Pollen derived low molecular compounds enhance the human allergen specific immune response in vivo

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Summary

Background Besides allergens, pollen release bioactive, low molecular weight compounds that modulate and stimulate allergic reactions. Clinical relevance of these substances has not been investigated to date.

Objective To elucidate the effect of a non-allergenic, low molecular weight factors from aqueous birch pollen extracts (Bet-APE < 3 kDa) on the human allergic immune response *in vivo*.

Methods Birch and grass pollen allergic individuals underwent skin prick testing with allergen alone, allergen plus Bet-APE < 3 kDa, or allergen plus pre-identified candidate substances from low molecular pollen fraction. Nasal allergen challenges were performed in non-atopic and pollen allergic individuals using a 3 day repeated threshold challenge battery. Subjects were either exposed to allergen alone or to allergen plus Bet-APE< 3 kDa. Local cytokine levels, nasal secretion weights, nasal congestion and symptom scores were determined.

Results Skin prick test reactions to pollen elicited larger weals when allergens were tested together with the low molecular weight compounds from pollen. Similar results were obtained with candidate pollen-associated lipid mediators. In nasal lining fluids of allergic patients challenged with allergen plus Bet-APE < 3 kDa, IL-8 and IgE was significantly increased as compared to allergen-only challenged patients. These patients also produced increased amounts of total nasal secretion and reported more severe rhinorrhea than the allergen-only challenged group.

Conclusions Low molecular compounds from pollen enhance the allergen specific immune response in the skin and nose. They are therefore of potential clinical relevance in allergic patients.

Keywords low molecular weight pollen fraction, nasal challenge, pollen-associated lipid mediator, prick test

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Introduction

Pollen are an important allergen source in outdoor air. When airway epithelia and skin are exposed to pollen grains, allergens are released and transported across the epithelia, where they are presented to the immune system [1]. Whereas the unnoticed outcome of such an event would be the induction of specific tolerance, in allergic sensitization, the result is a Th2-driven adaptive immune response, involving IgE production and allergic

SG and IB contributed equally to this work.

symptoms during forthcoming encounter of allergen. Why allergic sensitization to pollen occurs in a subset of susceptible people or why some people previously not reactive to pollen still may develop an allergy later in life remains incompletely understood.

Increasing evidence shows that 'allergenic potential' of pollen is not only a function of its allergen content [2], since pollen grains release a plethora of other protein and non-protein substances in excess to the allergen. These substances may act as danger signals, which recruit inflammatory cells or drive the adaptive immune system towards preferential induction of Th2 responses. Examples are oxidases, activating the epithelium by formation of reactive oxygen species [3–5], proteases, clipping tight junctions and facilitating the transport of allergens to sub-epithelial layers [6, 7], pollen-associated lipid mediators (PALMs) [8], attracting innate immune cells or inhibiting Th1-differentiation by dendritic cells [9–14], and adenosine [15], modulating the function of virtually every cell type involved in immune responses. We recently analysed the birch pollen metabolome in a protein-free, low molecular weight fraction of aqueous birch pollen extracts (Bet-APE < 3 kDa) and identified thousands of low molecular weight compounds, among them lipids, hormones, sugars and nucleosides [15]. Here we analsed whether these low molecular weight compounds of pollen actually have an effect on the allergic immune response *in vivo*, as suggested by several *in vitro* studies [9–15]. As it is virtually impossible to study allergic sensitization in humans, we focused on studying an already established allergic immune response. As models, we chose experimental skin prick tests and out-of-season nasal allergen challenges of patients with clinically relevant birch and/or grass pollen allergy. We sought to investigate intensifying effects of Bet-APE < 3 kDa on the outcome of allergic responses. Therefore, we performed skin prick tests, using adenosine and pollen-associated lipid mediators to test the hypothesis whether this would enhance weal reactions to allergen. We furthermore established a model of repetitive nasal allergen threshold challenges to evaluate effects of Bet-APE < 3 kDa on nasal priming, cytokine patterns and nasal symptoms.

Methods

Pollen sampling

Pollen was extracted from catkins by sieving as described [16]. Catkins were collected from birch (*Betula pendula*) and pine trees (*Pinus sylvestris*) during the flowering season of 2011 and 2012 in Munich. Timothy grass pollen (*Phleum pratense, Allopecurus pratensis*) was collected in 2013 on urban meadows in Munich. Grass pollen was extracted from flower heads by sieving. Pollen was stored at −80°C until further use.

Pollen extracts

Aqueous pollen extracts (APEs) of birch (*B. pendula;* Bet-APE) and timothy grass (*P. pratense*; Phl-APE) were prepared as previously described [11]. Fractions of aqueous pollen extracts were generated by ultra-filtration of APEs through a 3 kDa cut-off membrane (Amicon Ultra; Millipore, Schwalbach, Germany). The protein-enriched fractions, containing the allergens, are denoted 'Bet-allergen' or 'Phl-allergen'. The filtrated, low molecular weight fraction of aqueous birch pollen extract is termed 'Bet-APE < 3 kDa'. During ultracentrifugation, some of the pollen-derived lipids remain bound to the membrane. Therefore, Bet-APE enriched for lipids (Bet-APE-EtOH) was prepared from total Bet-APE by ultra-filtration through the 3 kDa cut-off membrane, subsequent elution of membrane-bound lipids with ethanol, speedvac-drying of the residuals and resuspension in PBS, 0.1% BSA.

