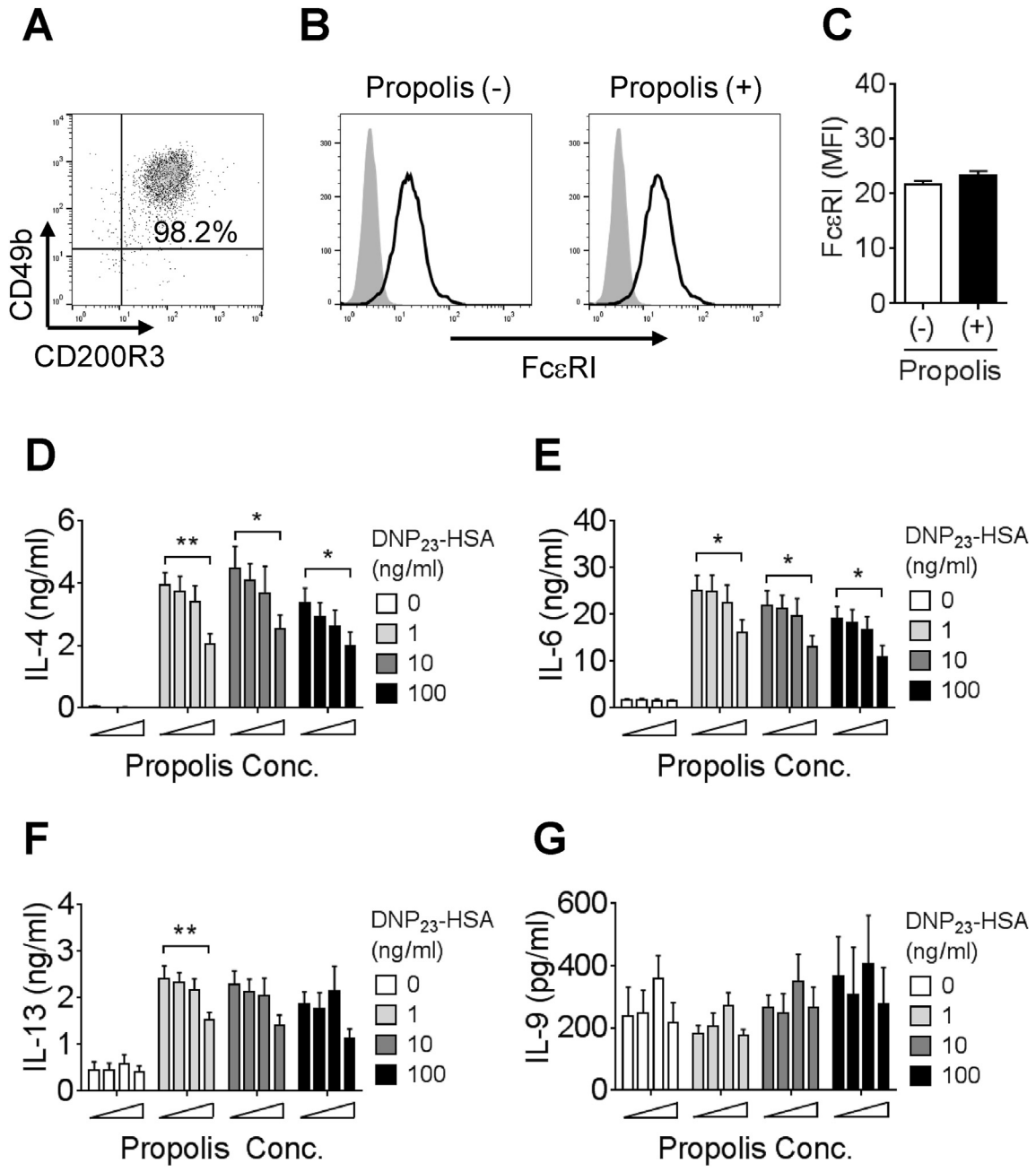

Fig 1. Propolis suppressed IgE/Ag-induced basophil cytokine production. **(A)** Bone marrow cells from a Balb/c mouse were cultured for 9 days in IL-3-containing RPMI 1640 medium for generation of BMBs. The purity of purified BMBs (CD200R3$^+$CD49b$^+$ cells) was analyzed by flowcytometric analysis. The dot plot shows representative data of the purity analysis. **(B)** BMBs were incubated with (100 μg/ml, Propolis [+]) or without (Propolis [-]) propolis for 6 h in the presence of anti-DNP IgE. FcεRI expression was analyzed by flowcytometric analysis using FITC-conjugated anti-mouse IgE mAb. Representative histogram plots of 2 independent experiments are shown. **(C)** Expression levels of FcεRI on basophils treated with or without propolis (100 μg/ml) are shown. White and Black bars show mean fluorescence intensity (MFI) of FcεRI expression on untreated (-) and propolis-treated (+) basophils, respectively. Data were shown mean + SEM of 2 independent experiments (n = 4). **(D–G)** BMBs were sensitized with anti-DNP IgE mAb for overnight. The BMBs were stimulated with DNP_{23}-HSA (0–100 ng/ml) in the presence of propolis (0–100 μg/ml) for 24 h. The levels of IL-4 **(D)**, IL-6 **(E)**, IL-13 **(F)** and IL-9 **(G)** in supernatants were measured by ELISA. Data **(D–F)** were shown mean + SEM of 3 independent experiments (n = 10–11). Data **(G)** were shown mean + SEM of 2 independent experiments (n = 4). *p < 0.05, **p < 0.01 by Mann–Whitney test.

treatment, were measured by ELISA. The levels of IL-4, IL-6 and IL-13 were increased in Ag-stimulated basophils compared with unstimulated basophils (Fig. 1D–F). However, although the BMBs produced IL-9 spontaneously, IL-9 production did not increase with Ag stimulation (Fig. 1G). The production of IL-4, IL-6 and IL-13 was suppressed by propolis treatment in Ag-stimulated basophils compared with untreated Ag-stimulated basophils, and this reduction was significant at 100 μg/ml propolis treatment (Fig. 1D–F). We also investigated whether propolis suppressed the spontaneous IL-9 production in basophils, but no suppressive effect was observed (Fig. 1G).

