Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells

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Abstract

Background: B cells play a central role in IgE-mediated allergies. In damaged airway epithelium, they are exposed directly to aeroallergens. We aimed to assess whether direct exposure of B cells to pollen constituents affects allergic sensitization.

Methods: B cells from murine splenocytes and from blood samples of healthy donors were incubated for 8 days under Th2-like conditions with aqueous ragweed pollen extracts (Amb-APE) or its constituents. Secreted total IgM, IgG, and IgE was quantified by ELISA. Additionally, birch, grass, or pine-pollen extracts were tested. The number of viable cells was evaluated by ATP measurements. B-cell proliferation was measured by CFSE staining. IgE class switch was analyzed by quantitation of class switch transcripts. In an OVA/Alum i.p.-sensitization mouse model, Amb-APE was intranasally instilled for 11 consecutive days.

Results: Upon Th2 priming of murine B cells, ragweed pollen extract caused a dose-dependent increase in IgE production, while IgG and IgM were not affected. The low-molecular-weight fraction and phytoprostane E1 (PPE1) increased IgE production, while Amb a 1 did not. PPE1 enhanced IgE also in human memory B cells. Under Th1 conditions, Amb-APE did not influence immunoglobulin secretion. The IgE elevation was not ragweed specific. It correlated with proliferation of viable B cells, but not with IgE class switch. *In vivo*, Amb-APE increased total IgE and showed adjuvant activity in allergic airway inflammation.

Conclusions: Aqueous pollen extracts, the protein-free fraction of Amb-APE, and the pollen-contained substance PPE1 specifically enhance IgE production in Th2-primed B cells. Thus, pollen-derived nonallergenic substances might be responsible for B-cell-dependent aggravation of IgE-mediated allergies.

Abbreviations
Amb, Ambrosia; APE, aqueous pollen extract; BAL, bronchoalveolar lavage; Bet, Betula; CSR, class switch recombination; OVA, ovalbumin; PGE2, prostaglandin E2; Phl, phleum; Pin, pinus; PPE1, phytoprostane E1; Th2, T helper cell 2.

Immunoglobulin E (IgE) is a fundamental player in atopic diseases and a hallmark of allergic sensitization (1). Upon type 2 T-cell help, B cells proliferate, undergo immunoglobulin isotype class switch recombination (CSR) toward IgE, and differentiate into antibody-secreting plasma cells. IgE is necessary for immediate phase reactions like mast cell and basophil degranulation. In damaged airway epithelium, B

cells can be exposed directly to aeroallergens, and despite their central role in allergic diseases, little is known about how B lymphocytes react upon exposure to pollen constituents.

In the past, we have shown that pollen grains not only are carriers of allergen, but also contain bioactive pollen-associated lipid mediators (PALMs) and other immunomodulatory substances (2). Therefore, allergic sensitization is not only due to genetic predisposition and molecular features of allergens, but is also facilitated by exposure to environmental cofactors that break immunotolerance (3–5): (i) Proinflammatory PALMs (e.g. oxylipins) exert chemoattractive effects on human neutrophils (6) and eosinophils (7) *in vitro*. (ii) Immunomodulatory PALMs such as E1 phytoprostanes generate a Th2-favouring milieu that is inhibitory for the synthesis of Th1-type chemokines by human dendritic cells (8–10). (iii) NADPH oxidases generate reactive oxygen species that damage airway epithelium and increase allergic lung inflammation (11, 12).

IgE is not only produced centrally, but also locally, and B cells undergo IgE CSR in nasal and mucosal tissue (13, 14). The concept of 'local allergic rhinitis' (LAR) with local IgE production in the airway mucosa suggests to analyze the direct influence of pollen extracts on B lymphocytes (15, 16). CSR requires the deletion of genomic DNA, containing constant gene regions of the IgM/IgG heavy chain, which is mediated by activation-induced cytidine deaminase (AID) and the sterile ε-germline transcript (17–19). IgE CSR results in an ε-postswitch transcript, a precursor of IgE (20–22). An unresolved question is whether adjuvants can facilitate CSR and thereby aggravate allergic diseases. Therefore, we analyzed the impact of pollen-derived extracts on T-cell-initiated CSR and subsequent IgE production.

The pollen extracts we used in our study originated from the highly allergenic plant species ragweed (*Ambrosia artemisiifolia*), birch (*Betula verrucosa*), and timothy grass (*Phleum pratense*), and from less allergenic pine (*Pinus strobus*). Additionally, protein-free fraction (<3 kDa) of ragweed pollen extract, which contains PALMs and other low-molecular-weight molecules, was generated (23–25).

In this study, we demonstrate that pollen extracts from various plant species enhance Th2-induced production of total IgE. Due to their capacity to specifically increase IgE-producing B cells, pollen-derived E1 phytoprostane might be a candidate substance responsible for the aggravation of IgE production. The IgE-adjuvant activity of ragweed pollen extract was verified *in vivo* by exacerbation of a mouse model of OVA-induced allergic lung inflammation.

Materials and methods

Preparation of pollen extracts

Aqueous pollen extracts (APE) of ragweed, birch, grass, and pine were generated from pollen grains (Allergon, Ängelholm, Sweden) incubated in PBS (10 mg/ml) for 30 min at 37°C. After pelleting (20 min at 3.345 g), supernatants were sterile-filtered (0.2 μm; Millipore, Schwalbach, Germany). To obtain a protein-free fraction, the extracts were passed three times over a 3-kDa cutoff filter (Amicon Ultra YM3; Millipore). The absence of proteins in Amb-APE <3 kDa was verified by Coomassie Assay (Invitrogen, Karlsruhe, Germany). LPS was measured by LAL assay and was below 1 EU/ml. Natural Amb a 1 was purified by ion exchange chromatography. See Data S1 for more details.