Patients and subjects

Sera of patients and subjects were screened by ImmunoCAP (Phadia laboratory systems, Uppsala, Sweden) for sensitizations against a standard clinical aeroallergen panel (including birch, hazel, timothy grass and mugwort pollen, house dust mite). Non-atopic subjects had no history of allergic disease, negative specific IgE (< 0.1 IU/mL) for the standard aeroallergen panel and total serum IgE levels < 10 IU/mL. Birch pollen-allergic patients had relevant seasonal allergic rhinitis symptoms for more than 2 years, elevated specific and total serum IgE levels and were mostly poly-sensitized to aeroallergens, especially tree and grass pollen. Grass-, but not birch pollen allergic patients were sensitized to timothy grass but not to birch pollen (specific IgE < 0.1 IU/mL). They had relevant seasonal allergic rhinitis symptoms for more than 2 years and elevated grass pollen-specific and total IgE levels in serum. For detailed information on patients and controls, see Table 1. All participants of the study gave their written, informed consent. The ethical board of the faculty of medicine, Technische Universität München, and the government of Upper Bavaria approved of the study.

Experimental skin prick tests

Skin prick tests were performed on the volar forearms with either the high molecular weight protein-containing fraction of aqueous birch pollen extracts ('Bet-allergen'; birch pollen-allergic patients) or isolated native grass pollen allergens (n Phl p 1 or n Phl p 6; grass

Table 1. Overview over patients enrolled in nasal challenges and skin prick tests. (A) Patients participating in skin prick tests are indicated by p1–p26. (B) Patients participating in the nasal allergen challenges are indicated by b1–b13 (birch pollen allergic) and g1–g4 (grass- but not birch pollen allergic)

		Specific IgE (IU/mL)	
Patient ID	Total IgE (IU/mL)	Birch	Timothy
Skin prick test study			
p1	67	71.20	1.05
p2	303	64.60	33.80
p3	964	26.50	> 100
p4	55	2.88	6.43
p5	549	0.00	34.00
p6	50	0.00	8.40
p7	42	0.00	12.90
p8	13	0.00	1.20
p9	485	42.50	10.80
p10	27	14.80	0.01
p11	315	8.90	20.90
p12	69	3.99	1.82
p13	14.5	0.00	0.77
p14	159	0.00	26.90
p15	158	0.16	30.20
p16	35	0.00	0.88
p17	155	25.90	2.33
p18	732	71.40	10.70
p19	151	1.32	2.19
p20	861	> 100	72.60
p21	123	8.36	52.3
p22	119	0.27	29.40
p23	409	5.06	1.71
p24	127	0.09	6.59
p25	56	12.60	4.38
Nasal allergen challenge study			
b1	67	71.20	1.05
b2	303	64.60	33.80
b3	964	26.50	> 100
b4*	160	3.67	0.86
b5	105	14.00	0.00
b6	238	27.90	1.02
b7	123	2.40	37.50
b8	311	38.80	21.80
b9	592	70.00	49.90
b10*	81	0.35	1.49
b11	445	> 100	> 100
b12	645	82.20	87.70
b13	55	2.88	6.43
g1	549	0.00	34.00
g2	50	0.00	8.40
g3	42	0.00	12.90
g4*	170	0.00	17.90

Some of the patients participated in both prick test study (IDs: p1, p2, p3, p4, p5, p6 and p20) and nasal allergen challenge study (IDs: b1, b2, b3, b13, g1, g2, g3).

NC, nasal challenge; SPT, skin prick test.

*Drop-out patients.

pollen-allergic patients). The relevant allergens/allergenic fractions were diluted in PBS, low molecular weight aqueous birch pollen extract (Bet-APE < 3 kDa), a lipid fraction of Bet-APE (Bet-APE-EtOH) or candidate substances, i.e. adenosine (ADO; 3 μM), linoleic acid derivatives (9(S)-HOTE, 13(S)-HOTE, 9(S)-HODE, 13(S)-HODE) (10^{-9}–10^{-5} M). Patients ($n = 25$) were either birch- and grass pollen-sensitized or sensitized to grass-, but not to birch pollen (for overview see Table 1). Standard prick test controls (0.9% NaCl, histamine; Allergopharma, Reinbek, Germany) were included in each experiment. Depending on the experiment, the following diluents were applied as additional controls: PBS, Ethanol, PBS + 1% BSA. In addition, test solutions (Bet-APE < 3 kDa, adenosine, Bet-APE-EtOH, HODEs and HOTEs – depending on the experiment) were applied without allergen. All solutions were applied in replicates of four, allocated mirrored and upside-down on each of the two forearms (see Fig. 1a). To have a chance to demonstrate possible aggravating effects of non-allergenic substances, sub-optimal concentrations of allergens were used throughout the experiments. Therefore, the allergen dose inducing a minimal cutaneous response was determined for each patient beforehand by titration of allergens. Notably, this minimal weal-and-flare response (mean weal size ± SEM: 15.3 cm^2 ± 1.9) was usually weaker than a positive response in standard diagnostic prick tests [17]. Mean values of the four intra-individual replicates were plotted as one independent experiment. Most patients were pricked more than once.

Study design of nasal challenges

All nasal challenges were performed out of pollen season. Patients and non-atopic controls were challenged 3× nasally, once a day, on three consecutive days. Each 'challenge cluster' consisted of three nasal challenges (Fig. 1b). Each patient and control passed through two challenge clusters, separated by 8-12 weeks (washout period) (Fig. 1b). On the first challenge cluster, birch pollen allergic patients were nasally challenged on three consecutive days (3×) with an allergen containing fraction of aqueous birch pollen extracts diluted in 0.9% NaCl (Bet-allergen/NaCl). The allergen dose inducing a 30–50% decline in nasal airflow or minimal nasal symptoms was determined by a cumulative threshold approach titrating patients up every 15 min until a TNSS of 6, and kept constant throughout the study. After the washout period, patients were split into two groups. The 'Bet-allergen/NaCl' group was re-challenged 3× with birch Bet-allergen/NaCl. The 'Bet-allergen/Bet-APE < 3 kDa' group was challenged 3× with Bet-allergen diluted in a low molecular weight fraction

