

In conclusion, we have found reversing trends in the prevalence of wheeze and eczema but not in rhino-conjunctivitis among Japanese children. One in three children still had one or more allergic disease, underlining the importance of further research into the risk factors and possible prevention strategies.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

Keywords

asthma, atopic dermatitis, epidemiology, pediatrics, rhinitis

ORCID

Mari Sasaki  <https://orcid.org/0000-0003-1590-3838>

Koichi Yoshida  <https://orcid.org/0000-0002-5041-7408>

Mari Sasaki¹ 

Emi Morikawa¹

Koichi Yoshida¹ 

Yuichi Adachi²

Hiroshi Odajima³

Akira Akasawa¹

¹Division of Allergy, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan

²Department of Pediatrics, University of Toyama, Toyama, Japan

³Department of Pediatrics, Fukuoka National Hospital, Fukuoka, Japan

Correspondence

Mari Sasaki, Division of Allergy, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan.

Email: mari.sasaki.md@gmail.com

REFERENCES

1. Asher MI, Montefort S, Bjorksten B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet*. 2006;368(9537):733-743.
2. Higuchi O, Adachi Y, Itazawa T, et al. Relationship between rhinitis and nocturnal cough in school children. *Pediatr Allergy Immunol*. 2012;23(6):562-566.
3. Asher MI, Keil U, Anderson HR, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J*. 1995;8(3):483-491.
4. Ronchetti R, Villa MP, Barreto M, et al. Is the increase in childhood asthma coming to an end? Findings from three surveys of schoolchildren in Rome, Italy. *Eur Respir J*. 2001;17(5):881-886.
5. Lee SL, Wong W, Lau YL. Increasing prevalence of allergic rhinitis but not asthma among children in Hong Kong from 1995 to 2001 (Phase 3 International Study of Asthma and Allergies in Childhood). *Pediatr Allergy Immunol*. 2004;15(1):72-78.
6. Kang SY, Song WJ, Cho SH, Chang YS. Time trends of the prevalence of allergic diseases in Korea: a systematic literature review. *Asia Pac Allergy*. 2018;8(1):e8.
7. Yamada T, Saito H, Fujieda S. Present state of Japanese cedar pollinosis: the national affliction. *J Allergy Clin Immunol*. 2014;133(3):632-639.
8. Bjorksten B, Clayton T, Ellwood P, Stewart A, Strachan D. Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood. *Pediatr Allergy Immunol*. 2008;19(2):110-124.
9. Morgenstern V, Zutavern A, Cyrys J, et al. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am J Respir Crit Care Med*. 2008;177(12):1331-1337.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Birch pollen allergen-specific immunotherapy with glutaraldehyde-modified allergoid induces IL-10 secretion and protective antibody responses

To the Editor,

Allergen immunotherapy (AIT) is the only disease-modifying therapy for immediate-type allergies with long-lasting effects.¹ Nonetheless, IgE-mediated side effects, potentially leading to severe anaphylactic

reactions, can occur during therapy. To improve the safety of AIT, chemically modified allergen extracts (so-called allergoids) are generated by either formaldehyde or glutaraldehyde (GA)-treatment of native allergens leading to inter- and intramolecularly cross-linked, high-molecular-weight allergen polymers.² By this means, the

Abbreviations: AIT, allergen immunotherapy; BPE, birch pollen extract; FAB, facilitated allergen binding; GA-BPE, glutaraldehyde-modified birch pollen extract; Tr1, type-1 regulatory T cells.

