

Cupressaceae Pollen Grains Modulate Dendritic Cell Response and Exhibit IgE-Inducing Adjuvant Activity In Vivo¹

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Pollen is considered a source of not only allergens but also immunomodulatory substances, which could play crucial roles in sensitization and/or the exacerbation of allergies. We investigated how allergenic pollens from different plant species (Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, and birch, ragweed, and grass) modulate murine bone marrow-derived dendritic cell (DC) responses and examined the effect of Cupressaceae pollen in vivo using mice. DCs were stimulated with pollen extracts or grains in the presence or absence of LPS. Cell maturation and cytokine production in DCs were analyzed by flow cytometry, ELISA, and/or quantitative PCR. Pollen extracts suppressed LPS-induced IL-12 production and the effect was greatest for birch and grass. Without LPS, pollen grains induced DC maturation and cytokine production without IL-12 secretion and the response, for which TLR 4 was dispensable, was greatest for the Cupressaceae family. Intranasal administration of Cupressaceae pollen in mice induced an elevation of serum IgE levels and airway eosinophil infiltration. Co-administration of ovalbumin with Cupressaceae pollen grains induced ovalbumin-specific IgE responses associated with eosinophil infiltration. The results suggest that modulation of DC responses by pollen differs among the plant families via (1) the promotion of DC maturation and cytokine production by direct contact and/or (2) the inhibition of IL-12 production by soluble factors. The strong DC stimulatory activity in vitro and IgE-inducing activity in mice support the clinical relevance of Cupressaceae pollen to allergies in humans. *The Journal of Immunology*, 2009, 183: 6087–6094.

Pollen is an important trigger of seasonal rhinitis, conjunctivitis, and/or asthma and an exacerbating factor in atopic dermatitis (1–4). Pollen grains of trees of the Cupressaceae family including the Taxodiaceae, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtuse*), *Cupressus* species, and *Juniperus* species, are relevant sources of allergens (2, 3, 5). In Europe and North America, birch of *Betula* species is the most important allergenic tree (5). Pollen grains of ragweed of *Ambrosia* species (6) and grasses of the Poaceae family, such as timothy (*Phleum pratense*), rye (*Lolium* spp.), Kentucky blue grass (*Poa pratensis*), orchard grass (*Dactylis glomerata*), Bermuda grass (*Cynodon dactylon*), and others (7), are also among the most clinically relevant sources of allergens.

In addition to the function of pollen grains as carriers of allergen proteins, pollen-derived substances could exhibit immunomodulatory effects. Ragweed pollen-derived NADPH oxidase (8–10) increases levels of reactive oxygen species in the epithelium and has critical roles in both sensitization to and the development of allergies in mouse models (8, 9). Very recently, we have demonstrated that allergenic pollen grains showed NADPH oxidase activity that differed in intensity and localization according to the plant families

and that the activity was mostly concentrated within insoluble fractions (11). Pollen grains release substances that have structural similarity with the inflammatory lipid mediators (12, 13). Lipid fractions from birch and grass pollen extracts induce chemotaxis and the activation of neutrophils (14) and eosinophils (15). Pollen extracts of birch, hazel, lilac, maple, and mugwort and birch pollen phytoprostanes inhibit LPS-induced IL-12 production (16) and birch pollen extract, and phytoprostanes enhance migratory and Th2-attracting capacities (17) in dendritic cells (DCs).³ CD1-restricted T cells and IgE in blood samples obtained from allergic subjects during the pollinating season have been reported to recognize cypress pollen-derived phospholipids (18). We have demonstrated that pollen grains of members of the Cupressaceae family, and birch, ragweed, and grass, release proteases (13, 19) that might be involved in the pathogenesis of allergic diseases, similar to house dust mite-derived and other protease allergens (20–29). Pollen extracts of grass, birch, giant ragweed, and Easter lily degrade tight junctions, and grass pollen extract does so in a protease-dependent manner (30). However, how these innate responses to allergenic pollen differ among plant species is unclear.

DCs are crucial for the initiation and maintenance of T cell-mediated adaptive immune responses (31, 32). Immature DCs, which reside in peripheral tissues, take up pathogens or allergens or are exposed to the milieu of proinflammatory cytokines provided by accessory cells, leading to the induction of DC maturation, which is characterized by the cell-surface expression of costimulatory and MHC class II molecules. Having matured, DCs migrate to lymph nodes, where they activate T cells. The type of

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³ Abbreviations used in this paper: DC, dendritic cell; BAL, bronchoalveolar lavage; MFI, mean fluorescence intensity; OVA, ovalbumin; rmGM-CSF, recombinant mouse GM-CSF.

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pathogen or proinflammatory cytokine milieu determines the phenotype of the mature DCs, which in turn determine the Th phenotype that naive Th cells adopt. DC-derived IL-12 is a crucial Th1-polarizing cytokine.

Inhalation of house dust mite extract (33) or fungal culture extract (34) with ovalbumin (OVA) induces production of IgE against OVA in mice. In mice sensitized i.p. with ragweed pollen extract plus alum, intranasal challenge with extract that includes ragweed pollen NADPH oxidase induces more specific IgE than does challenge with that without this enzyme (8). However, as far as we know, in vivo IgE-inducing adjuvant activity of inhaled pollen-derived substances without the use of alum has not been reported. Although one paper (35) described that the intranasal coadministration of birch pollen extract with OVA in OVA-specific TCR-transgenic mice resulted in Th2-skewed cytokine production in restimulated OVA-specific T cells in vitro, IgE production in vivo was not described.

In the present study, we compared how pollen grains and extracts of different plant species (Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, and birch, ragweed, and grass) affect cell maturation and cytokine production in murine bone marrow-derived DCs in vitro. Additionally, we examined the activity of Cupressaceae pollen grains administered intranasally to induce elevated serum IgE levels and airway eosinophil infiltration and also its adjuvant activity to induce an IgE response specific to a coadministered protein in vivo using mice.

Materials and Methods

Mice

Seven- to 10-wk-old female C57BL/6 and BALB/c mice purchased from Charles River Japan and TLR4-deficient mice (C57BL/6 background), which were a gift from S. Akira (Osaka University, Osaka, Japan), (36) were maintained in a specific pathogen-free animal facility at Juntendo University and used in accordance with the guidelines of the institutional committee on animal experiments.