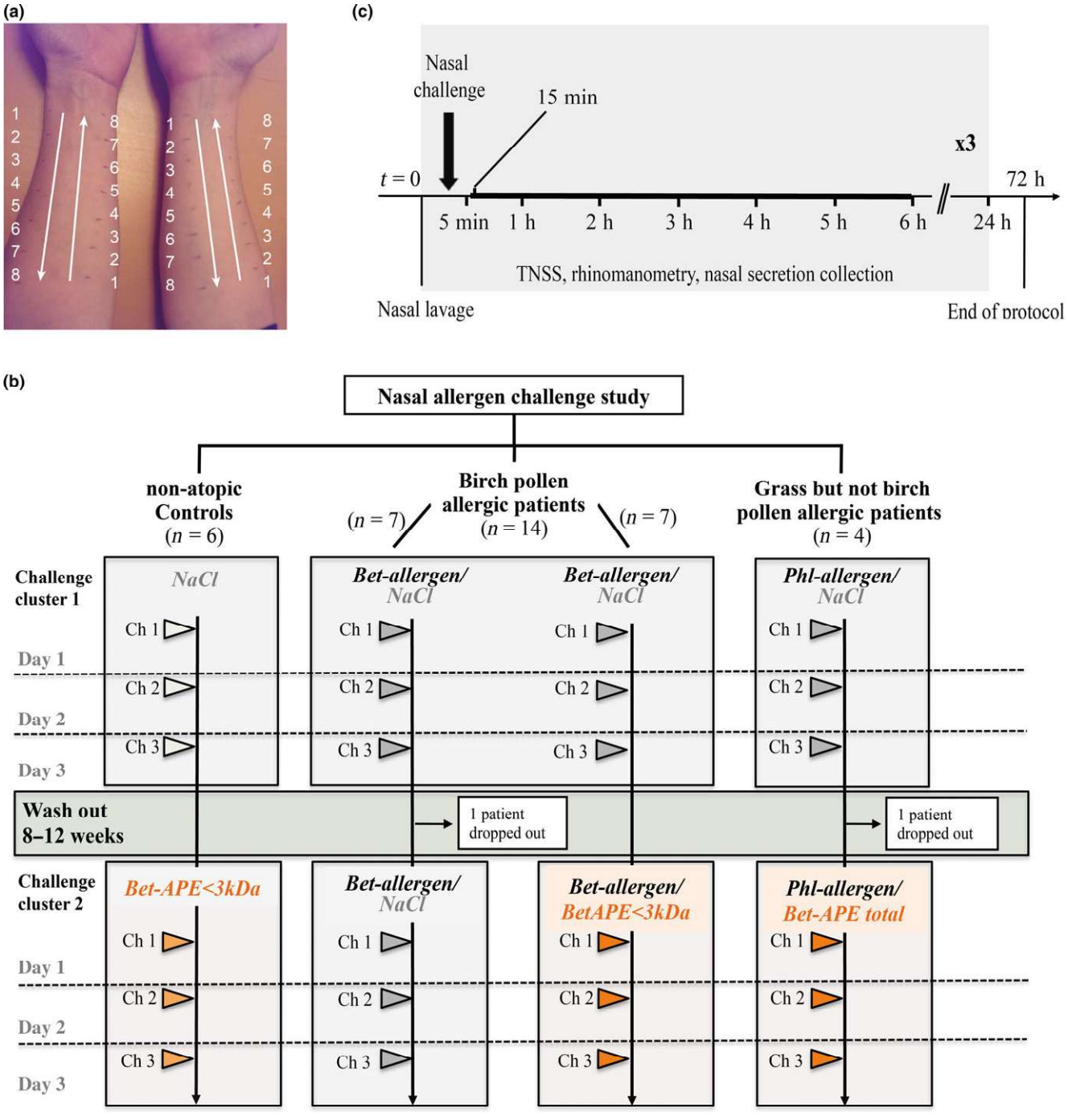

Fig. 1. Study design of skin prick tests and nasal challenges. (a) Design of experimental skin prick tests. Test solutions, positive and negative controls were applied in replicates of 4 on the volar forearms of patients in a mirrored, upside-down order. (b) Study design of nasal challenge. Non-atopic controls, birch pollen allergic patients and grass-, but not birch pollen allergic patients were nasally challenged on three consecutive days with indicated solutions (challenge cluster 1). After a washout period, they were re-challenged with indicated solutions (challenge cluster 2). ch = challenge. (c) Study protocol of nasal challenge. Patients and controls were nasally challenged once a day on three consecutive days. Shown is one single challenge including readouts and samplings. Prior to and at different time-points after each challenge, symptom score assessment, rhinomanometry and collection of nasal secretions took place. t0 = baseline.

of aqueous birch pollen extracts (Bet-allergen/Bet-APE < 3 kDa). Challenge of non-atopic controls: On the first challenge cluster, non-atopic controls were challenged 3× with NaCl. After washout, they were challenged 3× with Bet-APE < 3 kDa. Challenge of grass pollen allergic patients: Grass pollen sensitized patients lacking sensitization to birch pollen were challenged on the first challenge cluster 3× with grass pollen allergen

diluted in NaCl (Phl-allergen/NaCl). After washout, they were challenged 3× with grass pollen allergen diluted in total aqueous birch pollen extracts (Phl-allergen/*Bet*-APEtotal). For overview over study design, see Fig. 1b.

Study protocol of nasal challenges

Prior to and after each single nasal challenge, clinical and immunological readout parameters were assessed at time-points baseline (0), 5, 15 min, 1–6 h post challenge (Fig. 1c). Clinical readout parameters were nasal secretion, nasal airflow and symptom scores. Immunological readout was local cytokine production as measured in nasal secretions by ELISA.