Animals

Female, 6- to 10-week-old C57BL/6 and BALB/c mice were obtained from Charles River (Sulzfeld, Germany), housed under specific pathogen-free conditions in individually ventilated cages (VentiRack; BioZoneGlobal Ltd., Ramsgate, UK), and fed by standard pellet diet and water *ad libitum*. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (Munich, Germany).

Sensitization protocol

An established OVA-sensitization model was used (26, 27). Briefly, 6-week-old BALB/c mice were sensitized by intraperitoneal injections of 1 μg OVA (grade VI; Sigma-Aldrich, Munich, Germany) in 200 μl PBS adsorbed to 2.5 mg aluminum hydroxide (alum) (Thermo Fischer Scientific, Waltham, MA, USA) on days 0, 7, 14, and 42. Nonsensitized (NS) mice received PBS only in alum. From day 43 onward, mice were instilled on eleven consecutive days with 20 μl APE of ragweed (10 mg/ml) or PBS, followed by one aerosol challenge on day 52 for 20 min with 1% ovalbumin delivered by a Pari-Boy nebulizer (Pari, Starnberg, Germany). Bronchoalveolar lavage (BAL) analysis was performed 24 h (day 53) or 7 day (day 59) after OVA challenge. Blood samples were taken before sensitization and at the end of the experiment.

Analysis of bronchoalveolar lavage, lung histology, and serology

Bronchoalveolar lavage, lung histology, and immunoglobulin determination in plasma were performed as described previously (26, 27). See Data S1 for more details.

Murine B-cell isolation, culture, and stimulation

Spleens were taken aseptically from female, 7- to 10-week-old C57BL/6 mice, and single-cell suspensions were generated. Resting (mainly naïve) B cells were isolated by untouched magnetic cell sorting (MACS, B-cell Isolation Kit; Miltenyi, Bergisch Gladbach, Germany). Purity (anti-B220 staining) reached at least 97%. B cells were cultured in complete RPMI containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, 1 mM minimum essential media (MEM) sodium pyruvate, 0.1 mM

MEM nonessential amino acids, 20 mM HEPES, and 10% FCS (PAA, Coelbe, Germany) in triplicates during polyclonal stimulation with 5 μg/ml anti-CD40 mAb (eBioscience, San Diego, CA, USA) and 25 ng/ml murine IL-4 (PeproTech, Rocky Hill, NJ, USA) for a Th2-like condition or 25 ng/ml IFN-γ (PeproTech) for a Th1-like condition. Additionally, B cells were exposed to pollen extracts, Amb a 1, PPE1, or PGE2 for 8 days.

Human B-cell isolation, culture, and stimulation

Peripheral blood mononuclear cells were isolated from peripheral venous blood of healthy donors (commercially available buffy coats) by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation. Total $CD19^+$ B cells were isolated using untouched MACS sorting (B-cell Isolation Kit II human; Miltenyi). Purified B cells were stained with anti-CD19-APC-Cy7 and anti-CD27-PE (Biolegend, San Diego, CA, USA), and naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B-cell subsets were sorted using FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). B cells were cultured in RPMI1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME (GIBCO BRL, Basel, Switzerland), and 10% heat-inactivated FCS (Sera-Lab Ltd., Sussex, UK).

A total of 1000 naïve B cells and 5000 memory B cells were stimulated with human CD40-ligand-presenting mouse fibroblasts, 25 ng/ml human IL-21 (Novartis, Basel, Switzerland) and human IL-4 (Novartis), and increasing concentrations of Amb-APE or PPE1 for 12 days. To optimize the induction of IgE in our experiments, a titration of naïve and memory B cells was done prior to the stimulation experiment starting with 25 000 cells down to 195 cells per well in a 96-well plate (Fig. S2) (28).

Immunoglobulin determination

Total IgE, IgG, and IgM in cell culture supernatants were measured by ELISA (Jackson ImmunoResearch, West Grove, IA, USA), according to the manufacturer's instructions. See Data S1 for more details.

Murine B-cell proliferation and apoptosis assays

B-cell count was assessed by measuring intracellular ATP (CellTiter-Glo®; Promega Inc., Mannheim, Germany). Intracellular ATP concentration was correlated with viable B-cell count (trypan blue-negative cells in Neubauer chamber; data not shown). Proliferation was tested by CFSE dilution (flow cytometry) and apoptosis by annexin V and 7-AAD staining. Percentage of apoptotic single annexin V-positive cells was determined by flow cytometry.

RNA extraction and real-time PCR

Total RNA extraction of murine B cells and real-time PCR were performed according to the manufacturer's instructions (RNeasy Mini Kit; Qiagen, Hilden, Germany). Expression of IgE class switch-associated transcripts AID (activation-induced cytidine deaminase), ε-GLT (germline transcript), and ε-PST (postswitch transcript) was assessed. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as housekeeping gene. Primer sequences are listed in Table S1.

Data analysis

Results are shown as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum or as bar graphs indicating mean ± standard deviation. Statistical significance was determined by paired student's *t*-test if not stated otherwise. Results were considered significant as $P \leq 0.05$; $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Results

Aqueous ragweed pollen extract (Amb-APE) enhances IgE production in Th2-stimulated murine B cells

To mimic Th2-mediated activation and subsequent IgE production, MACS-sorted naïve B cells from murine C57BL/6 splenocyte suspensions were stimulated for 8 days with anti-CD40 and IL-4 or medium control. The influence of ragweed pollen on T helper 2 cell-induced IgE production was analyzed by an additional exposure to Amb-APE (Fig. 1A).