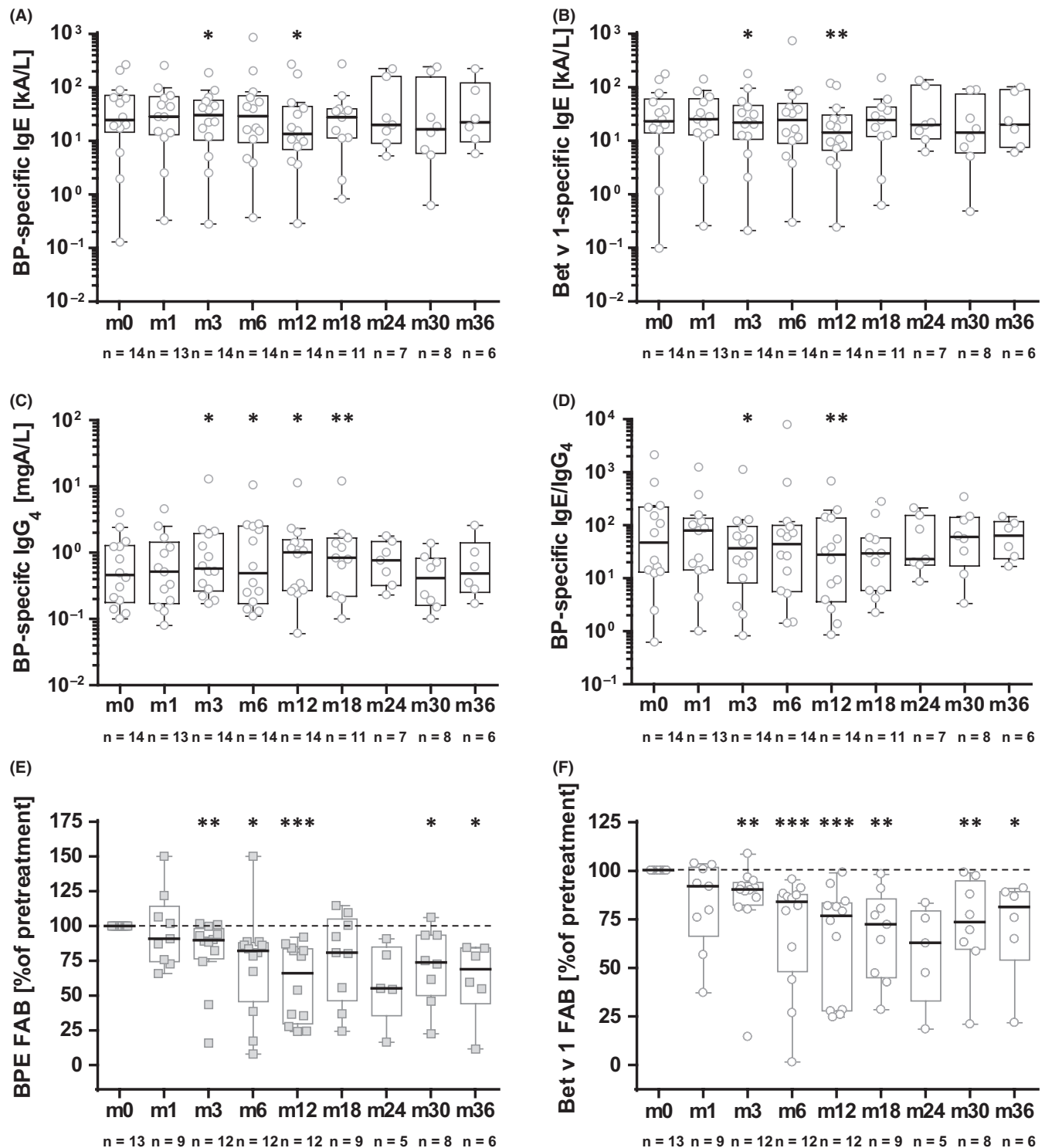


FIGURE 1 Specific IgE to (A) birch pollen (BP) and (B) Bet v 1 as well as (C) BP-specific IgG4 concentrations of patients treated with allergoid-IT. D, Ratio of BP-specific IgE to IgG4. IgE-facilitated allergen binding (IgE-FAB) during allergoid-IT was performed using either (E) BP extract (BPE) or (F) Bet v 1 for allergen-IgE complex formation. Differences between baseline (m0) and time-points during allergoid-IT were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$)

immunogenic linear T-cell epitopes should be conserved while conformational IgE epitopes, mainly responsible for potential side effects, are disrupted.