Nasal secretions

For collecting nasal secretions at different time-points prior to and after allergen challenges, a strip of absorbent filter paper (Pall, Leucosorb) was inserted ipsilaterally into the nostril and kept there for 45 s [18]. The filter paper strip was then placed into the insert of a 1.5 mL spinning filter tube (Costar, Corning, NY, USA). Secretion fluid was extracted by adding 500 μL of ice-cold 0.9% NaCl to the paper strip and spinning it down in a pre-cooled centrifuge (4°C) for 5 min at 10 000 g. Nasal secretion weights were assessed by weighing the tube plus filter paper before and after sample collection. Local cytokine release was calculated by normalizing cytokine concentration to nasal secretion volume.

Clinical readouts

At indicated time-points a total nasal symptom score (TNSS4: rhinorrhea, sneezing, nasal obstruction, ocular symptoms) ranging from 0 (no symptoms) to 3 (strong symptoms) was assessed. Additionally, anterior rhinomanometry was performed using a calibrated Rhinotest 2000 (EVG, Böhl-Iggelheim, Germany) by assessing the nasal flow in each nostril in cubic centimetres per second at an ambient air pressure of 150 Pa in the upper airways.

Reagents and ELISA kits

Adenosine was purchased from Sigma-Aldrich (Taufkirchen, Germany). Adenosine was diluted in PBS to a final concentration of 3 μM, corresponding to the concentration of adenosine in aqueous birch pollen extracts. Lipid mediators 9(S)-HOTE, 13(S)-HOTE, 9(S)-HODE and 13(S)-HODE (Biomol, Hamburg, Germany) were diluted in PBS, 0.1% BSA to final concentrations of 10^{-9}M to 10^{-5}M. ELISA kits used were BD OptEIA Human IL-8, BD OptEIA Human IL-1β and Human Total IgE (eBioscience, Frankfurt, Germany).

Statistics

The study presented here is an explorative study. Wilcoxon test for matched pairs was applied for pairwise comparisons of groups. Cytokine release data are expressed as percentages of initial release. To assess cumulative readout, area under the curve (AUC) was calculated. For comparison of AUC values between treatment groups Wilcoxon test for matched pairs was used.

Results

Low molecular weight fraction of aqueous birch pollen enhances the allergic response of the skin

To assess whether non-protein compounds from pollen impact on the cutaneous allergic response to pollen allergen, birch and grass pollen-allergic patients were pricked with their relevant allergen either diluted in PBS (allergen/PBS) or in a low molecular weight fraction of aqueous birch pollen extracts (allergen/Bet-APE < 3 kDa). The weal reactions were enhanced significantly (1.6-fold, $P < 0.005$) after testing with allergen/Bet-APE < 3 kDa as compared to allergen/PBS (Fig. 2a). This effect was independent of pollen-derived adenosine (ADO; 3 μM) at concentrations similar to that in Bet-APE (Fig. 2b). Bet-APE < 3 kDa or adenosine

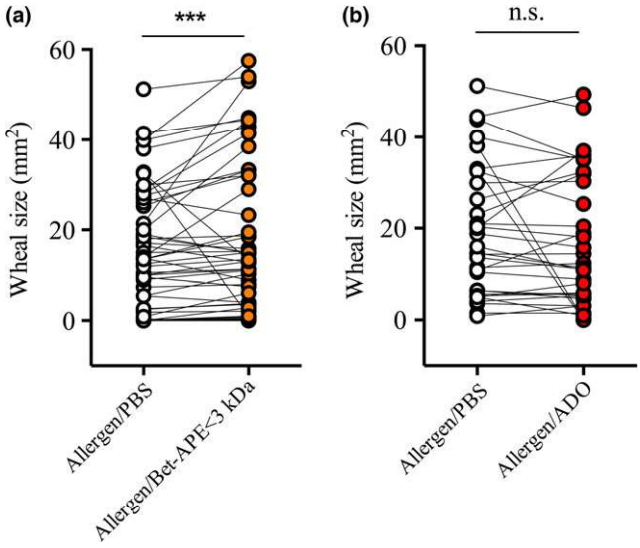

Fig. 2. Low molecular weight pollen extracts enhance the weal response to pollen allergen in skin prick tests. (a) Patients prick-tested with their respective pollen allergen diluted in PBS (allergen/PBS) and in an allergen-free fraction of aqueous birch pollen extract (allergen/Bet-APE < 3 kDa). Results of 50 independent experiments performed on nine patients. ***: $P < 0.005$, Wilcoxon test. (b) Patients prick-tested with relevant allergen diluted in PBS (allergen/PBS) or in 3 μM adenosine (allergen/ADO). Results of 32 independent prick tests performed on eight patients.

alone did not induce any cutaneous response in allergic patients. Likewise, non-atopic subjects did not react to allergen or any of the test substances (data not shown).

Pollen-associated lipid mediators enhance the cutaneous allergic response

The aggravating effect of Bet-APE < 3 kDa was dose-dependent and strongest (2-fold, $P < 0.05$) at a dose of 50 mg/mL (Fig. 3a). To identify bioactive mediators within Bet-APE < 3 kDa, allergen in NaCl was tested against allergen diluted in a lipid-fraction of Bet-APE (Bet-APE-EtOH). Bet-APE-EtOH significantly (1.5-fold at 12.5 mg/mL; $P < 0.05$) enhanced the allergic response to native Phl p 1 (Fig. 3b). This effect was reproduced to some extent with linoleic acid derivatives contained in Bet-APE, such as 9-HODE, 13-HODE, 9-HOTE and 13-HOTE (Fig. 3c–f), most prominently for 9- and 13-HOTE (Fig. 3e and f). Bet-APE-EtOH, HODEs and HOTEs did not induce any weal-and-flare responses in the absence of allergen, and non-atopic subjects did not show any cutaneous response (data not shown).