Propolis treatment inhibits FcεRI signal transduction in basophils

Next, we investigated the inhibitory mechanism of propolis in activated basophils using Western blot analysis to detect changes in the phosphorylation (activation) of FcεRI signaling molecules Lyn, Akt, and ERK. Sensitized BMBs were pretreated with 100 μg/ml propolis for 6 h and stimulated with 100 ng/ml Ag for indicated periods. The phosphorylation of Lyn was inhibited in propolis-treated basophils compared with untreated basophils, and the phosphorylation levels of Akt and ERK in were also reduced in propolis-treated basophils compared with untreated basophils (Fig. 2A). By densitometric analysis, we revealed that the levels of Lyn phosphorylation in propolis-treated BMBs were significantly reduced compared with the levels in untreated BMBs. It was also indicated that the reduced levels of Akt and ERK phosphorylation were statistically significant (Fig. 2B–D). We also tested phosphorylation of PLC-γ2, which is essential for calcium influx and degranulation, and found no inhibition of PLC-γ2 phosphorylation in propolis-treated basophils (data not shown). These results indicated that the inhibitory effect of propolis was specific to the FcεRI signaling molecules.

Propolis attenuates IgE- CAI without affecting the basophil population

We next analyzed whether propolis inhibited FcεRI-mediated basophil activation *in vivo*. We treated mice with propolis (0.3 mg/mouse) on each of three alternate days and monitored basophil populations by flow cytometric analysis using untreated mice as controls. The proportion of cells represented by basophils in the blood of mice treated with propolis was comparable to that of untreated mice (Fig. 3A, B), suggesting that propolis has no effect on the blood basophil population.