Pollens

Pollen grains of Japanese cedar (*Cryptomeria japonica*) were purchased from Wako Pure Chemical or generously provided by Torii Pharmaceutical. Pollen grains of Japanese cypress (*Chamaecyparis obtusa*) were purchased from Wako Pure Chemical. Pollen grains of white birch (*Betula alba*), water birch (*Betula fontinalis occidentalis*), and Kentucky bluegrass (*Poa pratensis*) were purchased from Sigma-Aldrich. Pollen grains of short ragweed (*Ambrosia artemisiifolia*) were purchased from Polysciences.

Preparation of pollen extracts

Pollen grains were suspended in Dulbecco's PBS without calcium and magnesium (pH 7.4) (100-mg grains/10 ml in 15-ml tubes). The suspension was rotated gently at 37°C for 1 h and centrifuged for 5 min at 490 × g. The supernatant was collected and filtered (0.22 μm). The filtered supernatants were stored at -80°C until used.

Measurement of endotoxin

Endotoxin contained in the pollen extracts and pollen grain suspensions was measured by using Endospecy (Seikagaku).

Generation and stimulation of DCs

C57BL/6 mouse bone marrow-derived DCs were generated as described (37). In brief, 2 × 10⁶ bone marrow cells prepared from tibia and femur of mice were cultured in 10 ml of RPMI 1640 medium (Sigma-Aldrich) supplemented with 200 U/ml recombinant mouse GM-CSF (rmGM-CSF) (Wako), 2 mM L-glutamine, 10% (v/v) heat-inactivated FCS, 0.05 mM 2-ME, and antibiotics (day 0). At day 3, another 10 ml of medium containing rmGM-CSF was added. At days 6 and 8, half the medium was exchanged for fresh medium containing rmGM-CSF. At day 9, DCs suspended in fresh medium containing rmGM-CSF were plated onto 24-well culture plates (5 × 10⁵ cells/400 μl/well). Finally, 100 μl of the suspension of LPS (Sigma-Aldrich or List Biological Lab), pollen extracts, or pollen grains was added to each of the wells.

Cell viability after the culture for 24 h with LPS, pollen extracts, or pollen grains, or without them was determined by the trypan blue exclusion test.

Flow cytometry

DCs stimulated for 24 h were collected and washed three times with PBS, then incubated with anti-mouse Fcγ receptor (CD16/CD32) mAb (2.4G2) (BD Biosciences) for 30 min at 4°C to avoid nonspecific binding of labeled mAbs. Cell-surface molecules were then stained by incubation of DCs with PE-conjugated anti-mouse CD11c (HL3) and FITC-conjugated anti-mouse I-A^b (AF6-120.1), CD80 (16-10A1) (BD Biosciences), or CD86 (GL1) (eBioscience) mAb for 20 min at 4°C. After being washed with PBS, the stained cells (live-gated on the basis of forward and side scatter profiles) were analyzed on a FACSCalibur (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences).

Cytokine ELISA

After stimulation for 24 h, culture supernatants were recovered by centrifugation at 490 × g for 5 min. Cytokine concentrations were measured with ELISA kits (Ready-SET-Go (eBioscience) for IL-23 and Quantikine or DuoSet (R&D Systems) for other cytokines).

Quantitative PCR

After stimulation for 3 h, total RNA was extracted from DCs using an RNeasy Plus Micro Kit (Qiagen). First-strand cDNA was synthesized from total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed with the TaqMan method using an ABI 7500 (Applied Biosystems). The mRNA levels of the target gene were normalized to the gene expression of GAPDH and are shown as relative expression levels to the control group.

Intranasal administration of Cupressaceae family pollen grains to mice

C57BL/6 or BALB/c mice were lightly anesthetized with an i.p. injection of pentobarbital (Nembutal; Abbott Laboratories) and allowed to inhale 20 μl of the Japanese cedar or Japanese cypress pollen grain suspension with or without OVA (grade V; Sigma-Aldrich) following application to the nares with a pipette twice per week for 6 wk for a total of 13 administrations. The day after the last intranasal administration, sera and bronchoalveolar lavage (BAL) cells were collected.

Bronchoalveolar lavage

At 24 h after the last intranasal administration, mice were terminally anesthetized, the tracheas were cannulated, and internal airspaces were lavaged with 500 μl of PBS with 10% FCS, followed by another 500-μl wash. Fluids were centrifuged at 1200 × g and the pellets were recovered for cellular analysis. Specimens were prepared on glass slides by Cytospin 4 (Thermo Shandon) followed by Diff-Quick (Sysmex) staining. Differential cell counts were performed with a minimum of 200 cells.

ELISA for serum total IgE and OVA-specific Abs

Serum total IgE was measured by a sandwich ELISA as described previously (38). OVA-specific Abs were detected on plates coated with 1 mg/ml OVA and blocked with BlockAce (Snow Bland) and developed with HRP-conjugated Abs specific to the murine IgE and IgG subclasses as described previously (39, 40) with some modifications as follows. Sera and detection Abs were diluted with solutions 1 and 2 of CanGetSignal (Toyobo), respectively. Serum dilutions were 1/200, 1/40,000, 1/200, and 1/4,000 for detecting OVA-specific IgE, IgG1, IgG2a, and IgG2b, respectively. For detecting OVA-specific IgE, incubation with serum or a detection Ab was for 15 h at 4°C or for 5 h at room temperature, respectively. For detecting OVA-specific IgGs, incubation with serum or a detection Ab was for 1.5 h at 37°C.

Statistical analysis

A one-way ANOVA with the Tukey post hoc test and the Mann-Whitney U test (two-tailed) were used to analyze the data obtained in the in vitro and in vivo experiments, respectively. A value of *p* < 0.05 was regarded as statistically significant.