Low molecular weight compounds from pollen do not affect nasal airflow but increase nasal secretion

To test the relevance of low molecular compounds from pollen in a human model of allergic rhinitis out of season, repetitive nasal challenges were performed on birch pollen allergic patients. As primary clinical end-points, the total amount of nasal secretion was determined by weighing and nasal airflow was measured by rhinomanometry. In all birch pollen-allergic patients, there was a net decrease in nasal airflow (i.e. an increase in nasal congestion) on challenge cluster 2 as compared to challenge cluster 1. This increase was similar between the Bet-allergen/NaCl and the Bet-allergen/Bet-APE < 3 kDa-group (Fig. 4a and b). Nasal secretion production did not differ between challenge clusters in the Bet-allergen/NaCl group (Fig. 4c). In contrast, nasal secretion production was significantly increased on challenge cluster 2 as compared to challenge cluster 1 in the Bet-allergen/Bet-APE < 3 kDa-group (Fig. 4d). Nasal hypersecretion was also observed in grass- but not birch pollen sensitized patients upon challenge with Phl-allergen/*Bet*-APEtotal as compared to Phl-allergen/NaCl challenge (Fig. S1).

Low molecular weight compounds from pollen differentially increase symptoms in birch pollen-allergic patients

Non-atopic controls did not report any significant symptoms throughout the study (data not shown). In birch pollen allergic patients, Bet-allergen/NaCl challenges led to a priming of total nasal symptom score (TNSS) during challenge cluster 1 (Fig. 5a and b). On challenge cluster 2, symptoms were increasing further in the Bet-allergen/Bet-APE < 3 kDa group, but not in the Bet-allergen/NaCl group (compare Fig. 5d and c). Whereas priming of TNSS during the early-phase response was comparable in both treatment groups, priming of the late-phase response was only

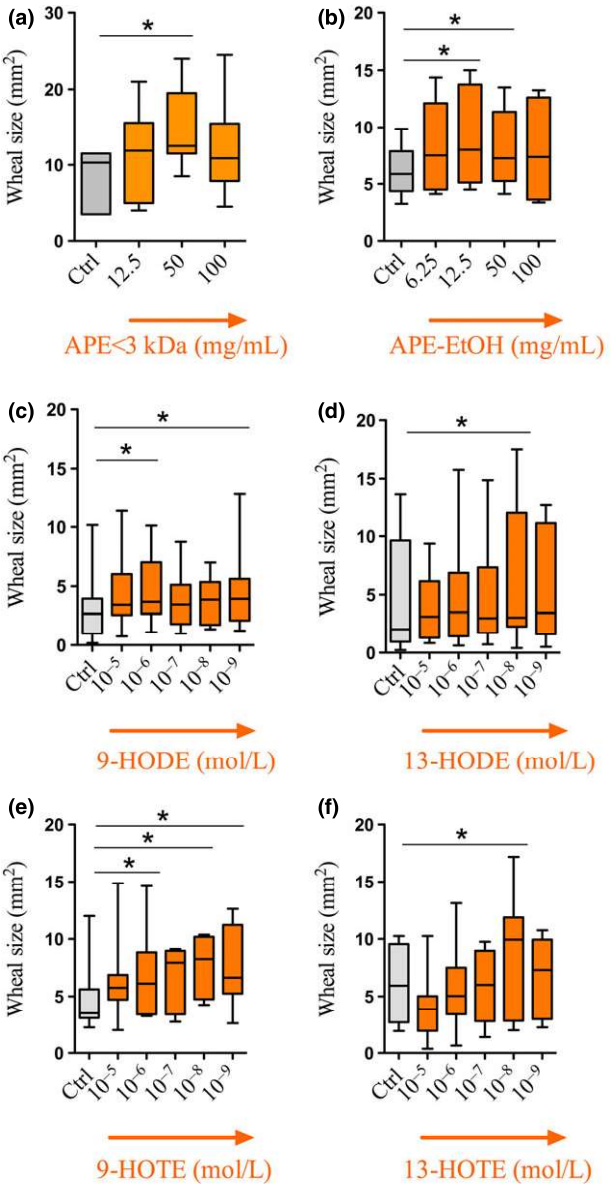

Fig. 3. Pollen-associated lipid mediators enhance the cutaneous weal response to pollen allergen. (a) Effect of an allergen-free aqueous birch pollen fraction (Bet-APE < 3 kDa) on the cutaneous weal reaction of patients ($n = 6$) to pollen allergen. (b) Effect of a lipid-enriched fraction of Bet-APE (Bet-APE-EtOH) on the cutaneous weal reaction of patients ($n = 7$) to pollen allergen. (c–f) Effects of candidate pollen-associated lipid mediators, 9-HODE (c), 13-HODE (d), 9-HOTE (e), 13-HOTE (f) on the cutaneous weal response to pollen allergen ($n = 8$). *: $P < 0.05$, Wilcoxon test.