Next, we employed IgE-CAI to determine the effect of propolis on basophil activation *in vivo*. Mice were intragastrically administered 0.3 mg propolis on each of three alternate days. After the third propolis treatment, mice were intravenously injected with 50 μg anti-DNP IgE mAb and intradermally challenged with Ag 24 h after the IgE injection. In untreated mice, ear thickness increased measurably two days after Ag administration and peaked on the

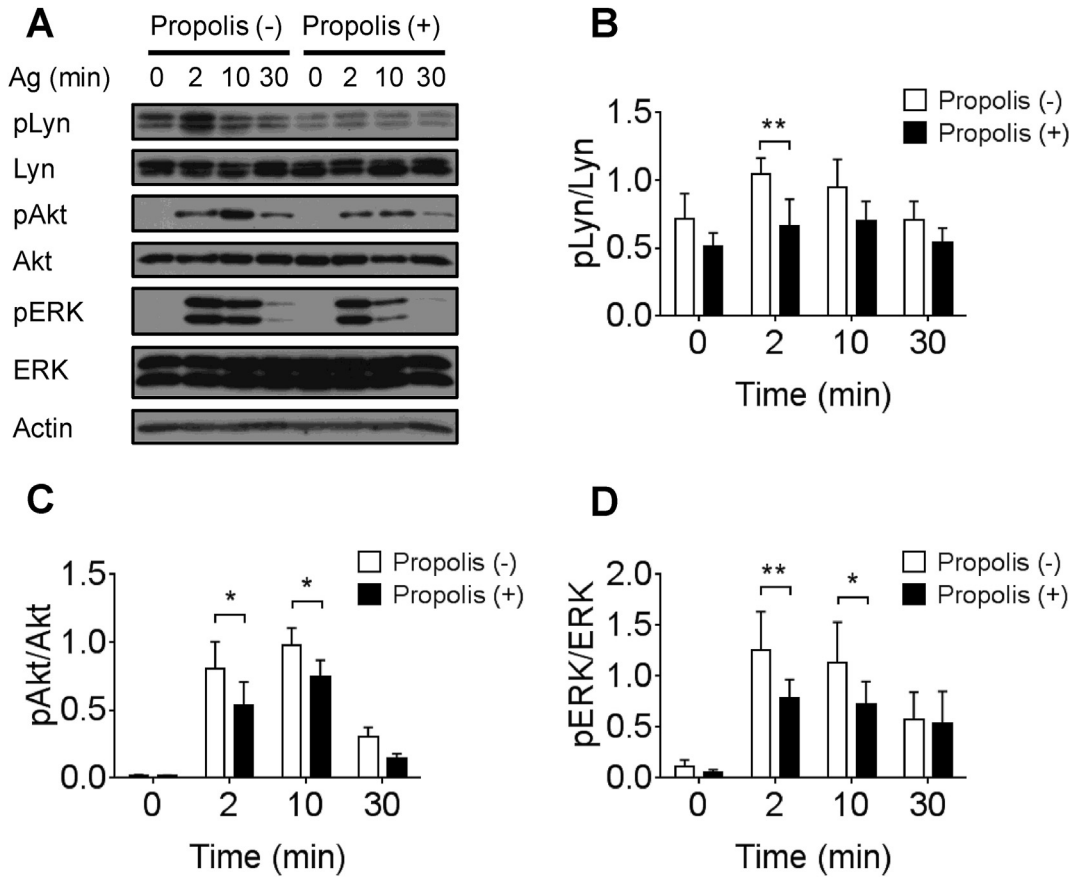

Fig 2. Propolis inhibited FcεRI signal transduction in basophils. BMBs were sensitized with anti-DNP IgE mAb for overnight. The IgE-bound BMBs were incubated with propolis (100 μg/ml, Propolis [+]) for 6 h and, then, stimulated with DNP_{23}-HSA (100 ng/ml) for indicated time points. For control, untreated BMBs (Propolis [-]) were used. Phosphorylated and whole Lyn, Akt and ERK proteins were analyzed by western blotting. **(A)** Representative images of phosphorylated and whole Lyn, Akt and ERK proteins in propolis-treated and untreated BMBs are shown. Actin was detected as a loading control. **(B–D)** Phosphorylation intensity of Lyn **(B)**, Akt **(C)** and ERK **(D)** are shown. White and Black bars show untreated (Propolis [-]) and propolis-treated (Propolis [+]) BMBs, respectively. Data were shown mean + SEM of 4 independent experiments ($n = 5$). *$p < 0.05$, **$p < 0.01$ by Bonferroni's multiple comparisons test.