While AIT with allergoids is clinically effective,^{3,4} its immunological mechanisms leading to allergen tolerance are poorly

characterized. Here, we followed 14 birch pollen (BP)-allergic patients treated with depigmented GA-modified BP extract (BPE) for up to 3 years analyzing allergen-specific humoral and T cellular immune parameters (Figure S1). The details on patient characteristics, study design and methods are provided in this article's Data S1.

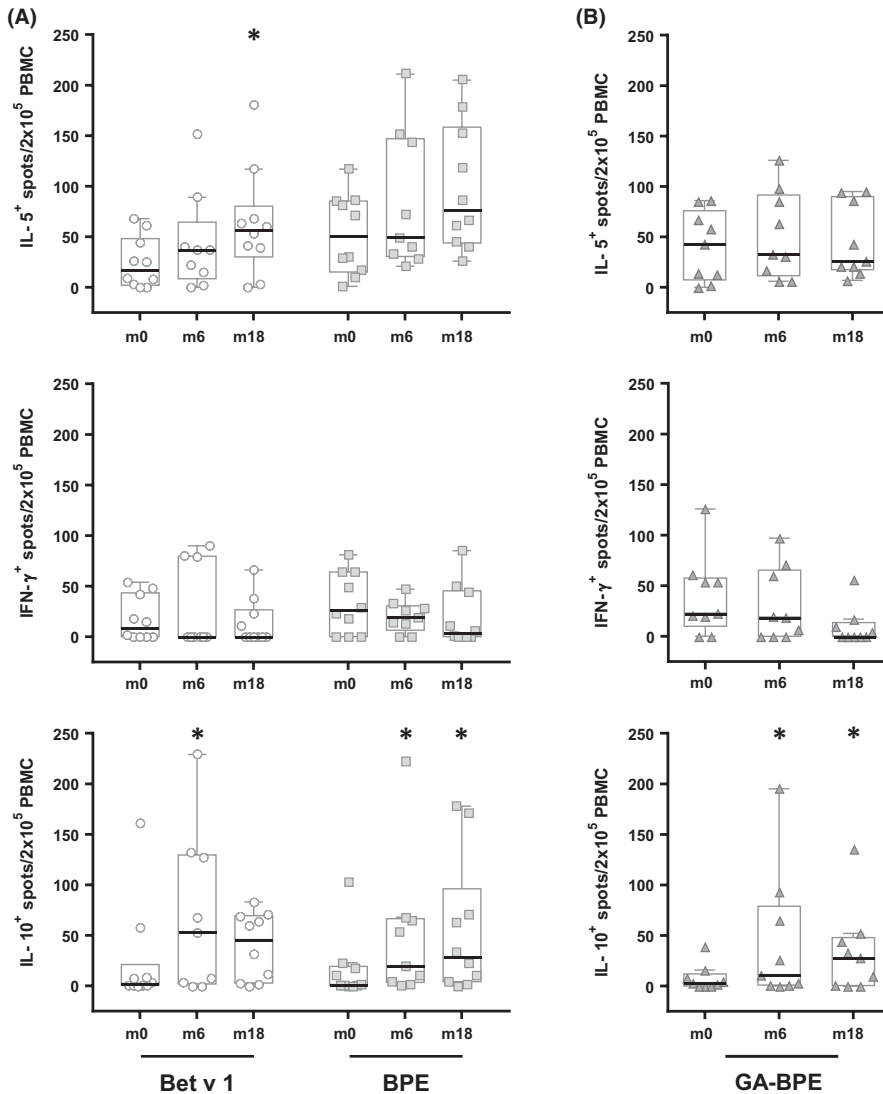


FIGURE 2 ELISpot analysis of (A) Bet v 1- and birch pollen extract (BPE)-specific and (B) glutaraldehyde-modified (GA-)BPE-specific T-cell frequencies during allergoid-IT. Baseline frequencies (m0) of specific IL-5-, IFN- γ - and IL-10-secreting T cells were compared to subsequent BP seasons (m6 and m18) during allergoid-IT. Differences between baseline and time-points during allergoid-IT were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$)

In agreement with previously published randomized, double-blind, placebo-controlled trials utilizing depigmented GA-modified pollen extracts,^{3,4} allergic symptoms of patients included in this open, uncontrolled, longitudinal study improved within the first year of allergoid-IT and remained decreased over the 3-year observation period (mean clinical improvement compared with baseline [m-6]: 31.5% [m6], 26.4% [m18] and 44.1% [m30]; Table S1).