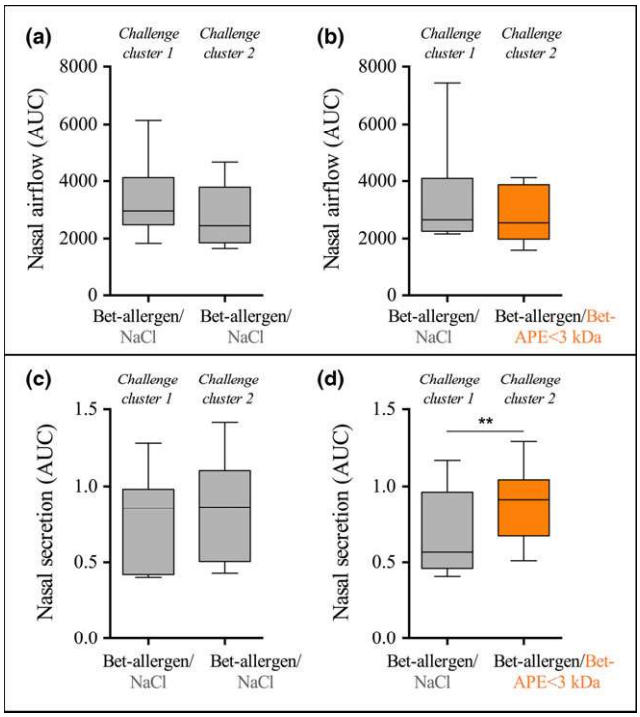

Fig. 4. Low molecular weight pollen extract induces nasal hypersecretion but not nasal congestion. Nasal airflow assessed by rhinomanometry over the course of two challenge series in patients of the Bet-allergen/NaCl (a) and Bet-allergen/Bet-APE < 3 kDa group (b). Nasal secretions collected and weighed over the course of two challenge clusters in patients of the Bet-allergen/NaCl (c) and Bet-allergen/Bet-APE < 3 kDa group (d). AUC, area under the curve. **: $P < 0.01$, Wilcoxon test.

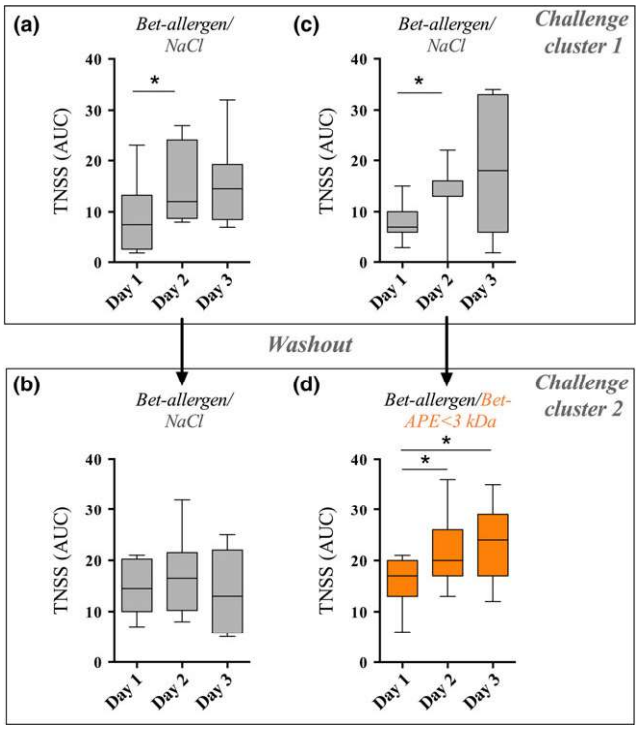

Fig. 5. Low molecular weight pollen extract differentially enhances nasal symptoms. Cumulative total nasal symptom scores (TNSS) assessed throughout challenge clusters 1 (upper panels) and 2 (lower panels). (a) TNSS of patients repetitively challenged with Bet-allergen/NaCl. (b) TNSS of the same patients after repetitive re-challenge with Bet-allergen/NaCl. (c) TNSS of a second group of allergic patients after repetitive challenge with Bet-allergen/NaCl. (d) TNSS of the same group of patients after repetitive re-challenge with Bet-allergen/Bet-APE< 3 kDa. AUC, Area under the curve. *: $P < 0.05$, Wilcoxon test.

observed in the Bet-allergen/Bet-APE < 3 kDa group (Figs S2 and S3). We also evaluated symptom scores differentially. Whereas net nasal congestion increased from challenge cluster 1–2 in both groups, conjunctival symptoms, sneezing and rhinorrhea did so only in the Bet-allergen/Bet-APE < 3 kDa group (Fig. S4). In the grass pollen group, two of three grass- but not birch pollen allergic patients reported stronger symptoms after receiving Phl-allergen/*Bet*-APEtotal as compared to Phl-allergen/NaCl (Fig. S5).

Low molecular weight compounds from pollen increase priming of nasal IL-8/CXCL8 release in atopic patients

As experimental readout, IL-8/CXCL8 was measured in nasal secretions of atopic patients at different time-points prior to and after the nasal challenges. On challenge cluster 1, all patients were nasally challenged 3× with allergen diluted in NaCl (Bet-allergen/NaCl; Fig. 6a and c). On challenge cluster 2, patients were split into two groups: one group was re-challenged 3× with Bet-allergen/NaCl (Fig. 6b), the other group received 3× Bet-allergen/Bet-APE < 3 kDa (Fig. 6d). During challenge cluster 2, local release of IL-8/CXCL8 increased from day 1 to day 3 (1.6-fold, $P < 0.01$) only in the group of patients receiving Bet-allergen/Bet-APE < 3 kDa, (compare Fig. 6d).

Low molecular weight compounds from pollen have no effect on non-specific, NaCl induced priming of nasal IL-8/CXCL8 release in non-atopic control persons

To test for sensitization-independent effects of low molecular weight pollen compounds, local IL-8/CXCL8 release was compared in non-atopic controls between challenge clusters 1 (3× NaCl) and 2 (3× Bet-APE < 3 kDa) (Fig. 6e and f). A priming in local IL-8/CXCL8 release from day 1 to 2 was observed after NaCl challenges during challenge cluster 1 (1.5-fold, $P < 0.05$, Wilcoxon test). A comparable priming of IL-8 release was observed during challenge cluster 2 after challenges with Bet-APE < 3 kDa (1.5-fold, $P < 0.01$, Wilcoxon test; Fig. 6f).