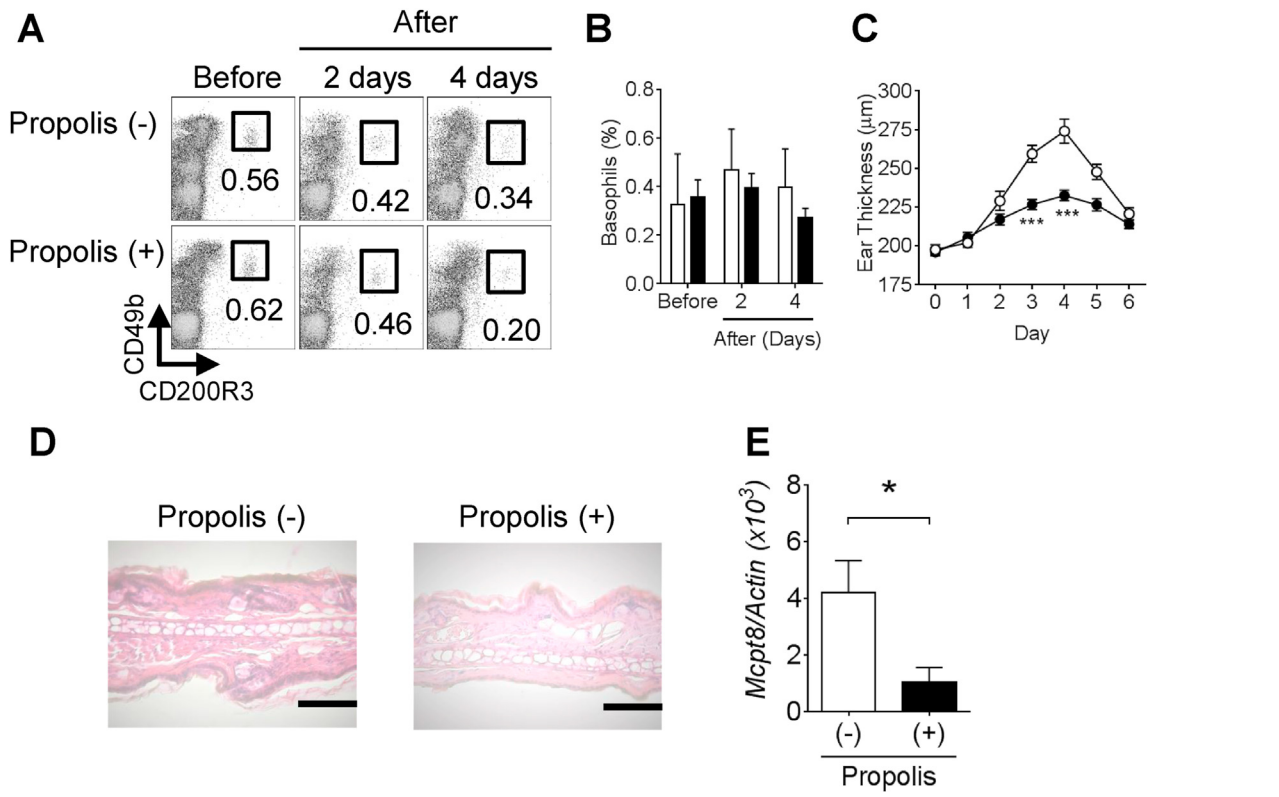

Fig 3. IgE-CAI was inhibited by propolis treatment without any effects of basophil maintenance. **(A)** Before propolis administration, basophil population was monitored by flowcytometric analysis. Then, Balb/c mice were intragastrically administered with (Propolis [+], lower panels) or without (Propolis [-], upper panels) 0.3 mg propolis on each of 3 alternate days. Two and four days after first treatment of propolis, basophil populations in mice were analyzed by flowcytometric analysis. Gated populations ($CD200R3^+CD49b^+$ cells) were basophils. **(B)** Blood basophil populations were shown mean + SEM of 2 independent experiments. White and Black bars show untreated and propolis (0.3 mg)-treated mice, respectively (untreated: n = 12, propolis-treated: n = 10). **(C)** Balb/c mice were intragastrically treated with (black cycles) or without (white cycles) 0.3 mg propolis on each of 3 alternate days. Four days after first propolis administration, the mice were intravenously injected with 50 μg anti-DNP IgE mAb. Twenty-four h after IgE injection, the mice were intradermally injected with 10 μg DNP_{11}-OVA and dermal thickness was measured once daily for 6 days. Data were shown mean + SEM of 3 independent experiments (untreated: n = 15, propolis-treated: n = 12). ***$p < 0.001$ by Tukey's multiple comparisons test. **(D)** Ear Inflammation and leukocyte infiltration in mice treated with (Propolis [+]) or without (Propolis [-]) propolis were analyzed by H&E staining. Representative images of the ears are shown. Bars = 100 μm. **(E)** Expression of *Mcpt8* in ear skins from untreated and propolis-treated IgE-CAI mice. Ear skin samples at 4 days after IgE-CAI induction were collected and mRNA was purified. The *Mcpt8* expression levels were analyzed by qPCR. Relative expression after normalizing to *Actin* expression was shown mean + SEM of two independent experiments (Propolis [-]: n = 7, Propolis [+]; n = 6). *$p < 0.05$ by Mann–Whitney test.

fourth day. Compared with untreated mice, propolis-treated mice showed significant attenuation of the IgE-CAI as measured by changes in ear thickness (Fig. 3C). We also observed decreased infiltration of leukocytes in the ears of propolis-treated mice compared with the ears of untreated mice (Fig. 3D). We purified mRNA from skin samples at 4 days after Ag administration and *Mcpt8* expression was analyzed by qPCR to reveal whether basophil migration into the ear was suppressed by propolis treatment because it has been reported that IgE-CAI induces basophil migration into the skin.