After the build-up phase, specific IgE concentrations to BP and its major allergen Bet v 1 temporarily increased but returned to pretreatment values in the later course of therapy (Figure 1A,B). In contrast, a significant induction of BP-specific IgG4 was observed albeit lasting for no longer than 18 months after starting allergoid-IT (Figure 1C). Likewise, the BP-specific IgE/IgG4 ratio decreased during this period (Figure 1D).

In view of AIT-induced humoral changes, it has been reported that clinical efficacy correlated rather with the allergen-blocking capacity of patients' sera than with merely increasing concentrations of allergen-specific IgG4.⁵ To investigate the competition of IgE and IgG4 for allergen binding and the influence of allergoid-IT on the patient's ability to form allergen-IgE complexes, we made use

of the IgE-facilitated allergen-binding (FAB) assay (Figure S2A,B). As previously shown for grass pollen,⁶ the IgE-FAB was dependent on both the low-affinity IgE receptor CD23 and the BP-specific IgE (Figure S2C-E). Similar to a recent study on patients treated with formaldehyde-modified grass pollen extract,⁷ IgE-FAB was significantly reduced after 3 months of treatment utilizing either BPE (Figure 1E) or Bet v 1 as allergen (Figure 1F). Interestingly, despite BP-specific IgG4 concentrations ceased to be increased after 18 months, the allergen-blocking effect remained enhanced until the end of the 3-year period of allergoid-IT and, remarkably, its extent seemed to correspond with the clinical improvement of patients after finishing allergoid-AIT (Figure S3). Moreover, comparing BP-specific IgG4 concentrations with IgE-FAB results during allergoid-IT revealed significant inverse correlations only within the first year of treatment (Figure S4). Because allergen-specific IgG4 concentrations seem to not reflect the capacity of patients' sera to inhibit allergen-IgE complex formation, at least during later time-points of allergoid-IT, other factors, such as epitope specificity and accessibility, affinity maturation and/or Ig clonality, might contribute to functionally relevant IgE-blocking antibodies.^{5,7,8}

In addition, we investigated different allergen preparations on stimulating T cells of GA-modified allergoid-treated patients by ELISpot analysis. Since BPE is a mixture of several allergens and nonallergenic components in different amounts, we included recombinant Bet v 1 as a well-standardized major BP allergen to evaluate the impact of allergoid-IT on distinct T-cell subsets. The frequencies of allergen-specific T cells stimulated with Bet v 1 and BPE, respectively, were determined before the initiation of allergoid-IT (m0) and during the two consecutive BP seasons (m6 and m18; Figures 2A and S1). Regardless of the type of BP allergen utilized for PBMC stimulation, all patients exhibited a predominance of allergen-specific IL-5-secreting T-cell numbers prior to allergoid-IT, which further increased during the following BP seasons. Thus, in contrast to AIT with native BPE characterized by a gradual loss of allergen-specific Th2-cell reactivity,⁸ allergoid-IT did not prevent the induction of a Th2-cell response during the observation period of 18 months. Accordingly, a recent study showed that a significant decrease in the Th2 response was detectable not until the third year of allergoid-IT.⁹

Although a shift to a more Th1-cell-dominated immune response has been reported as a consequence of allergen tolerance induced by AIT, no increases in allergen-specific IFN- γ -producing T cells were noticed during treatment. However, we observed a significant induction of Bet v 1- and BPE-specific T cells secreting IL-10 during the first BP season (m6)—also referred to as type-1 regulatory T (Tr1) cells—which have been identified as a common hallmark for the initiation of AIT-induced allergen tolerance. Here, in most patients treated with allergoid-IT frequencies of IL-10-secreting cells remained enhanced until the second BP season, which is contrary to the results from our group and others showing a return of allergen-specific Tr1 cells to pretreatment levels already within the first year of AIT.⁸