Without Th2 stimulation, Amb-APE had no effect on immunoglobulin production. Neither IgE, nor IgG or IgM levels were increased in supernatants from B-cell cultures (Fig. 1B). However, when anti-CD40/IL-4 was added, Amb-APE dose-dependently enhanced the production of IgE (Fig. 1C). In contrast, Amb-APE did not increase the production of IgG or IgM. Thus, under Th2 conditions Amb-APE selectively enhanced IgE production. When B cells were stimulated with the IgG-promoting Th1 cytokine IFN-γ (instead of IL-4), IgG, IgM, and IgE production were not enhanced (Fig. 1D).

Intranasal instillation of Amb-APE elevates levels of total IgE in plasma of OVA-sensitized mice

The IgE-enhancing effects seen *in vitro* prompted us to analyze whether these effects have physiological relevance *in vivo*. Thus, OVA-sensitized BALB/c mice were instilled intranasally with Amb-APE or PBS for eleven consecutive days. Nonsensitized mice instilled with Amb-APE were used as additional controls. On the last day of intranasal instillation, all mice were challenged with OVA aerosol (Fig. 2A). One or seven days after the aerosol challenge, plasma was analyzed for total IgE levels. In nonsensitized mice, instillation of Amb-APE followed by OVA-aerosol challenge had no influence on total IgE levels (NS/APE/OVA; Fig. 2B). In OVA-sensitized mice, intranasal instillation of Amb-APE followed by OVA-aerosol challenge (S/APE/OVA) resulted in significantly increased total IgE levels compared to

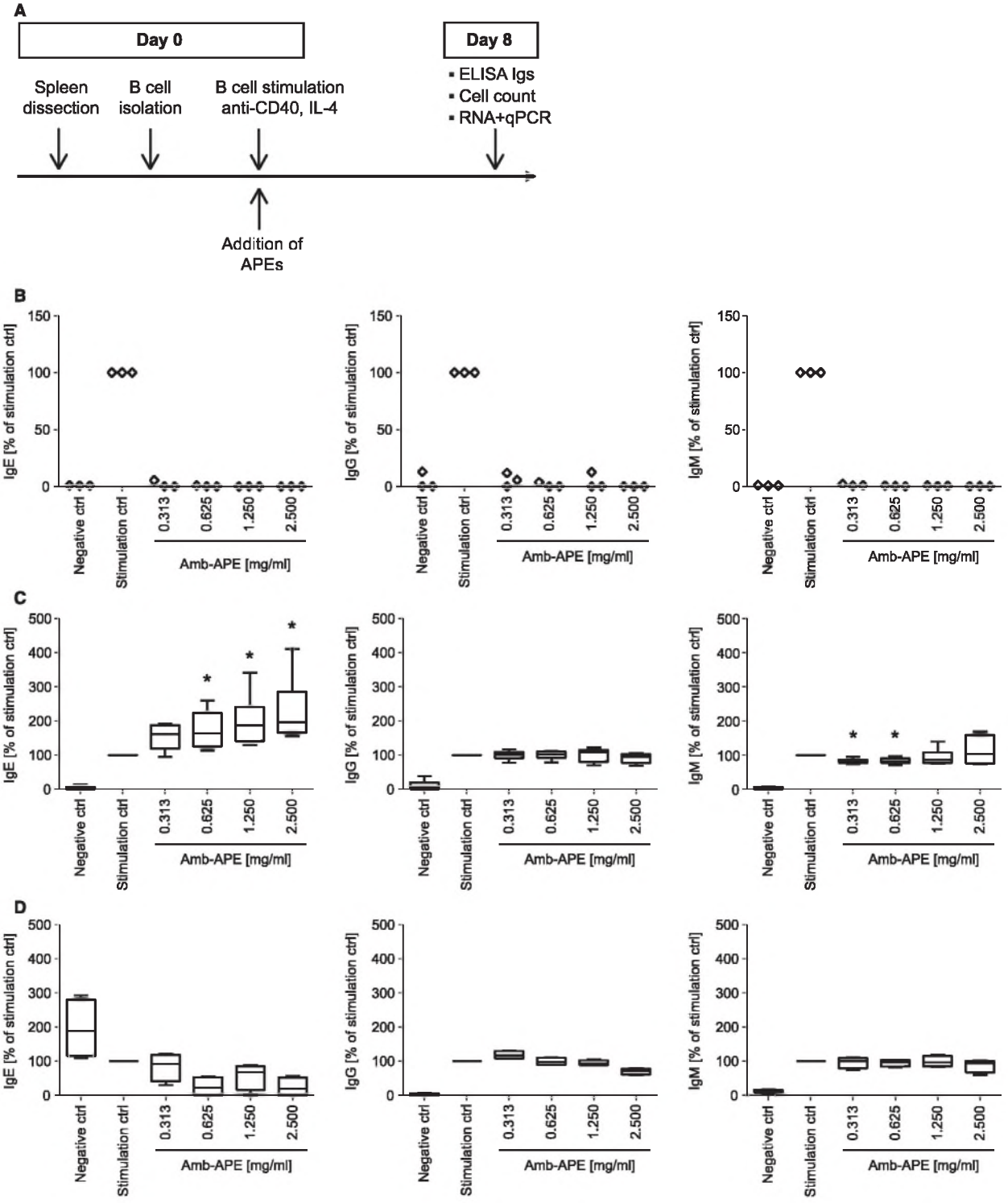

Figure 1 Experimental setup and IgE-enhancing effect of Amb-APE. (A) Total IgE, IgG, and IgM were measured on day 8 after exposure of naïve B cells from C57BL/6 mice to increasing concentrations of Amb-APE without (B) or with stimulation with anti-CD40 and IL-4 (C) or with stimulation with anti-CD40 and IFN-γ (D). Negative ctrl. = untreated cells, stimulation ctrl. = cells stimulated with anti-CD40 and IL-4 (B, C) or anti-CD40 and IFN-γ (D). Absolute values: (C) 579 ng/ml IgE, 37.4 ng/ml IgG, 2520.6 ng/ml IgM; (D) 1 ng/ml IgE, 52.7 ng/ml IgG, 1213.3 ng/ml IgM. Data are shown as individual data points (B, D) or medians ± quartiles and maximum/minimum. $n = 3$ (B), 6 (C), or 4 (D) independent experiments. $^*P \leq 0.05$ compared to stimulation control (Wilcoxon's signed-rank test).