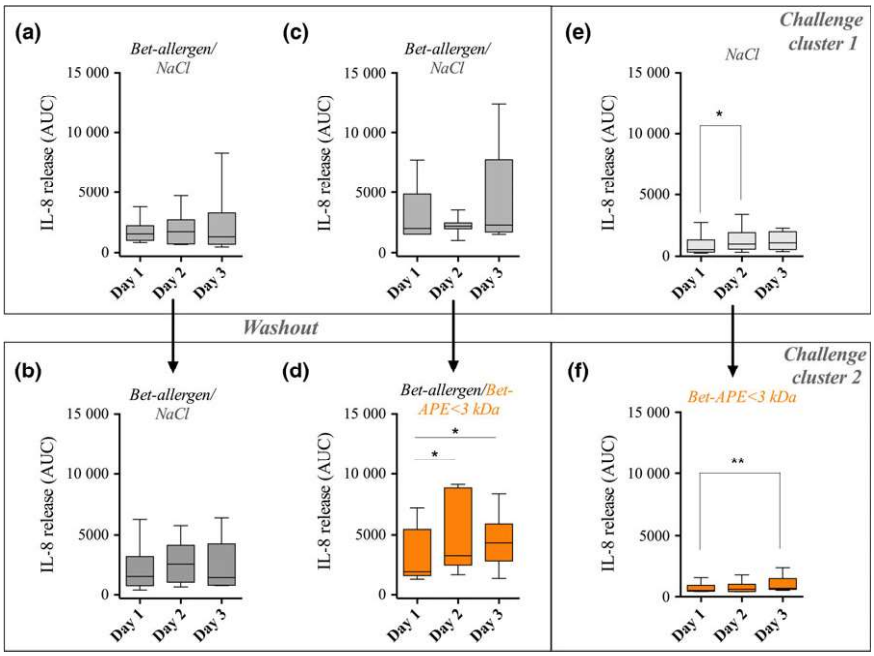

Fig. 6. A low molecular weight fraction of aqueous birch pollen extract induces priming of nasal IL-8/CXCL8 release. (a) IL-8 in nasal secretions of birch pollen allergic patients repetitively challenged with Bet-allergen/NaCl on challenge cluster 1. (b) IL-8 in nasal secretions of the same patients repetitively re-challenged with Bet-allergen/NaCl (challenge cluster 2). (c) IL-8 in nasal secretions of a second group of allergic patients after repetitive challenge with Bet-allergen/NaCl (challenge cluster 1). (d) Priming of nasal IL-8 release in the same patients repetitively challenged with Bet-allergen/Bet-APE < 3 kDa (challenge cluster 2). (e) Unspecific priming of IL-8 in nasal secretions of non-atopic control subjects after repetitive challenge with NaCl. (f) Unspecific priming of IL-8 in nasal secretions of the same non-atopic control subjects after repetitive challenge with Bet-APE < 3 kDa. AUC, area under the curve. *: $P < 0.05$; **: $P < 0.01$, Wilcoxon test.

No regulation of IL-1β, Th2 and ILC2 related cytokines by low molecular weight pollen

We also assessed the local release of the inflammasome-related cytokine IL-1β since nasal challenges were reported to go along with up-regulation of IL-1β in allergic patients [19]. However, no differences were seen in the release of IL-1β between allergic patients of both groups (Bet-allergen/NaCl and Bet-allergen/Bet-APE < 3 kDa) (see Fig. S6). The same applied to non-atopic controls (data not shown). Cytokines IL-33 and IL-25, implicated in the propagation of Th2-inducing, innate lymphoid cells (ILC2), were not detected in nasal samples, neither were the Th2 cytokines IL-4, IL-5 and IL-13 (data not shown).

Low molecular weight compounds from pollen enhance priming of nasal IgE production in atopic patients

To address the question whether low molecular weight substances from pollen enhance IgE-mediated allergic responses in the nose, total IgE was measured in nasal secretions of patients challenged with either allergen alone (Bet-allergen/NaCl) or allergen plus Bet-APE < 3 kDa (Bet-allergen/Bet-APE < 3 kDa; see Fig. S7). We observed a slight but significant priming of local IgE release only in the Bet-Allergen/Bet-APE < 3 kDa group on challenge cluster 2 (compare Fig. S7b and d). No total IgE was measured in nasal secretions of non-atopic subjects upon repetitive challenge with APE < 3 kDa (data not shown).

Bet-APE < 3 kDa enhances IgE-dependent and -independent degranulation of basophils

To assess whether APE < 3 kDa has any direct effect on allergen- and IgE-mediated processes, Basophil granulocytes from birch pollen allergic patients were stimulated with recombinant Bet v 1 and different concentrations of Bet-APE < 3 kDa, either alone or in combination (see Fig. S8). At a Bet v 1 concentration of 0.1 µg/mL, approximately 10% of basophils showed an up-regulation of CD63, which is a surface marker for degranulation. In the presence of high doses of APE < 3 kDa, CD63 up-regulation occurred in the absence of Bet v 1 in up to 40% of basophils (Fig. S8a and b). In the presence of Bet v 1, simultaneous stimulation with APE < 3 kDa also led to enhanced basophil activation, an effect that seems to be dose-dependent (Fig. S8c).

Discussion

This study gives the first indication of clinical relevance of non-protein substances from pollen in allergic

immune responses *in vivo*. In skin prick tests, APE < 3 kDa significantly enhanced the weal response to sub-clinical amounts of pollen allergen. In nasal challenges, APE < 3 kDa enhanced local mediator release and nasal symptoms in response to a sub-maximal allergen dose.

Pollen-derived adenosine, shown to be adjuvant in the lung [20], did not have any consistent effect on weal size. This is in line with previous reports pointing out distinct roles for adenosine in the lung and skin, with predominant inhibitory effects on skin mast cells via A2 receptors and activating effects in the lung via A3A receptor [21, 22]. At high concentrations, adenosine can even be transported into cutaneous mast cells via equilibrative nucleoside transporter 1 (ENT1) and specifically inhibit intracellular FcεRI signalling [21].