[28] The *Mcpt8* expression levels in the ear skins from propolis-treated mice were significantly reduced compared with the ear skins from untreated mice, indicating that inhibition of basophil migration by treatment with propolis is responsible for suppression of IgE-CAI response. Taken together, these data indicate that propolis has the ability to inhibit IgE-dependent basophil activation both *in vitro* and *in vivo*.

Propolis prophylactic treatment suppresses the Ag-induced food allergy reaction

Finally, to investigate whether propolis inhibits Ag-induced intestinal anaphylaxis, which involves basophils,[13] we compared the intestinal anaphylaxis of propolis-treated mice with that of untreated and PBS-treated mice. In OVA-sensitized (food allergy) mice, diarrhea occurred after the repeated oral administration of OVA. Similar diarrheal symptoms occurred in the PBS-treated food allergy mice (Fig. 4A). However, the occurrence of diarrhea was significantly reduced in propolis-treated mice compared with untreated ($p < 0.001$) and PBS-treated mice ($p < 0.001$), respectively (Fig. 4A, Mantel–Cox Log-rank test). The clinical scores of propolis-treated mice were also significantly reduced compared with the scores of untreated and PBS-treated mice (Fig. 4B, C). Because serum mMCP-1 levels reflect the severity of Ag-induced intestinal inflammation, we compared the serum mMCP-1 levels in propolis-treated food allergy mice with the levels in untreated and PBS-treated food allergy mice. Consistent with the results shown in Figure 4A–C, mMCP-1 serum levels were significantly lower in propolis-treated mice compared with untreated and PBS-treated mice (Fig. 4D).

We next tested antibody production in food allergy mice prophylactically treated with propolis compared with untreated and PBS-treated food allergy mice. As measured by ELISA, the levels of total IgE and IgG1 as well as OVA-specific IgE and IgG1 in sera from propolis-treated mice were comparable to those in sera from untreated and PBS-treated mice (Fig. 4E–H). Because we revealed inhibitory effect of propolis on FcεRI-mediated basophil IL-4 production *in vitro* (Fig. 1D), we compared IL-4 expression levels in jejuna of propolis-treated food allergy mice with the levels of