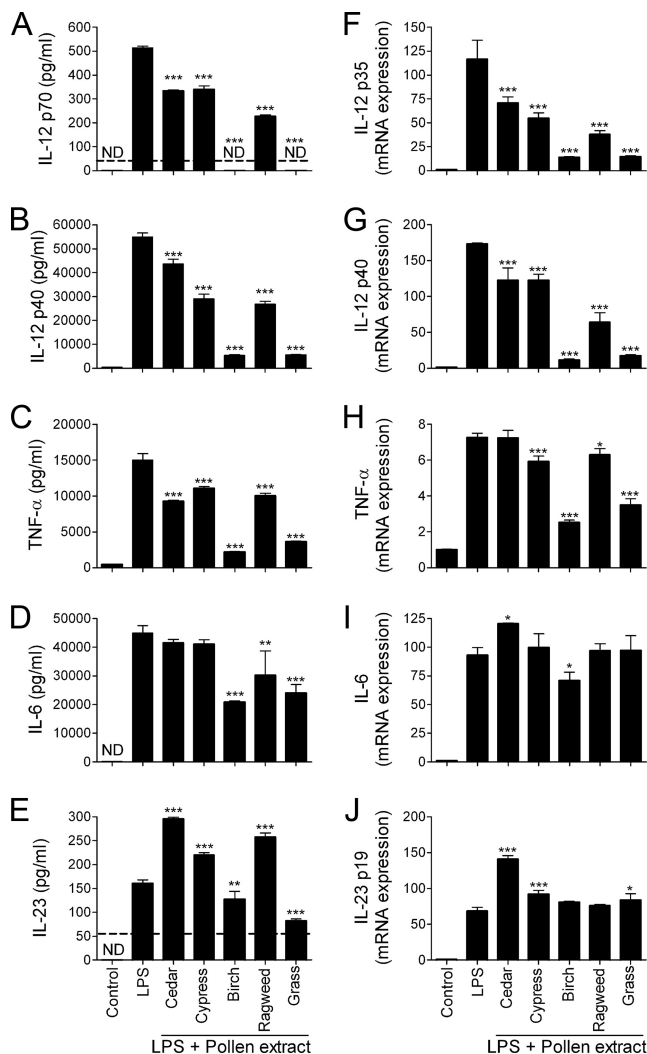


FIGURE 1. Effect of pollen extracts on LPS-induced cytokine expression in DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated with LPS (Sigma-Aldrich) (100 ng/ml) in the absence or presence of pollen extracts. *A–E*, At 24 h, culture supernatants were collected and cytokine concentrations were measured by ELISA. *F–J*, At 3 h, DCs were collected and mRNA expression was examined by quantitative real-time PCR. ND, Not detected. Broken line indicates minimum detection limit. Data are indicated as the means $\pm$ SD for three wells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs cells stimulated with 100 ng/ml LPS alone by one-way ANOVA with the Tukey post hoc test. Data shown are representatives of three independent experiments with similar results.

Results

Pollen extracts modulated LPS-induced cytokine production by DCs

We examined the effect of extracts containing substances released from pollen on the response of DCs to LPS. LPS-induced cytokine production in DCs was analyzed (Fig. 1). The extracts of birch and grass pollen diminished the LPS-induced production of IL-12 (IL-12 p70), which is a heterodimer composed of the IL-12 p40 and IL-12 p35 subunits (Fig. 1A), and inhibited IL-12 p40 and TNF- α remarkably (Fig. 1, B and C) and IL-6 moderately (Fig. 1D). The pollen extracts other than those of birch and grass moderately inhibited the LPS-induced production of IL-12 p70, IL-12 p40, and TNF- α (Fig. 1, A–C). Pollen extracts exhibited little or no inhibition of the LPS-induced production of IL-23, a heterodimer composed of the IL-12 p40 and IL-23 p19 subunits (Fig. 1E). The

results for protein levels (Fig. 1, A–E) were supported by the analyses at the mRNA level (Fig. 1, F–J). LPS-induced DC maturation in terms of the expression of CD80, CD86, and a MHC class II molecule (I-A^b) was not affected by the addition of pollen extracts (data not shown).

In the absence of LPS, the pollen extracts had no effect on the production of the cytokines examined, although they slightly enhanced the cell surface expression of a MHC class II molecule, and Japanese cedar or Japanese cypress pollen extracts marginally enhanced CD80 expression (data not shown). Endotoxin amounts released into the pollen extract samples were 26, 32, 2.3, 440, and 510 pg of endotoxin from 1 mg of pollen grains of Japanese cedar, Japanese cypress, birch, ragweed, and grass, respectively. Therefore, endotoxin concentrations in the DC stimulation, where the pollen extracts originally prepared at 10 mg of grains/ml were used at the dilution factor of 5 (2 mg of grains/ml), were 52, 64, 4.6, 870, and 1000 pg of endotoxin/ml, respectively.

The viabilities of DCs after the 24-h culture with LPS, pollen extracts, or both, or without them, were similarly $\sim 95\%$ (data not shown), indicating that the pollen extracts showed no toxicity at the concentrations tested and suggesting that the inhibitory effect of birch and grass pollen extracts (Fig. 1), particularly on the IL-12 p70 production, is not due to their cytotoxicity.

Thus, soluble substances released from pollen grains inhibit the LPS-induced production of IL-12 p70 in DCs with an efficiency that differs among plant species, and the effect was greatest for birch and grass pollen extracts. These substances themselves, at least at the concentration tested, have little or no activity to induce DC maturation or cytokine production.

Pollen grains promoted DC maturation in the absence of LPS

To mimic the contact of DCs with pollen grains in peripheral tissues during the initial sensitization process, we tested the effect of pollen on immature DCs in the absence of LPS (Figs. 2 and 3 and supplemental Fig. S1).⁴ Pollen grains induced the cell-surface expression of CD80, CD86, and a MHC class II molecule (Fig. 2A). Pollen of Japanese cedar and Japanese cypress showed maturation-inducing activity at much lower doses than pollen of birch, ragweed, and grass (Fig. 2B).

The viabilities of DCs after the 24-h culture with or without the pollen grains were similarly $\sim 95\%$ with an exception of 2.5 mg of birch pollen grains/ml that showed a low viability ($\sim 25\%$) (data not shown), indicating that the pollen grains other than birch at the highest density and birch at lower densities (0.5 mg of grains/ml and 0.1 mg of grains/ml) showed no toxicity.