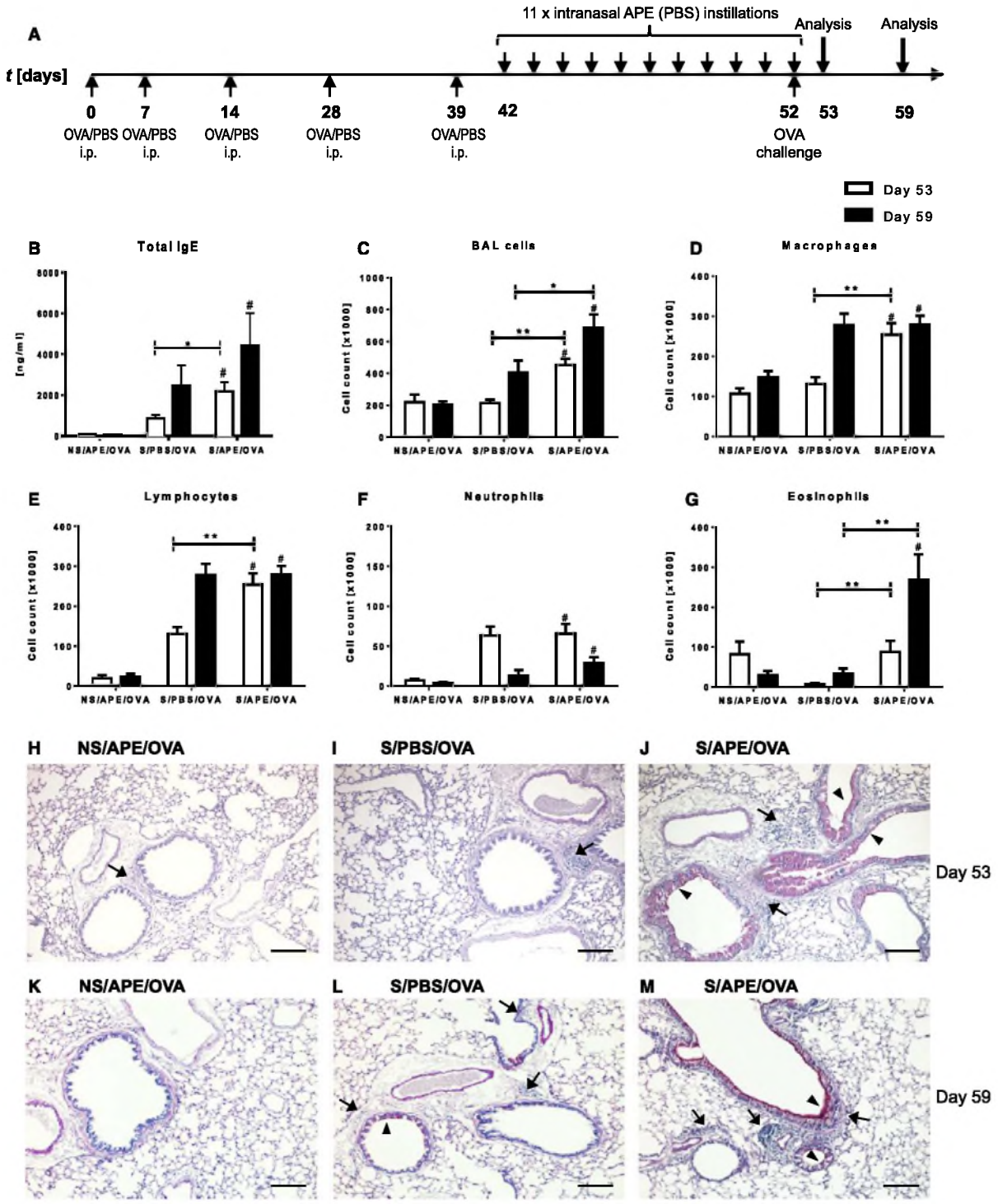

Figure 2 Adjuvant activity of intranasal Amb-APE on OVA-specific airway inflammation. (A) BALB/c mice were i.p. sensitized with OVA/alum (S) or control solution (PBS/alum [NS]). Prior to OVA-aerosol challenge on day 52, mice received i.n. Amb-APE (APE) or PBS for eleven consecutive days. Mice were killed 1 day (day 53) or 7 days (day 59) after OVA challenge. Levels of total IgE in plasma (B), BAL total cell count (C), total macrophages (D), lymphocytes (E), neutrophils (F), and eosinophils (G) at day 53 (white bars) and at day 59 (black bars). Data are expressed as mean + SEM. $n = 6$–12 mice per group. $^{\#}P < 0.05$ *vs* NS/APE/OVA; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. Representative pulmonary sections (PAS-staining) at day 53 (H–J) and at day 59 (K–M). Arrows: inflammatory infiltrate; arrowheads: mucus hypersecretion; scale bar: 100 μm.

nonsensitized mice ($P < 0.05$ *vs* NS/APE/OVA, Fig. 2B) and compared to PBS instillation in the early time-point ($P < 0.05$ *vs* S/PBS/OVA for day 53). In the late time-point (day 59), the increase of total IgE in S/APE/OVA *vs* S/PBS/OVA did not reach statistical significance (Fig. 2B).