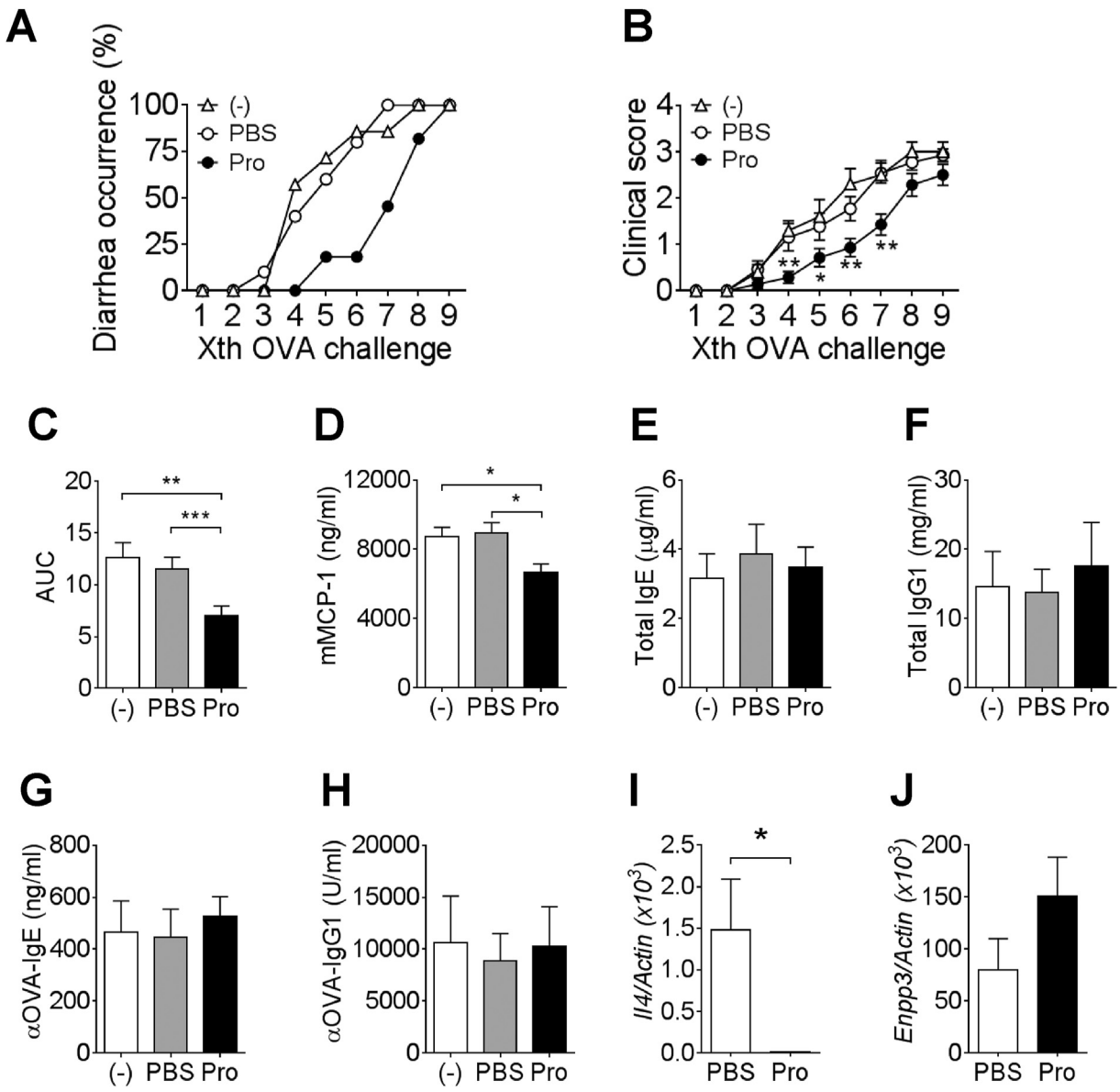

Fig 4. Propolis treatment resulted in reduction of basophil-mediated food allergy reaction. Balb/c mice were sensitized with OVA (50 μg) with Alum (1 mg) at days 1 and 15. From day 29, the sensitized mice were intragastrically challenged with 25 mg OVA 3 times in a week for 3 weeks. Some mice were administered with 0.3 mg propolis 2 times in a week for 3 weeks from day 28. PBS-treated mice were used as a negative control. **(A)** Diarrhea occurrence was monitored. Data were shown mean + SEM of 3 independent experiments (untreated [-]; n = 10, PBS-treated [PBS]; n = 13, Propolis-treated [Pro]; n = 14). **(B)** Clinical scores of food allergy mice treated with or without propolis were shown. Data were shown mean + SEM of 3 independent experiments (untreated [-]; n = 10, PBS-treated [PBS]; n = 13, Propolis-treated [Pro]; n = 14). *p < 0.05, **p < 0.01 by Mann–Whitney test compared with PBS-treated mice **(C)** AUC of clinical scores in each food allergy mice were shown. Data were shown mean + SEM of 3 independent experiments (untreated; n = 10, PBS-treated; n = 13, Propolis-treated; n = 14). **p < 0.01, ***p < 0.001 by Mann–Whitney test. **(D)** Serum level of mMCP-1 was measured by ELISA. Data were shown mean + SEM of 3 independent experiments (untreated [-]; n = 7, PBS-treated [PBS]; n = 11, Propolis-treated [Pro]; n = 13). *p < 0.05 by Mann–Whitney test. **(E–H)** Total IgE **(E)**, total IgG1 **(F)**, OVA-specific IgE **(G)** and OVA-specific IgG1 **(H)** levels in sera from food allergy mice were measured by ELISA. Data were shown mean + SEM of 3 independent experiments (untreated [-]; n = 8, PBS-treated [PBS]; n = 12, Propolis-treated [Pro]; n = 13). **(I)** Expression of *Il4* mRNA in jejuna from food allergy mice treated with or without propolis was measured by qPCR. Relative expression after normalizing to *Actin* expression was shown mean + SEM of two independent experiments (PBS-treated [PBS]: n = 9 Propolis-treated [Pro]; n = 7). *p < 0.05 by Mann–Whitney test. **(J)** Expression of *Enpp3* mRNAs in jejuna from food allergy mice treated with or without propolis was measured by qPCR. Relative expression after normalizing to *Actin* expression was shown mean + SEM of two independent experiments (PBS-treated [PBS]: n = 7, Propolis-treated [Pro]; n = 9).