Pollen grains induced cytokine production in DCs in the absence of LPS

Next, we analyzed the production of proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) and cytokines related to the differentiation of Th cells (IL-12 and IL-23) (Fig. 3 and supplemental Fig. S1). Pollen grains induced production of TNF- α , IL-6, and IL-1 β (Fig. 3, A–C). Similar to the results of the analysis of DC maturation (Fig. 2), Cupressaceae family (Japanese cedar and Japanese cypress) pollen had a strong stimulatory effect at much lower doses than did the other pollen species tested (Fig. 3, A–C). Birch pollen grains induced a lesser response (Fig. 3 and supplemental Fig. S1). None of the pollen grains induced IL-12 p70 production (Fig. 3D), although all of the species induced production of IL-12 p40 (Fig. 3E and supplemental Fig. S1). Pollen grains induced production of IL-23 (Fig. 3F) in accordance with expression of the IL-12 p40 subunit (Fig. 3E).

⁴ The online version of this article contains supplemental material.

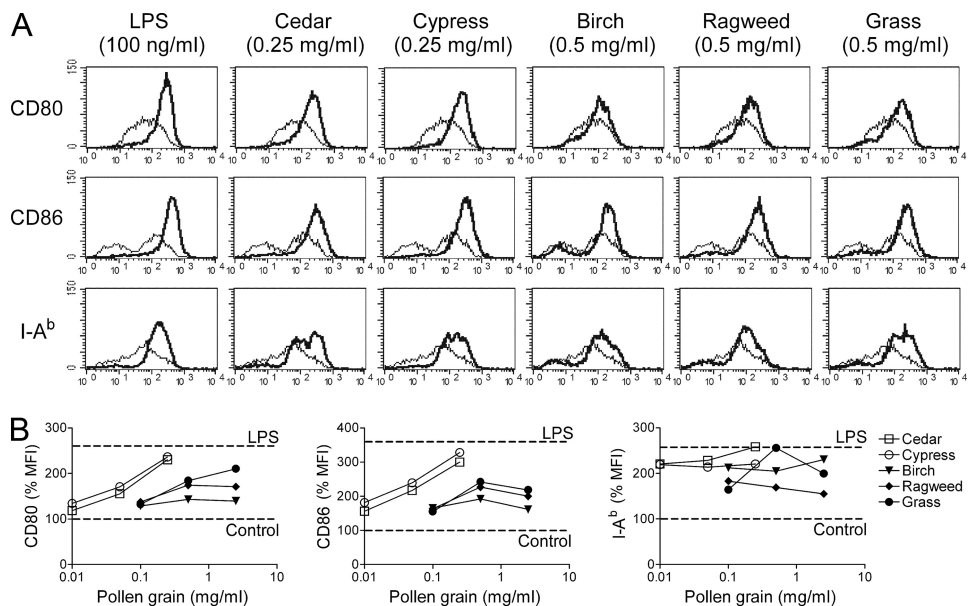


FIGURE 2. Effect of pollen grains on maturation of DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cell-surface expression of costimulatory molecules (CD80 and CD86) and a MHC class II molecule (I-A^b) were analyzed by flow cytometry. **A**, Histograms of pollen grain- or LPS-stimulated cells (bold lines) are overlaid on histograms of control cells (fine lines). The histograms indicated are gated on the CD11c⁺ population. **B**, Relative cell-surface expression indicated as a percentage of mean fluorescence intensity (MFI). Values were calculated as follows: (% MFI) = 100 × (MFI of stimulated cells/MFI of control cells without stimulation). LPS indicates cells stimulated with LPS (Sigma-Aldrich) (100 ng/ml); broken lines, values of negative (Control) and positive (LPS) controls. Data shown are representatives of three independent experiments with similar results.

Endotoxin contents in the pollen grains were 100, 260, 23, 1500, and 1100 pg of endotoxin/mg of pollen grains of Japanese cedar, Japanese cypress, birch, ragweed, and grass, respectively.

Pollen grains induced maturation and cytokine production in TLR4-deficient DCs

In TLR4-deficient DCs, pollen grains other than birch induced maturation (Fig. 4) and cytokine production but not IL-12 p70 production (Fig. 5), and LPS induced no response (Figs. 4 and 5, LPS). Pollen of Japanese cedar and Japanese cypress induced the similar levels of DC maturation (Fig. 4) and ~50–100% production of TNF- α , IL-12 p40, and IL-23 (Fig. 5, A, E, and F), and <50% but significant production of IL-6 and IL-1 β (Fig. 5, B and C) compared with those in wild-type DCs (Figs. 2 and 3 and supplemental Fig. S1), indicating that most of the

DC-maturing activity and the activity to induce TNF- α , IL-12 p40, and IL-23 and a part of the activity to induce IL-6 and IL-1 β are not via the TLR4 pathway in Japanese cedar and Japanese cypress.

Pollen of ragweed and grass induced the similar levels of DC maturation (Fig. 4) and lower cytokine production (Fig. 5) compared with those in wild-type DCs (Figs. 2 and 3 and supplemental Fig. S1), indicating that the TLR4 pathway is dispensable for the DC maturation but partially contributes to the cytokine response in ragweed and grass. Endotoxin concentrations in the DC stimulation were 25 and 65 pg of endotoxin/ml for 0.25 mg of pollen grains/ml in Japanese cedar and Japanese cypress and 3800 and 2800 pg of endotoxin/ml for 2.5 mg of pollen grains/ml in ragweed and grass. The higher endotoxin concentrations in ragweed and grass at the tested pollen grain