As one further allergen preparation, we utilized GA-modified BPE for its T-cell stimulatory capacity. In contrast to Bet v 1 and native BPE, seasonal increases in IL-5-secreting T cells were absent and Th2-cell frequencies even tend to diminish (Figure 2B). On the other hand, courses of both GA-BPE-specific IFN- γ -secreting T cells and IL-10-secreting Tr1 cells did not differ from the frequencies in allergoid-treated patients after stimulation with nonmodified BP (Figure 2B), suggesting either a differentiated Th2-cell reactivity to the antigen used for in vitro stimulation or a preferential deletion of GA-BPE-specific Th2 cells in patients during allergoid-IT.¹⁰

In summary, we here show that AIT with GA-modified allergoid results in distinct immunological effects on the humoral and T cellular levels. While allergen-specific IgG4 concentrations temporarily increased, the capacity to block allergen-IgE complex formation was continuously elevated over the 3-year treatment period. Furthermore, allergoid-IT resulted in augmented IL-10-producing Tr1-cell numbers, presumably counter-balancing increases in allergen-specific Th2 cells during the seasonal BP exposure. Thus, similar to treatment with native allergen extracts, the induction of both allergen-blocking capacity and regulatory T cells seems to be the main immunological alteration associated with AIT utilizing GA-modified allergens.

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ORCID

Michèle Myriam Rauber  <https://orcid.org/0000-0001-8022-7421>

Barbara Bohle  <https://orcid.org/0000-0002-5105-7985>

Mohamed H. Shamji  <https://orcid.org/0000-0003-3425-3463>

Wolfgang Pfützner  <https://orcid.org/0000-0002-8721-724X>

Christian Möbs  <https://orcid.org/0000-0002-5197-7669>

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Michèle Myriam Rauber^{1,2} 

Hoi Ka Wu³

Britta Adams¹

Julia Pickert¹

Barbara Bohle⁴ 

Mohamed H. Shamji³ 

Wolfgang Pfützner¹ 

Christian Möbs¹ 

¹Clinical & Experimental Allergology, Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

²Experimental Dermatology and Allergy Research Group, Department of Dermatology and Allergology, Justus-Liebig University Giessen, Giessen, Germany

³Allergy and Clinical Immunology, National Heart and Lung Institute, Imperial College London, London, UK

⁴Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

Correspondence

Christian Möbs, Clinical & Experimental Allergology, Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany.

Email: moeb@med.uni-marburg.de

REFERENCES

1. Muraro A, Roberts G, Halken S, et al. EAACI guidelines on allergen immunotherapy: executive statement. *Allergy*. 2018;73(4):739-743.
2. Marsh DG, Lichtenstein LM, Campbell DH. Studies on "allergoids" prepared from naturally occurring allergens. I. Assay of allergenicity and antigenicity of formalinized rye group I component. *Immunology*. 1970;18(5):705-722.
3. Hoiby AS, Strand V, Robinson DS, Sager A, Rak S. Efficacy, safety, and immunological effects of a 2-year immunotherapy with Depigoid birch pollen extract: a randomized, double-blind, placebo-controlled study. *Clin Exp Allergy*. 2010;40(7):1062-1070.
4. Pfaar O, Biedermann T, Klimek L, Sager A, Robinson DS. Depigmented-polymerized mixed grass/birch pollen extract immunotherapy is effective in polysensitized patients. *Allergy*. 2013;68(10):1306-1313.
5. Shamji MH, Ljorring C, Francis JN, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy*. 2011;67(2):217-226.
6. Shamji MH, Wilcock LK, Wachholz PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods*. 2006;317(1-2):71-79.
7. Reithofer M, Böll SL, Kitzmüller C, et al. Alum-adsorbed allergoids induce functional IgE-blocking antibodies. *Clin Exp Allergy*. 2018;48(6):741-744.
8. Möbs C, Ipsen H, Mayer L, et al. Birch pollen immunotherapy results in long-term loss of Bet v 1-specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. *J Allergy Clin Immunol*. 2012;130(5):1108-1116.
9. Zissler UM, Jakwerth CA, Gueth FM, et al. Early IL-10 producing B-cells and coinciding Th/Tr17 shifts during three year grass-pollen AIT. *EBioMedicine*. 2018;36:475-488.
10. Wambre E. Effect of allergen-specific immunotherapy on CD4 + T cells. *Curr Opin Allergy Clin Immunol*. 2015;15(6):581-587.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Sublingual immunotherapy with recombinant Mal d 1 downregulates the allergen-specific Th2 response