Intranasal instillation of Amb-APE aggravates OVA-specific lung inflammation in OVA-sensitized mice

In OVA-sensitized BALB/c mice, intranasal instillation of Amb-APE followed by OVA challenge caused a significant increase in inflammatory lung cell infiltrate as analyzed by bronchoalveolar lavage compared to PBS treatment ($P < 0.01$ and $P < 0.05$ *vs* S/PBS/OVA for days 53 and 59, respectively; Fig. 2C). The aggravated response in S/APE/OVA was characterized by significantly elevated numbers of macrophages (Fig. 2D), lymphocytes (Fig. 2E), and eosinophils (Fig. 2G) on day 53, which increased even more on day 59 compared to S/PBS/OVA. In contrast, neutrophil numbers in S/APE/OVA increased slightly, but not significantly, only on day 59 compared to S/PBS/OVA (Fig. 2F).

The exacerbation of lung inflammation was also evident in the histopathological analysis of lung specimen. NS/APE/OVA at day 53 showed a slight inflammatory cell infiltration, which disappeared completely by day 59 (Fig. 2H,K, respectively). S/PBS/OVA showed a mild inflammatory infiltrate at both time-points and mucus hypersecretion at day 59, although both to a lesser extent compared to S/APE/OVA (Fig. 2I,L). In sensitized mice, intranasal instillation of total Amb-APE followed by a single OVA challenge leads to exacerbated and persisting peribronchial and perivascular inflammation and strong mucus hypersecretion (S/APE/OVA, Fig. 2J,M). This finding indicates an adjuvant activity of Amb-APE on an unrelated bystander inflammation.

Protein-free pollen fractions are sufficient to increase Th2-induced IgE *in vitro*

Pollen extracts increased total IgE in a dose-dependent manner in isolated B cells from C57BL/6 mice (Fig. 1). Nevertheless, we could not detect Amb a 1-specific IgE, or Amb-APE-specific IgE in supernatants from anti-CD40/IL-4/Amb-APE-stimulated B cells (data not shown). We then investigated whether factors of Amb-APE other than allergens (or proteins in general) might cause the IgE enhancement. Therefore, murine C57BL/6 B cells were incubated under Th2 conditions with Amb-APE total, Amb a 1, with an allergen-free fraction of Amb-APE (<3 kDa), or with the pollen-associated compounds E1 phytoprostane (PPE1) and prostaglandin E2 (PGE2). To exclude an effect of LPS contamination of pollen on IgE induction, we also incubated with LPS. The major ragweed allergen Amb a 1 (Fig. 3A), LPS (Fig. S1), and PGE2 (data not shown) did not affect IgE levels, but the low-molecular-weight fraction enhanced IgE production to a similar extent as total Amb-APE (Fig. 3A). Also, the pollen-associated mediator PPE1 (Fig. 3B) increased IgE in a dose-dependent manner. Thus, IgE enhancement by Amb-APE does not require the major ragweed allergen or other proteins, but is elicited by low-molecular-weight substances such as PPE1.

APEs from various plant species enhance IgE production

Because PPE1 had also been detected in birch pollen (3), we investigated whether the *in vitro* IgE enhancement is a common effect of pollen extracts independent from plant species. Therefore, we compared the effects of pollen extracts from a variety of plant species. Besides Amb-APE, we tested pollen extracts from birch, timothy grass, and pine.

Extracts of ragweed (Amb-APE), birch (Bet-APE), timothy grass (Phl-APE), and pine pollen (Pin-APE) were used as

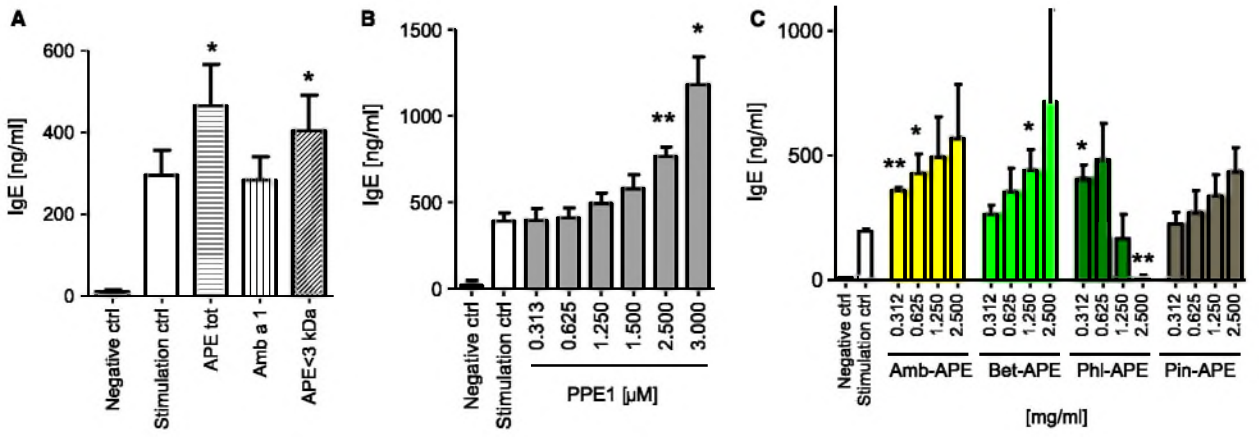

Figure 3 IgE enhancement caused by allergen-free pollen fraction, allergen-free compound PPE1, and other pollen species. Naïve B cells from C57BL/6 mice were stimulated with anti-CD40 and IL-4. (A) IgE enhancement by 1.25 mg/ml of Amb-APE, allergen-free APE fraction (Amb-APE <3 kDa), but not by Amb a 1 alone (same concentration as in 1.25 mg/ml Amb-APE). (B) IgE enhancement by PPE1. (C) IgE enhancement by pollen extracts from ragweed (Amb-APE), birch (Bet-APE), grass (Phl-APE), and pine (Pin-APE). Data are displayed as mean + SD. $n = 3$–4 independent experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$ compared to stimulation control.