An aggravating effect on weal size was obtained with a lipid fraction of aqueous pollen extracts (Bet-APE-EtOH). Pollen-associated lipid mediators (PALMs) showing the most obvious activities were 9-HOTE and 13-HOTE, previously identified as constituents of birch pollen extracts and shown to be functionally analogous to mammalian LTB_4 [23]. The rapid, aggravating effect of PALMs in the acute inflammatory response of the skin, as observed in the present study, is unexpected. Leukotrienes – to which PALMs resemble functionally and structurally – recruit neutrophils, eosinophils and Th2 cells to sites of inflammation [24, 25]. Thus, they are supposed to play an important role in the late-phase allergic response. However, immediate-type reactions to allergen are mediated mainly by FcεRI-induced mast cell degranulation. The role of LTB_4 and its receptors on mast cell degranulation is controversial. Data on the effect of leukotrienes applied by direct intradermal injection are also conflicting. Several studies reported weal and flare reactions or itch-sensation induced by intradermal LTB_4 or analogues in the absence of allergen [26, 27], whereas others found no effect [28]. In prick tests presented herein, we never observed any response to Bet-APE < 3 kDa, PALMs, or adenosine in the absence of allergen (data not shown). Likewise, non-atopic control subjects did not respond to allergen alone nor to allergen plus Bet-APE < 3 kDa or candidate low molecular weight substances (data not shown). This discrepancy might be explained by the fact that all candidate substances were tested only at a single concentration in the absence of allergen, missing out possible dose-dependent effects. Given the high relevance of leukotriene receptors in the allergic late-phase response, it would be interesting to analyse the effects of PALMs on the late-phase response of the skin.

In nasal allergen challenges, Bet-APE < 3 kDa increased local release of IL-8, nasal secretion production and reported symptoms. Recently, low molecular weight compounds of aqueous ragweed extracts was shown to induce IL-8 secretion and neutrophil chemotaxis of human airway epithelial cells and neutrophilic lung infiltration in a murine model of allergic asthma [20]. In this model, neutrophil recruitment to the lung occurred after only three intranasal instillations, preceding sensitization. This is in line with our human model of repetitive challenge. In our study, IL-8 was induced in non-atopic controls upon repetitive NaCl challenge, indicating non-specific mechanisms such as mechanical trauma. Nevertheless, Bet-APE < 3 kDa further induced IL-8 in non-atopic control persons, as well as in challenged allergic patients. The findings that APE < 3 kDa enhanced nasal IgE production as well as IgE-induced basophil activation seems to argue for an aggravation of allergic responses in atopic patients rather than a mere irritant effect. The fact that no Th2 cytokines were measurable in nasal secretions of atopic patients in response to allergen challenges could be explained by either high dilution of nasal samples or by the sub-maximal allergen doses used in our experimental design.

Patients nasally challenged with allergen plus Bet-APE < 3 kDa reported increased rhinorrhea and consistently showed nasal hypersecretion as determined experimentally. Nasal hypersecretion by Bet-APE was observed in two different patient groups – in birch pollen allergic patients upon exposure to allergen plus Bet-APE < 3 kDa and in grass- but not birch pollen allergic patients upon exposure to allergen plus allergologically irrelevant total extract (Bet-APEtotal). In contrast with rhinorrhea, Bet-APE < 3 kDa did not have any effect on nasal congestion. The present study might be underpowered for the detection of differences in nasal airflow between treatment groups by rhinomanometry. However, nasal congestion, as assessed by TNSS, did not differ between treatment groups either, which supports the experimental data. Selective enhancement of nasal symptoms by Bet-APE < 3 kDa could reflect the activity of a specific compound within the low molecular weight fraction, acting on solute or water transport or mucus secretion.

Taken together, our study provides evidence of the clinical relevance of non-protein substances from pollen in allergic immune responses *in vivo*. The presence of low molecular weight compounds enhancing allergic inflammation is of potential relevance for commercial pollen extracts used for immunotherapy, as they may have an adjuvant role in the formation of immune memory in desensitization process. The present study opens the door for studies fractionating the pollen content and identifying single substances in regard to their contribution to allergic pathogenesis. This knowledge will help to understand mechanisms of sensitization as well as sharpen therapeutic strategies involving pollen extracts.

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Conflict of interest

The authors declare no conflicts of interest.

Author's contributions

SG designed the study, recruited patients for nasal challenges and skin prick tests, performed *in vitro* experiments and wrote the manuscript. IB recruited patients for skin prick tests and performed *in vitro* experiments. AC contributed to design of the nasal challenge study, recruited patients, performed nasal challenges and provided essential methods. MB gave logistic support for the nasal challenge study. MM helped with skin prick tests. AP supplied the native grass pollen allergens used in skin prick tests. LC performed basophil activation assays. JG, CSW, and HB provided discussion and critical revision of the manuscript. CTH co-designed the study, discussed results and critically revised the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Time-course of nasal secretion production after repetitive nasal challenges of patients.

Figure S2. Reported nasal symptoms in birch pollen allergic patients of both study groups.

Figure S3. Priming of nasal symptoms differs during early and late phase responses.

Figure S4. Differential symptom aggravation by low molecular weight factors from pollen after nasal challenge of birch pollen allergic patients.

Figure S5. Symptoms of grass pollen mono-sensitized patients after nasal challenges.

Figure S6. IL-1β release after nasal challenges of birch pollen allergic patients.

Figure S7. Priming of IgE in nasal secretions of birch pollen allergic patients.

Figure S8. Basophil activation in response to Bet v 1 and a low molecular weight pollen fraction.

Data S1. Low molecular weight substances from pollen enhance priming of nasal IgE release.