To the Editor,

Birch pollen-related food allergy (BPRFA) is the most prevalent food allergy in adolescents/adults and affects more than 70% of birch pollen-allergic patients. Following sensitization to the major birch pollen allergen Bet v 1, allergic symptoms to food occur due to immunological cross-reactivity to homologous proteins. Mal d 1, the Bet v 1-homologue in apple, is among the most frequent triggers of BPRFA. Allergen-specific immunotherapy (AIT) with birch pollen extract is established as effective treatment for birch pollinosis. However, its benefit for the concomitant food allergy is controversial. In search of alternative and more efficient treatment options for BPRFA, we conducted a randomized double-blind placebo-controlled sublingual immunotherapy (SLIT) study with standardized doses of recombinant (r) Mal d 1 in patients with birch pollen-related apple allergy. After 16 weeks, patients who received rMal d 1 showed clinical improvements, tolerating higher amounts of the apple allergen in oral challenges and developing smaller wheal sizes in skin prick tests.¹ Moreover, their Mal d 1-specific IgG₄/IgE ratios increased and their post-SLIT sera contained antibodies with IgE-blocking activity (manuscript in preparation). None of these changes were observed in the patients who received placebo.¹ Here, we characterized for the first time the specific T-cell response to a sublingually administered recombinant food allergen.

We have previously reported a significant downregulation of allergen-specific T-cell proliferation already after 4 weeks of SLIT.²

Accordingly, we stimulated PBMC from 20 rMal d 1-treated individuals collected before, at 4 and 16 weeks of treatment with rMal d 1. We also included Bet v 1, as the majority of Mal d 1-reactive T cells are originally Bet v 1-specific cells that cross-react with the apple allergen. Tetanus toxoid (TT) served as control antigen. Figure 1A shows that rMal d 1- and rBet v 1-induced proliferation was significantly decreased at 4 weeks and 16 weeks, whereas TT-induced proliferation was not affected. No altered proliferative responses to either allergen or TT were observed with PBMC of 19 individuals receiving placebo (Figure 1A). These results confirmed the early decrease in allergen-specific T-cell proliferation in SLIT.² Moreover, the results confirmed our expectations deduced from a previous study demonstrating that two sublingual administrations of 50 µg of rMal d 1 reduced proliferative responses to both Mal d 1 and Bet v 1.³ Thus, SLIT performed with recombinant apple allergen also suppressed Bet v 1-specific, cross-reactive T cells.

To assess the possible induction of Tregs and of a shift from Th2 towards Th1 responses,² we monitored the key cytokines of allergen-specific Th2, Th1 and Treg cells before and after 4 and 16 weeks of SLIT with rMal d 1. PBMC were stimulated for 6 hours with rMal d 1, CD3⁺ T cells were isolated and mRNA expression levels of IL-4, IL-5, IL-13, IFN-γ, IL-10 and TGF-β were measured by qPCR (see Data S1 for detailed Methods). The post-treatment to baseline comparison revealed a continuous decline of IL-4 which was