adjuvant in the anti-CD40/IL-4 B-cell stimulation assay (C57BL/6). Besides Amb-APE, also Bet-APE, Phl-APE, and Pin-APE dose-dependently increased IgE production (Fig. 3C). Low-dose Phl-APE augmented IgE production, but led to lower IgE secretion at concentrations of 1.25–2.5 mg/ml. A highly statistically significant ($P < 0.01$) IgE increase was detected for anti-CD40/IL-4/Amb-APE costimulation, while this effect was significant but less pronounced ($P < 0.05$) also with Bet-APE and Phl-APE and was reversed at higher concentrations of Phl-APE. Pin-APE showed a similar tendency to enhance IgE from Th2-primed murine B cell. Therefore, the IgE-enhancing effect of pollen extracts is not specific to ragweed pollen, but is also caused by pollen from various plant species, including trees and grasses.

IgE enhancement by pollen extracts depends on B-cell proliferation

To investigate the mechanism of APE-induced augmentation of IgE production, we first evaluated immunoglobulin isotype switching in B cells, as it is a prerequisite for IgE production. We measured transcripts of IgE CSR by quantitative real-time PCR following aCD40/IL-4 ± Amb-APE stimulation of C57BL/6 naïve B cells. The IgE CSR-facilitating transcripts AID and ε-GLT were not induced upon Amb-APE exposure compared to stimulation control, but the ε-postswitch transcript (IgE precursor) was up-regulated by Amb-APE (Fig. 4A). Thus, Amb-APE did not enhance IgE CSR, but increased the expression of IgE transcript. We further investi-