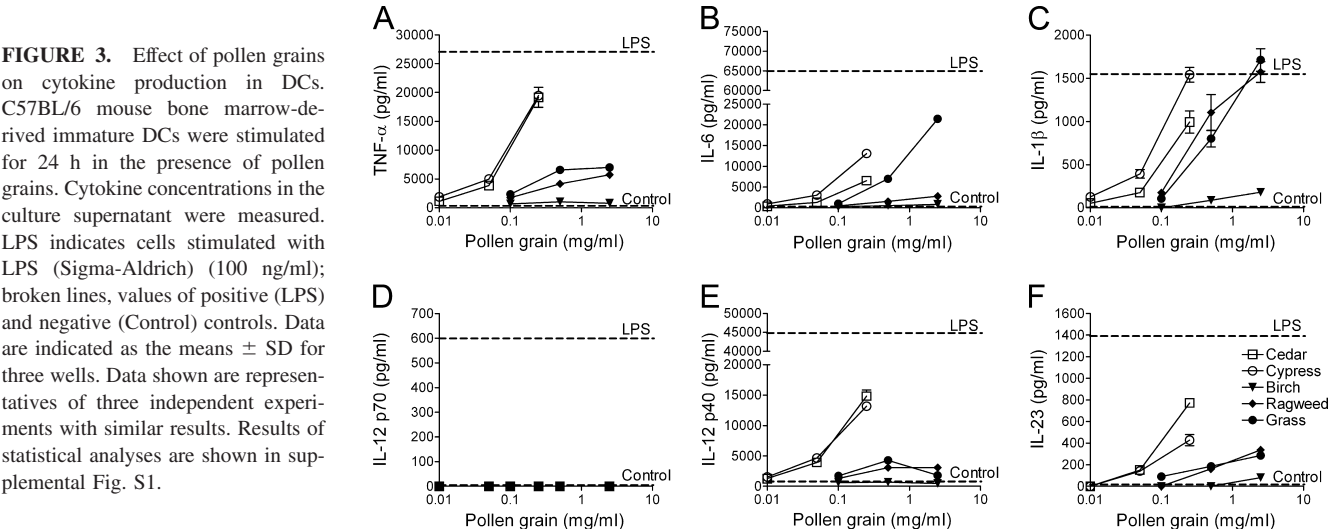
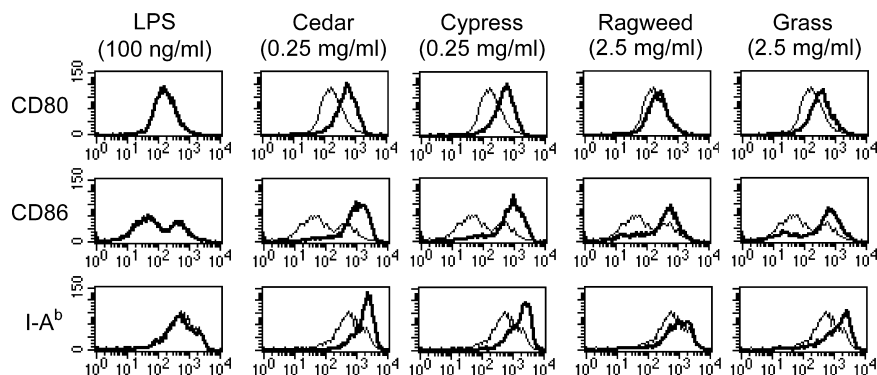


FIGURE 3. Effect of pollen grains on cytokine production in DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cytokine concentrations in the culture supernatant were measured. LPS indicates cells stimulated with LPS (Sigma-Aldrich) (100 ng/ml); broken lines, values of positive (LPS) and negative (Control) controls. Data are indicated as the means $\pm$ SD for three wells. Data shown are representatives of three independent experiments with similar results. Results of statistical analyses are shown in supplemental Fig. S1.

FIGURE 4. Effect of pollen grains on maturation of TLR4-deficient DCs. TLR4-deficient mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cell-surface expression of costimulatory molecules (CD80 and CD86) and a MHC class II molecule (I-A^b) were analyzed by flow cytometry. Histograms of pollen grain- or LPS (List Biological Lab)-stimulated cells (bold lines) are overlaid on histograms of control cells (fine lines). The histograms indicated are gated on the CD11c⁺ population.



dose could explain the partial contribution of the TLR4 pathway to the cytokine response in ragweed and grass (Figs. 3 and 5 and supplemental Fig. S1).

Inhalation of Cupressaceae pollen grains induced increases in serum IgE and airway eosinophil infiltration

Pollen grains of Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, induced DC maturation (Fig. 2) and cytokine production (Fig. 3 and supplemental Fig. S1) more efficiently than did pollen of the other plant species tested. Next, we examined the effect of Cupressaceae pollen on IgE induction and airway eosinophil infiltration in vivo (Figs. 6 and 7).

In C57BL/6 mice, inhalation of Japanese cedar pollen grains resulted in an elevation of serum total IgE levels (Fig. 6A) and the infiltration of inflammatory cells, mainly eosinophils, into the airway (Fig. 6B). Similar results were obtained in BALB/c mice (Fig. 6, C and D). Infiltration of macrophages/monocytes was also observed in BALB/c mice exposed to Japanese cedar pollen (data not shown). Inhalation of Japanese cypress pollen grains induced similar effects (data not shown).

Coadministration of Cupressaceae pollen grains with OVA induced an OVA-specific IgE response in vivo

In C57BL/6 mice, while the administration of OVA alone induced little or no elevations of total IgE and OVA-specific Abs and air-

way eosinophil infiltration (Fig. 7A–C, OVA), coadministration of Japanese cedar pollen grains with OVA (Fig. 7A–C, Cedar + OVA) induced elevations of serum total IgE and OVA-specific IgE (Fig. 7A) and IgG (Fig. 7B) levels, which were associated with airway eosinophil infiltration (Fig. 7C). Similar results were obtained in BALB/c mice (Fig. 7D–F) except for OVA-specific IgG2a. Coadministration of Japanese cypress pollen grains with OVA induced similar effects (data not shown).

Discussion

Exposure to pollen or house dust mite triggers a Th2-skewed immune response toward allergic diseases, which are associated with IgE production and eosinophilic inflammation. Although little is known about the initial sensitization process after first contact with innocuous environmental allergens, recent studies have suggested that molecules produced by allergen-producing organisms are involved in the pathogenesis through sensitization and/or exacerbation via IgE-independent mechanisms and the modification of IgE-dependent responses (8–30, 41–43). In the present study, we compared the response of DCs to pollen of different plant species. We found that pollen grains of members of the Cupressaceae family, Japanese cedar and Japanese cypress, exhibited the greatest stimulatory effect (Figs. 2 and 3 and supplemental Fig. S1), for which the TLR4 is dispensable (Figs. 4 and 5), while birch and grass pollen extracts exhibited the most prominent inhibition of