untreated food allergy mice. We found that expression of IL-4 in jejuna was significantly down-regulated by propolis treatment (Fig. 4I). It has been previously reported that E-NPP3 expression is increased in basophils after IgE/Ag stimulation and is involved in suppression of mast cell/basophil activation that is required for antigen-induced intestinal anaphylaxis.[26] We therefore compared expression levels of *Enpp3* in jejuna of food allergy mice treated with or without propolis. We found that expression levels of *Enpp3* in propolis-treated food allergy mice were similar to those in untreated food allergy mice (Fig. 4J). Taken together, these results suggest that propolis has the ability to attenuate food allergy reactions by repressing basophil activation.

Discussion

In the current study, we show that propolis treatment suppresses FcεRI-mediated basophil cytokine production without affecting FcεRI expression. Investigation into the molecular mech-

anisms of this observation revealed that the activation of FcεRI signaling molecules Lyn, Akt and ERK, as measured by their phosphorylation, was inhibited in basophils by propolis treatment *in vitro*. Finally, using IgE-CAI, which is dependent on basophils, we investigated the inhibitory effect of propolis on basophil activation *in vivo* and found that the Ag-induced intestinal inflammatory reaction was attenuated in mice prophylactically treated with propolis. These results suggest that propolis might serve as a novel anti-allergic supplement for patients with food allergy.

Cho *et al.* report that CAPE inhibits degranulation and the production of certain cytokines in the activated human mast cell line HMC-1 by suppressing JNK and NF-κB phosphorylation but not p38 and ERK phosphorylation. The same study also showed that CAPE administration attenuates IgE/Ag-induced passive cutaneous anaphylaxis in mice.[29] CAPE has a similar inhibitory effect on degranulation and leukotriene release from rat peritoneal mast cells.[30] In the current study, propolis treatment reduced cytokine production in basophils by inhibiting Lyn, Akt and ERK activation. However, we did not observe a reduction of degranulation or PLC-γ2 phosphorylation in propolis-treated basophils (data not shown). Therefore, we assume the inhibitory mechanism(s) of propolis in basophils may differ from its mechanism(s) in mast cells. Understanding the mechanisms of the propolis inhibitory effect on basophil activation events in more detail will be the subject of a future study.