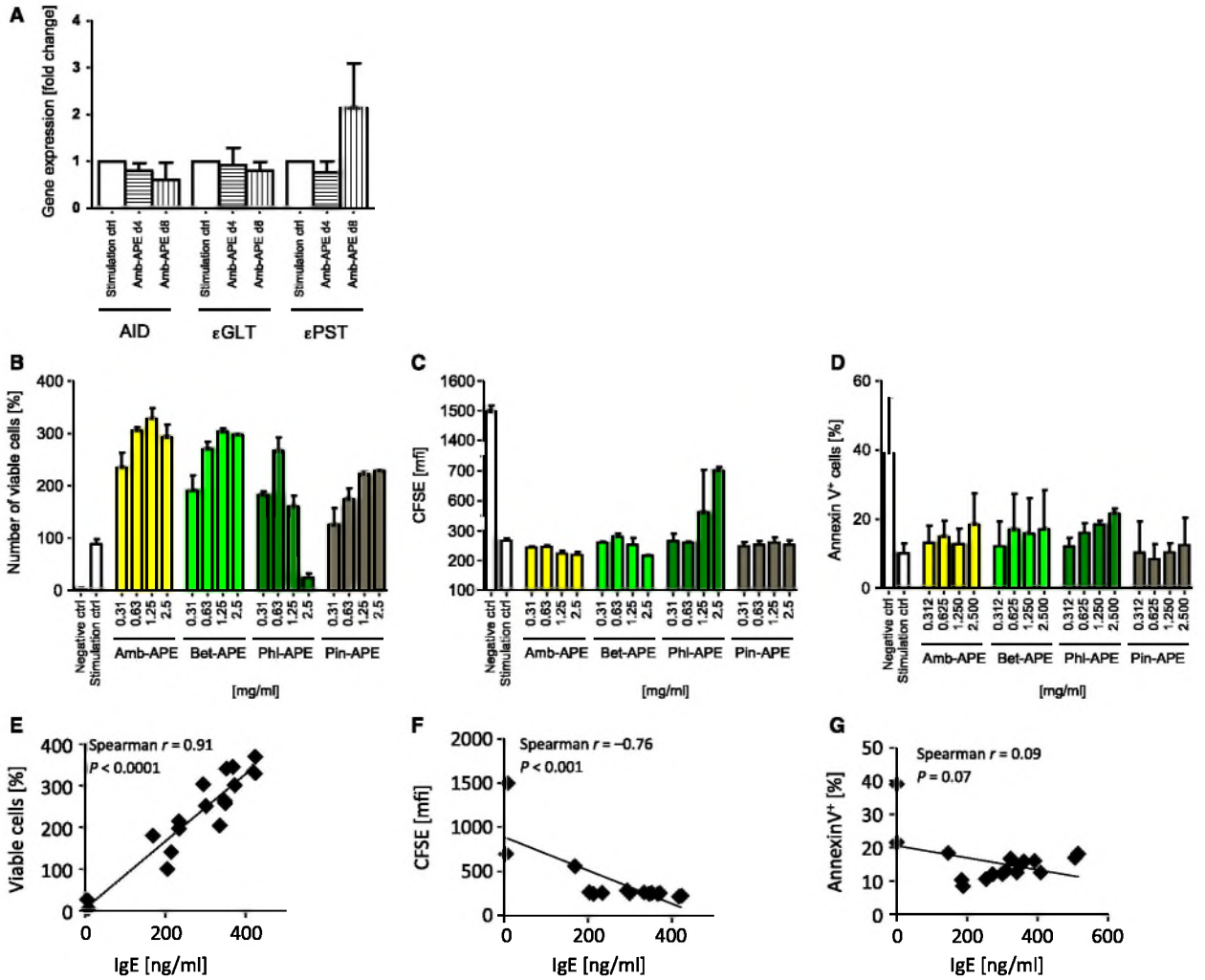

Figure 4 Mechanism of pollen-induced IgE augmentation. Naïve B cells from C57BL/6 mice were stimulated with anti-CD40 and IL-4 together with or without pollen extracts. (A) Expression of IgE class switch-associated transcripts AID (activation-induced cytidine deaminase), ε-GLT (ε germline transcript), and the precursor of IgE transcript ε-PST (ε-postswitch transcript) in B cells 0, 4, and 8 days after stimulation. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. HPRT: housekeeping gene. (B) Number of viable B cells on day 8 measured by intracellular ATP content. (C) Proliferation of B cells measured as mean fluorescence intensity (mfi) of CFSE. (D) Proportion of apoptotic cells (7-AAD$^-$/annexin V$^+$). (E, F, G) correlation with IgE level in supernatant for B, C, and D, respectively. Data are expressed as mean + SD. $n = 3$ (A) and 1 representative experiment (B–G).

gated whether the pollen extract-induced increase in IgE production was correlated with the number of B cells at the end of the experiment. Count of viable B cells was assessed by measuring intracellular ATP on day 8 of culture following increasing concentrations of APEs. The number of viable cells increased dose dependently with APE exposure (Fig. 4B) and followed the same pattern as the IgE production (Fig. 3C). Correlation of cell count and IgE levels was highly significant ($P < 0.0001$; correlation coefficient: 0.91 [Fig. 4E]). Because higher number of viable cells can be achieved by either increased proliferation or decreased apoptosis, both mechanisms were analyzed. Proliferation of CFSE-stained B cells showed the same coherency with IgE levels (Fig. 4C/F). In contrast, apoptosis (annexin V staining) did not correlate with IgE production (Fig. 4D/G).

Human memory B cells increase IgE production after PPE1 exposure

To translate our findings into the human system, we stimulated human naïve and memory B cells from healthy donors ($n = 6$) with increasing concentrations of PPE1 and Amb-APE together with IL-4, IL-21, and sCD40L (Fig. 5). In naïve B cells, the response to PPE1 demonstrated high donor variability, and there was no significant effect of Amb-APE (Fig. 5A). A significant increase in IgE secretion was observed in memory B cells after incubation with PPE1, while IgG secretion remained unchanged with increasing concentrations of PPE1 (Fig. 5B). Incubation of memory B cells with Amb-APE induced a slight increase in IgE secretion, which did not reach statistical significance (Fig. 5B).

Discussion

Pollen extracts showed the ability to enhance IgE production in both the *in vitro* Th2-like stimulated B cells and an *in vivo* OVA-sensitization model. This IgE enhancement depended on an increase in B-cell proliferation and number, but not of IgE class switch recombination. Causing factors, such as PPE1, were present in the protein-free fraction of ragweed pollen extracts.

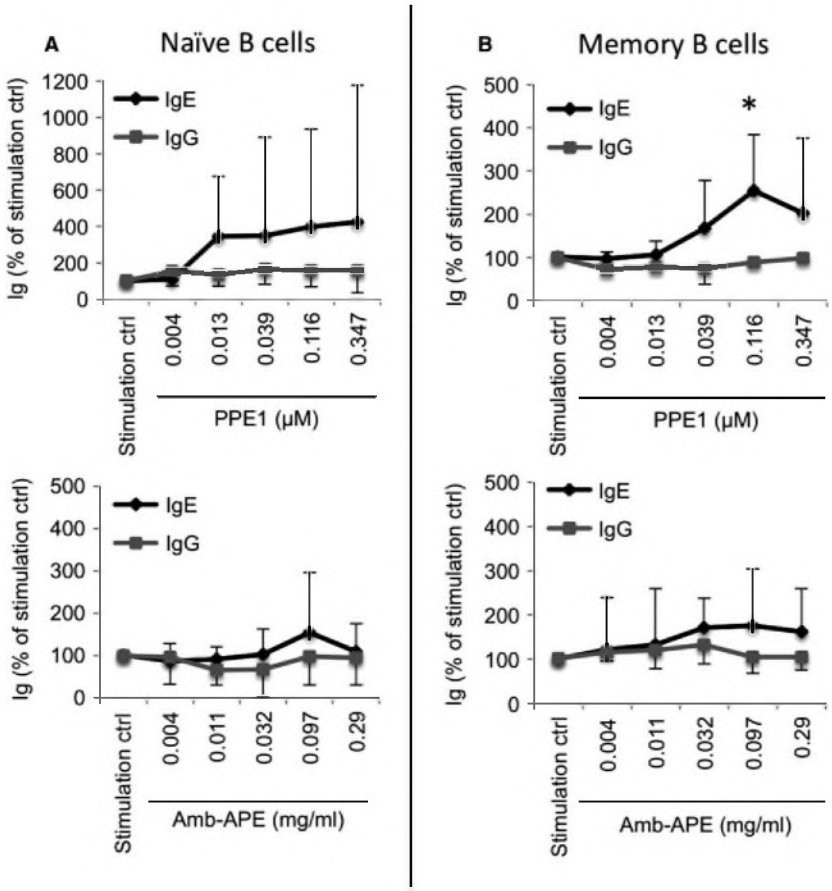

Figure 5 Modulation of immunoglobulin secretion in human naïve and memory B cells by PPE1 and Amb-APE. Naïve (A) and memory (B) B cells were stimulated with IL-4 and IL-21 and incubated with increasing concentrations of PPE1 and Amb-APE. Displayed are IgE and IgG concentrations in B-cell supernatants as percentage of stimulation control (mean + SD). $n = 5$–6 donors. Absolute values of stimulation control: naive B cells: 44.9 ng/ml IgE, 27 699 170 ng/ml IgG; memory B cells 27.1 ng/ml IgE, 87 616 650 ng/ml IgG. $^*P < 0.05$ compared to stimulation control.