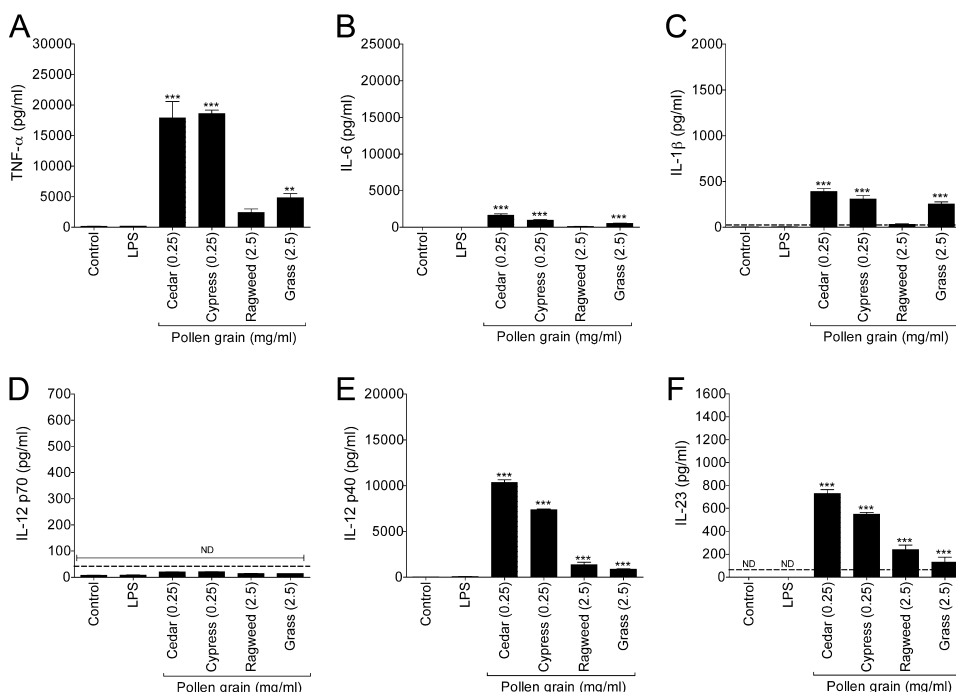


FIGURE 5. Effect of pollen grains on cytokine production in TLR4-deficient DCs. TLR4-deficient mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cytokine concentrations in the culture supernatant were measured. LPS indicates cells stimulated with LPS (List Biological Lab) (100 ng/ml); ND, not detected; broken line, minimum detection limit. Data are indicated as the means $\pm$ SD for three wells. **, $p < 0.01$ and *** $p < 0.001$ vs control by one-way ANOVA with the Tukey post hoc test.

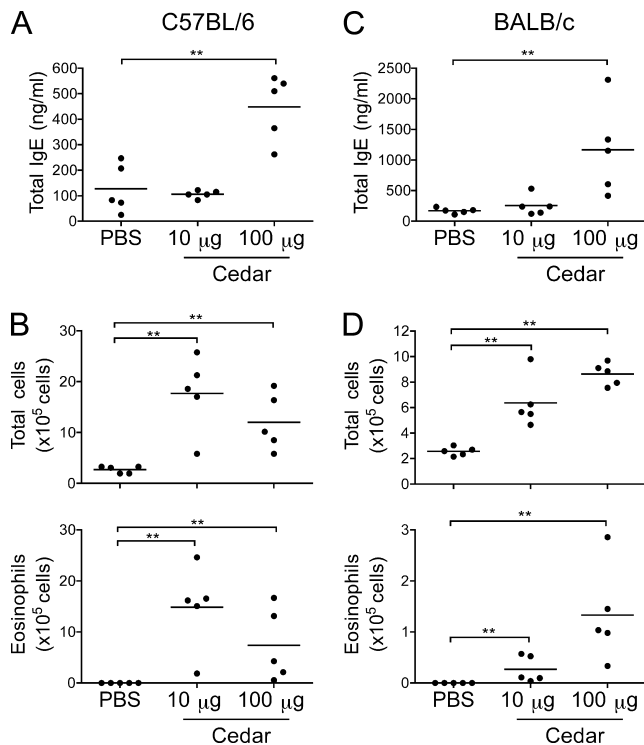


FIGURE 6. Induction of IgE production and airway eosinophil infiltration by inhalation of Japanese cedar pollen grains. C57BL/6 (A and B) or BALB/c (C and D) mice were intranasally administered with Japanese cedar pollen grains (10 or 100 $\mu\text{g}/\text{head}$) twice a week for 6 wk. Serum and BAL fluid were collected at 1 day after the last intranasal administration. A and C, Serum total IgE. B and D, Total number of cells and number of eosinophils in BAL fluid. Data are values for five mice per group. Bars indicate means. **, $p < 0.01$ vs PBS by the Mann-Whitney U test. Data shown are representatives of three independent experiments with similar results.

LPS-induced IL-12 (IL-12 p70) production (Fig. 1). Furthermore, we demonstrated that inhaled Cupressaceae pollen grains increased levels of serum IgE and airway eosinophilic infiltration (Fig. 6) and had adjuvant activity to induce production of IgE specific to coadministered OVA in mice (Fig. 7).

A research group reported that birch pollen extract reduced LPS-induced IL-12 p70 production in DCs, leading to Th2-biased priming of naive CD4^+ T cells in both human (16) and mouse (35) in vitro systems. Consistent with their results, birch pollen extract strongly suppressed the LPS-induced production of IL-12 p70 and p40 at the protein level (Fig. 1A), although a difference was observed between the murine bone marrow-derived DCs used in the present study and human monocyte-derived DCs (16), namely, the birch pollen extract inhibited both IL-12 p40 and p35 mRNA expression in the present study (Fig. 1) while it inhibited p40 but not p35 mRNA expression in the human monocyte-derived DCs (16). We demonstrated that grass pollen also inhibited LPS-induced IL-12 production. Birch and grass pollen extracts inhibited LPS-induced production of $\text{TNF-}\alpha$ significantly and IL-6 moderately. Pollen extracts of Japanese cedar, Japanese cypress, and ragweed showed moderate suppression of LPS-induced IL-12 p70 and $\text{TNF-}\alpha$ production, although the possibility that they could show more effective suppression if concentrated cannot be excluded. These results suggest that pollen-derived substances released into pollen extracts act as suppressors of immune responses by inhibiting production of IL-12 p70, $\text{TNF-}\alpha$, and so on, although the extent of the effect differs among the plant families.