Supporting Information

METHODS

Patients: The cohort included in this open, uncontrolled, longitudinal study comprised 14 patients with BP allergy (median age 37 (23-54) years; 7 female). Inclusion criteria for the participants were a history of BP allergy with moderate-to-severe allergic rhinoconjunctivitis (14/14) and/or mild-to-moderate allergic asthma (8/14),^{E1,E2} positive skin prick test (SPT) reactivity to BPE (ALK Prick SQ, ALK-Abelló, Hørsholm, Denmark), and specific IgE to BP and Bet v 1 (Phadia ImmunoCAP System, Thermo Fisher Scientific, Uppsala, Sweden). All patients were polysensitised with additional sensitisations to pollen other than early flowering trees (9/14), animal epithelial (9/14), house dust mites (5/14) and/or moulds (1/14). Patients with perennial symptoms and chronic and/or non-allergic asthma were not included. Overall, the treatment with allergoid-IT was well-tolerated. Immediate local reactions occurred at the site of injection, especially during the build-up phase, in about one third of patients (4/14). Moderate-to-severe systemic adverse reactions were not reported. Due to a loss of patients in the follow-up phase of allergoid-IT, cellular analyses could not be completed for the last pollen season (month 30) of the observation period. Withdrawal of patients occurred because of non-treatment-related events, i.e. patients left the study because of relocation, were excluded because of non-adherence to study-related appointments or refused additional blood drawings at later time-points of the observation period although continuing allergoid-IT. The study was approved by the Ethics Committee of the Medical Faculty of Philipps University, Marburg, Germany. All patients provided written informed consent to participate in the trial.

Study design: Patients were treated with a standardised depigmented GA-modified BPE (Depigoid, Laboratorios LETI, S.L., Barcelona, Spain) according to the manufacturer's recommendation. Increasing amounts of allergoid were injected subcutaneously every week until the maintenance dose (1,000 DPP/ml) was reached followed by monthly applications of the maintenance dose for a total of 3 years (Fig. S1). Symptoms were assessed by a clinical score of 1 (no symptoms) to 7 (most severe symptoms) at defined time points during seasonal BP exposure, i.e. 6 months (m) before the initiation of AIT (m-6), and at m6, m18 and m30 of treatment. The begin of the local birch pollen season was defined as at least 3 consecutive days with a daily mean birch pollen concentration of ≥ 11 pollen/m³ according to the data of the German Meteorological Service (DWD). Accordingly, the end of the local birch pollen season was specified as 3 consecutive days with a daily mean birch pollen concentration of ≤ 11 pollen/m³. Additionally, SPT reactivity to BPE was tested out of season before (m0) and during AIT (m12, m24, m36) according to the guidelines of the German Society for Allergology and Clinical Immunology on a scale of 0-4.^{E3} Mean wheal diameters (in millimetres) were graded semiquantitatively on a scale of 0 to 4. Skin tests results were defined as follows: 0, wheal less than 3 mm; 1, wheal of 3 to less than 4

mm; 2, wheal of 4 to less than 5 mm; 3, wheal of 5 to less than 6 mm; and 4, wheal of 6 mm or greater. Test results were exclusively evaluated by 2 specially instructed technicians to avoid interphysician variation in scoring and interpretation of SPT reactivity.

Determination of allergen-specific antibody concentrations: Concentrations of total IgE, specific IgE, IgG and IgG4 to BP, and specific IgE to Bet v 1 were quantified by the Phadia ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

IgE-facilitated allergen binding (FAB) assay: The IgE-FAB was performed as previously described.^{E4} In brief, 20µl indicator serum containing a high BP-specific IgE concentration (>100kU/ml) plus an equal volume of RPMI-1640 were incubated with 5µl of allergen (0.03µg/ml Bet v 1 or 0.3µg/ml BPE, respectively; Fig. S2A and B) and incubated for 1h at 37°C (5% CO₂). When testing for inhibition of IgE-FAB, RPMI-1640 was replaced by test serum of patients (20µl) either before initiation of therapy or during the course AIT. After allergen-IgE complex formation, 5µl of EBV-transformed B cells (1x10⁶) were added and incubated for additional 30min on a shaker at room temperature. Cells were finally stained with PE-labelled anti-human IgE (Miltenyi Biotec, Bisley, UK). A total of 5,000 viable EBV-transformed B cells was analysed by flow cytometry (FACS Canto II; BD Biosciences, San Jose, USA). All samples were prepared in triplicates.

Blood sampling and preparation of PBMCs: Peripheral blood was drawn in citrate-phosphate-dextrose-adenine (CPDA)-containing tubes at defined time points before and during AIT. PBMCs were isolated by Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. Initial gradual freezing of 10⁷ PBMCs/ml in 90% heat-inactivated fetal calf serum (FCS, PAA Laboratories, Pasching, Austria) and 10% dimethyl sulfoxide (Sigma, Schnelldorf, Germany) was performed in freezing containers (decrease by 1°C/min; Nalgene Nunc International, Rochester, USA) to a temperature of -80°C followed by long-term storage in liquid nitrogen.