Pollen extracts alone or under Th1-like conditions did not affect immunoglobulin production, while the polyclonal Ig secretion under Th2-like conditions was enhanced. The increase was specific to the allergy-associated antibody isotype IgE. Using an OVA mouse model, we demonstrated an Amb-APE-induced IgE increase with an adjuvant effect of ragweed pollen, which was characterized by an early recruitment of BAL macrophages, lymphocytes, and eosinophils, that increased further in the later time-point. The adjuvant effect of ragweed was also confirmed in the histological analysis by an increased inflammatory infiltrate and mucus hypersecretion.

The pollen extract-enhanced IgE production is mediated by a variety of plant pollen including those of weeds, grass, and trees and even pine, which are very low in protein but high in lipid content (29). Together with the observation that only nonspecific IgE production was measurable upon Amb-APE exposure, this lead to the hypothesis that substances of the pollen extracts other than allergens or proteins are sufficient to enhance IgE production. In fact, incubation with a protein-/allergen-free fraction containing low-molecular-weight substances caused the same enhancing effects as observed with total pollen extract. In contrast, the major allergen of ragweed Amb a 1 applied in the same concentration as present in Amb-APE did not influence IgE production. Low-molecular-weight substances [PALMs, (2)] were suitable candidates with adjuvant activity. PALM E_1-phytoprostanes signal via PPAR-γ (8), which enhances B-cell antibody production and differentiation (30). Our results show for the first time that PPE1 has a direct influence on B cells by enhancing ongoing IgE production. A further candidate substance might be LPS, which is described to enhance Th2 responses in low concentrations (31) and in combination with IL-4 to induce CSR to IgE (32). In our pollen extracts, LPS was measurable in low concentrations (data not shown), but LPS without pollen extract did not increase IgE production when applied together with anti-CD40 and IL-4 (Fig. S1). Thus, LPS is not related to pollen-enhanced IgE production. The allergen alone is not only unnecessary for IgE enhancement, but can even have the opposite effect. In human memory B cells, a grass pollen allergen-mediated inhibition of IgE production has been reported via increased IL-10 production (33). This observation supports our finding that other substances of the pollen extracts than the allergen itself, such as nonallergenic proteins or small molecules like PPE1, can exert the IgE-enhancing effect.

The mechanism of APE-mediated enhancement of IgE production seems to be an increased proliferation rate in B cells that switch their immunoglobulin isotype toward IgE. This effect was detected with pollen extracts from four different plant species. However, there are also plant species, like *Typha angustata* (cattail), that produce pollen compounds with antiproliferative properties (34). In contrast, rapeseed pollen-derived polysaccharides were found to increase the proliferation of lymphocytes (35). While proliferation of IgE-generating B cells was increased *in vitro*, IgE class switch recombination itself was not. Takhar et al. (14) found that allergen exposure directly induces local IgE class switch recombination outside of lymphoid tissue. Local IgE CSR was found in noses of allergic rhinitis patients and in bronchial mucosa of asthmatics, but not in atopic patients without asthma (13). Thus, local IgE CSR seems to be dependent on a specific local micromilieu. In certain patients, the IgE-enhancing effects of pollen that we found in our experiments might then be potentiated by an additional increase in IgE CSR.

Pollen-derived phytoprostanes have structural similarity to endogenous prostaglandins that were previously found to increase IgE in B cells, such as PGE2 (36). PGE2-induced IgE enhancement was caused by an increase in the proportion of IgE-secreting B cells (37) caused by enhanced IgE CSR that depends on increased levels of AID (37, 38). In our system, CSR was not affected and PGE2 did not influence IgE synthesis.

In our human B-cell experiments, we evaluated the effect of Amb-APE and PPE1 on both naïve and memory B cells. We observed high donor variability, especially in naïve B cells. This might be due to the small number of cells per well used in the experiments. Therefore, small differences in the cell number might have a massive effect on the IgE production.

Because the effect of the pollen extracts is higher in memory B cells compared to nonswitched naïve B cells, we hypothesize that these pollen-derived substances have a stronger effect on proliferation of preexisting IgE-switched memory B cells than they have on class switch recombination toward IgE in naïve B cells, similarly to what was seen in mouse. It is plausible that the higher concentration of IgE in memory B cells might also simply arise from preexisting IgE-switched B cells in this fraction.

This study provides for the first time data on the direct influence of pollen extracts on B cells that result specifically in increased IgE secretion. This finding further emphasizes the impact of pollen-derived nonallergenic low-molecular-weight substances in allergic sensitization and inflammation and might be of special importance for patients suffering from local allergic rhinitis.

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Conflicts of interest

The authors have declared no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. IgE-enhancing effect of Amb-APE is not LPS-dependent. Naïve B cells were stimulated with anti-CD40/IL-4 ± LPS. On day 8 total IgE (A), IgG (B), and IgM (C) were determined. Data are shown as medians ± quartiles and maximum/minimum. n = 5–7 independent experiments.

Figure S2. Titration of B cells for optimal IgE-secretion. Titration of naïve and memory B cells was done starting with 25 000 cells down to 195 cells per well in a 96-well plate. IgE secretion was induced by stimulation with human CD40-ligand-presenting mouse fibroblasts, 25 ng/ml human IL-21 and human IL-4 and measured by ELISA.

Table S1. Primers used for real-time PCR.

Data S1. Materials and Methods.

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