Stimulation of DCs with pollen grains in the absence of LPS promoted the maturation of DCs (Fig. 2) and induced the production of cytokines (Fig. 3A–C), but not the secretion of IL-12 p70 (Fig. 3D). Pollen grains of Japanese cedar and Japanese cypress, part of the Cupressaceae family, exhibited the greatest capacity to induce DC responses even at lower doses compared with the other pollen species tested (Figs. 2 and 3 and supplemental Fig. S1). As stimulation with pollen extracts alone had little effect on the response (data not shown), contact with the surface of pollen grains seemed crucial in the induction of maturation and cytokine production. Grass and ragweed pollen-induced maturation of human monocyte-derived DCs has been reported to be contact-dependent (44). APCs, including DCs, express a diversity of surface receptors, such as scavenger receptors, mannose receptors, and C-type lectins, for binding to exogenous ligands (45, 46). In addition to these receptors, the interaction of CD1 molecules on human monocyte-derived DCs and phospholipids on the surface of pollen has been reported (18). The mechanisms behind the induction of the DC responses by pollen grains (Figs. 2 and 3) should be addressed in future studies.

The exposure of DCs to pollen grains induced no detectable production of IL-12 p70 (Fig. 3D), but it significantly induced the production of IL-12 p40 (Fig. 3E). The p40 subunit seemed to contribute to the formation of IL-23, a heterodimer of the IL-12 p40 and IL-23 p19 subunits (Fig. 3F). IL-23 and IL-6 are cytokines involved in the differentiation of Th17 cells (47). Although it was reported that serum IgE levels and airway eosinophil infiltration were reduced in IL-17 receptor-deficient mice (48), several studies indicated that IL-17 is related to neutrophil-dominant, rather than eosinophil-dominant, inflammation (47, 49). DCs stimulated with pollen grains produced both IL-23 and IL-6 (Fig. 3, B and F). Whether the inhalation of pollen grains leads to Th17 cell-mediated inflammation via the stimulation of DCs is yet to be investigated.

In Japan, a common seasonal allergic disease posing a major public health problem is caused by inhalation of Cupressaceae pollen of Japanese cedar and Japanese cypress, affecting $\sim 20\%$ of the total population (2, 3). Cupressaceae pollen has also been identified as a source of pollinosis in Mediterranean countries (2, 50) and the United States (51, 52). Large studies on unselected young adults in France and Italy estimated the prevalence of allergies to cypress pollen to be $\sim 2.4\text{--}8\%$ of the general population (1, 2, 50). As Cupressaceae pollen exhibited the greatest stimulatory effect on DCs, even at lower doses (Figs. 2 and 3), we examined their effect in vivo using mice (Figs. 6 and 7). Inhalation of Cupressaceae pollen grains increased serum IgE levels and airway eosinophil infiltration (Fig. 6). The coadministration of OVA with Cupressaceae pollen grains induced increases in OVA-specific IgE and IgGs associated with airway eosinophil infiltration (Fig. 7). The lack of IL-12 p70 production by DCs in vitro (Fig. 3) suggests a contribution to the elevation in levels of IgE (Fig. 7, A and D), IgG1, and IgG2b rather than IgG2a (Fig. 7, B and E) in mice exposed to pollen of the Cupressaceae family. The results indicate that pollen grains of the Cupressaceae family have adjuvant activity in vivo that promotes Ag-specific IgE production associated with airway eosinophil infiltration. As far as we know, this is the first demonstration of in vivo IgE-inducing adjuvant activity of inhaled pollen-derived substances without using alum.

Hashiguchi et al. (53) reported that artificial exposure to Japanese cedar pollen at 2500 pollen grains/ m^3 , which is equivalent to the airborne pollen amount in the early pollinating season, caused penetration of 250 and 14 grains/h to the nose and eyes in humans. Airborne pollen amount during the middle and late pollinating season is ~ 5000 pollen grains/ m^3 (53) and it could be estimated to