It is thought that, as in mast cells, the activation of Lyn, a Src family member, is necessary for the initiation of FcεRI signaling in basophils.[31,32] However, Rivera and colleagues have reported that Lyn-deficient basophils are more proliferative than wild type basophils, and expression of GATA3, a transcription factor important for inflammatory and allergic responses, is significantly increased in IgE/Ag-stimulated Lyn-deficient basophils compared with wild type basophils.[33] These observations indicate that Lyn also has a negative function in the regulation of basophil homeostasis and FcεRI signaling as had been previously reported in mast cells.[34] In this study, we show that propolis treatment significantly attenuates Lyn phosphorylation and cytokine production in basophils *in vitro*. Thus, we speculate that treatment with propolis may affect only the positive function(s) of Lyn in basophils.

Basophils are involved in the pathogenesis of food allergy at both the sensitization and elicitation phases. Epicutaneous sensitization induces TSLP secretion from epithelial cells which in turn activates basophils to produce IL-4. This TSLP-basophil-IL-4 axis is important for the manifestation of Ag-specific IgE and Ag-induced intestinal anaphylaxis.[12] We previously demonstrated that basophil depletion at the elicitation phase resulted in reduced occurrence of diarrhea, lower clinical scores and reduced numbers of intestinal mast cells in food allergy mice.[13] Our present data also demonstrate that propolis-treated mice are more resistant to Ag-induced intestinal anaphylaxis. These results and previous reports suggest that propolis directly inhibits basophil function in the food allergy model. A recently published study demonstrated that Allergin-1 on basophils is involved in Ag-induced intestinal anaphylaxis in an OVA-induced food allergy mouse model.[35] Our preliminary experiments suggest that expression levels of *Allergin1* in small intestines of propolis-treated food allergy mice were comparable to those of untreated food allergy mice. Therefore, other mechanisms may be involved in the suppression of basophil activation by propolis treatment, although this requires further investigation.

It has been reported previously that mice deficient in IL-9, a Th2 cell-associated cytokine, were resistant to Ag-induced mast cell-dependent intestinal anaphylaxis.[36] The MMC9 is the key cell type involved in susceptibility to Ag-induced intestinal inflammation in food allergy mice.[37] IL-4 produced by Th2 cells is required for MMC9 generation, and MMC9-derived IL-9 is essential for the accumulation of mast cells in the intestines, resulting in the exacerbation of Ag-induced intestinal anaphylaxis. Propolis reportedly inhibits IL-9 expression in mast cells after activation.[38] Given the recent report from Mark H. Kaplan's group demonstrating that basophils produce IL-9 after IL-33 stimulation through the control of the *Il9* CNS-25 regulatory element,[39] we hypothesized that propolis might suppress IL-9 production in Ag-activated basophils, resulting in the attenuation of food allergic reaction observed in our study. Our results indicated that basophils spontaneously produce IL-9; however, no increase in IL-9 production was observed in basophils activated through FcεRI. We also found that propolis does not suppress the spontaneous IL-9 production in basophils. Therefore, the attenuation of the food allergic reaction in propolis-treated mice is most likely not due to a reduction in IL-9 production in basophils but rather the suppression of IL-4 production in basophils, which results in the inhibition of both MMC9 generation and mastocytosis. In addition to this inhibitory effect of propolis on basophil activation, it is possible that propolis directly inhibits IL-9 production in MMC9 resulting in the attenuation of Ag-induced intestinal anaphylaxis in food allergy mice.

In conclusion, this study demonstrates that propolis inhibits FcεRI-mediated cytokine production in basophils without altering FcεRI expression. In propolis-treated basophils, the levels of Ag-induced phosphorylation of FcεRI signal molecules are significantly reduced compared with the levels in untreated basophils. Propolis also inhibits basophil activation *in vivo* with no effect on the basophil population in the food allergy mouse model. Finally, prophylactic treatment with propolis results in the attenuation of the intestinal food allergy reaction in these mice. Therefore, propolis could be a useful supplement for the prevention of food allergen-induced anaphylaxis in patients with food allergy.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

JK, MY and KS performed experiments and analyzed the data. All authors contributed discussion. TM supervised the project. JK and TM designed the project and wrote the manuscript with input from all coauthors. All authors read and approved the final manuscript

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