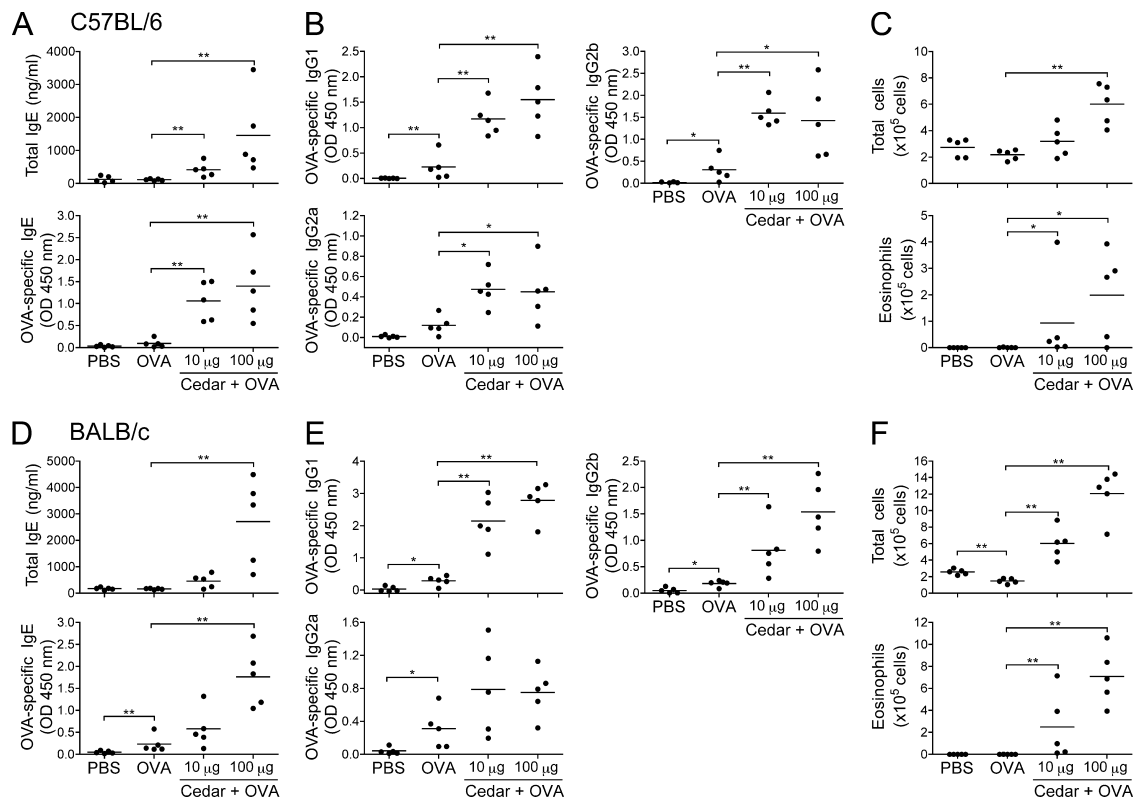


FIGURE 7. In vivo IgE-inducing adjuvant effect of Japanese cedar pollen grains. C57BL/6 (A–C) or BALB/c (D–F) mice were intranasally administered with OVA (25 µg/head) and Japanese cedar pollen grains (10 or 100 µg/head) twice a week for 6 wk. Serum and BAL fluid were collected at 1 day after the last intranasal administration. A and D, Serum total IgE and OVA-specific IgE. B and E, OVA-specific IgGs. C and F, Total number of cells and number of eosinophils in BAL fluid. Data are values for five mice per group. Bars indicate means. *, $p < 0.05$ and **, $p < 0.01$ vs OVA by the Mann-Whitney U test. Data shown are representatives of three independent experiments with similar results.

cause penetration of 500 grains/h to the nose, which is equivalent to 5 µg of grains/h (13, 54). Therefore, everyday exposure for 3 h/day could achieve inhalation of 100 µg of grains/wk in the middle and late pollinating season. In mice, administration of 20 µg of grains/wk with OVA exhibited adjuvant activity to induce OVA-specific IgE (Fig. 7, A and D) and eosinophil infiltration (Fig. 6, B and D, and Fig. 7, C and F). In our preliminary experiments, lower dose administration of 2 or 0.2 µg of grains/wk without OVA-induced eosinophil infiltration in mice (data not shown), suggesting the relevance of the natural exposure during the pollinating season to the sensitization toward Cupressaceae pollen allergy in humans. Although administration of 20 µg (or less) of grains/wk without OVA did not increase serum total IgE levels (Fig. 6, A and C, 10 µg; and data not shown), it does not exclude the possibility that Ag-specific IgE was increased (39). An assay system for IgE specific to pollen-derived Ags with high sensitivity should be established in a future study.

In summary, the results of the present study suggest that the modulation of DC responses to pollen differs among plant families in two ways: (1) the promotion of maturation and cytokine production through direct contact, which is greatest for Cupressaceae pollen, and (2) the inhibition of IL-12 production by soluble factors, which is greatest for birch and grass pollen. The strong stimulatory effect on DCs in vitro and IgE-inducing adjuvant activity in mice supports the clinical relevance of Cupressaceae pollen to allergies in humans, which are prominent in diverse geographic areas (2, 3, 50–52). Pollen grains contain various releasable or insoluble substances including lipids (12–18), proteases (13, 19, 30), NADPH oxidase (8–11), subpollen particles (10), and starch granules (55), which could be involved in the pathogenesis of allergic

diseases. Mechanisms of the induction of DC responses by pollen and induction of Th2-inducing cytokines in other types of cells (22, 56, 57) by pollen-derived substances should be addressed in future studies.

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Disclosures

The authors have no financial conflicts of interest.

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1 **Legend of Supplementary Figure S1**

2

3 **Supplementary Figure S1. Effect of pollen grains on cytokine production in DCs.**

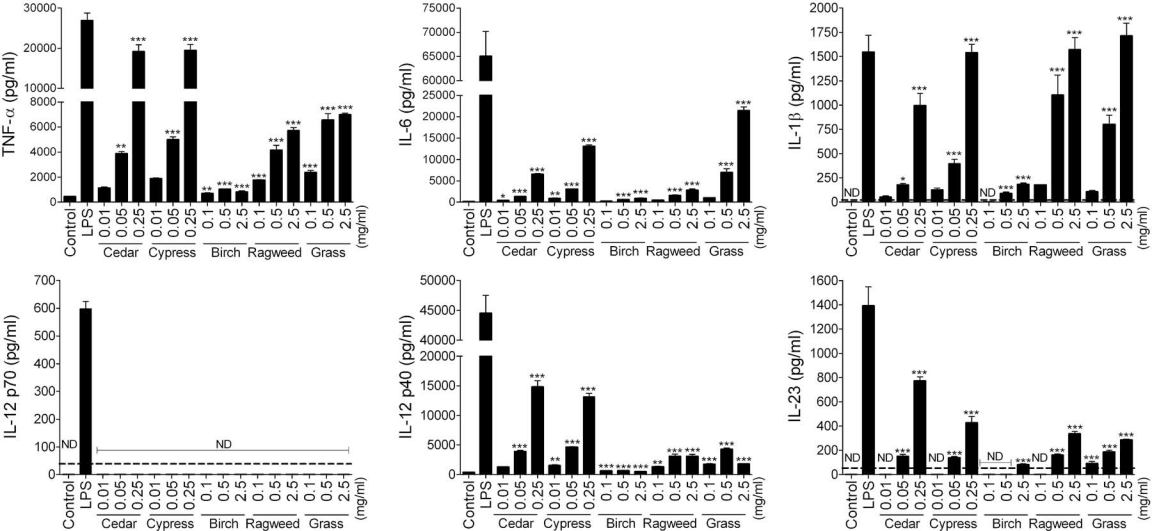
4 Data shown in **Fig. 3** are shown here as bar graphs and statistical analyses were

5 performed. Data are indicated as the mean $\pm$ SD for three wells. * $p < 0.05$, ** $p <$

6 0.01, *** $p < 0.001$ versus *Control* by one-way ANOVA with the Tukey *post hoc* test

7 among the four groups including *Control* and the three suspensions of each pollen.

8 Data shown are representatives of three independent experiments with similar results.



Kamijo, S. et al., SUPPLEMENTARY FIGURE S1