ELISpot assay: The ELISpot assay was performed as described previously.^{E5,E6} Briefly, frozen PBMCs were thawed and cultured at a concentration of 10⁶ PBMCs/ml with different allergen preparations, i.e. 10µg/ml recombinant Bet v 1a (Biomay, Vienna, Austria), 10µg/ml BPE and 10µg/ml GA-BPE (both kindly provided by ALK-Abelló, Hørsholm, Denmark), or left unstimulated. After 48h of culture, IL-2 (10U/ml; Roche, Mannheim, Germany) and IL-7 (10ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) were added to expand allergen-specific T cells. On day 8, PBMCs were restimulated with 10µg/ml of the respective allergen preparation on the ELISpot plate coated with either anti-IFN-γ, anti-IL-5, or anti-IL-10 monoclonal antibodies. After an additional incubation for 20h, plates were analysed according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). Spot numbers were counted by an ELISpot plate reader

(A.EL.VIS, Hannover, Germany). All samples were prepared in triplicates and median spot numbers were determined after subtracting the unspecific spots (unstimulated control) from the spots of allergen-stimulated wells.

Statistical Analysis: Because of the nonparametric distribution of the data, medians and ranges were calculated. Comparison of paired samples was performed using the nonparametric Wilcoxon signed-rank test. The relationship between allergen-specific IgG concentrations and the IgE-FAB was evaluated by the two-tailed Spearman's rank correlation coefficient. Differences were considered as statistically significant at P values <0.05 . Statistical analysis was performed using Graph Pad Prism software (Graph Pad Software, La Jolla, USA).

REFERENCES

- E1. Bousquet J, Khaltaev N, Cruz AA, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy*. 2008;63(Suppl 86):8-160.
- E2. Buhl R, Berdel D, Criée CP, et al. [Guidelines for diagnosis and treatment of asthma patients]. *Pneumologie*. 2006;60(3):139-177.
- E3. Rueff F, Bergmann KC, Brockow K, et al. [Skin tests for diagnostics of allergic immediate-type reactions. Guideline of the German Society for Allergology and Clinical Immunology]. *Pneumologie*. 2011;65(8):484-495.
- E4. Shamji MH, Wilcock LK, Wachholz PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods*. 2006;317(1-2):71-79.
- E5. Möbs C, Ipsen H, Mayer L, et al. Birch pollen immunotherapy results in long-term loss of Bet v 1-specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. *J Allergy Clin Immunol*. 2012;130(5):1108-1116.
- E6. Möbs C, Slotosch C, Löffler H, Jakob T, Hertl M, Pfützner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol*. 2010;184(4):2194-2203.

Table S1: Clinical characteristics of patients treated by AIT with GA-allergoid

	Before AIT		During AIT	
	Year 0	Year 1	Year 2	Year 3
Clinical Score				
In season	m-6	m6	m18	m30
n	13	14	11	8
Median (range)	5 (3-6)	3 (1-6)**	3 (1-5)*	3 (1-4)*
SPT				
Out of season	m0	m12	m24	m36
n	14	12	6	5
Median (range)	2 (1-3)	1 (0-3)*	2 (1-3)	2 (1-2)
Total IgE [kU/l]				
	m0	m6	m18	m30
n	14	14	11	8
Median (range)	167 (20-842)	146.3 (18.1-790)	93.7 (19.3-706)	117.4 (26.4-1,134)
Specific IgE / total IgE				
	m0	m6	m18	m30
n	14	14	11	8
Median (range)	0.184 (0.007-0.717)	0.227 (0.009-1.47)	0.238 (0.016-0.392)	0.159 (0.022-0.33)

Birch pollen-allergic patients treated with allergoid-IT: n=14; sex (female/male): 7/7; median age (range): 37 years (23-54 years).

Differences between time points during the course of allergoid-IT and pre-treatment values were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$ and ** $P < 0.01$).

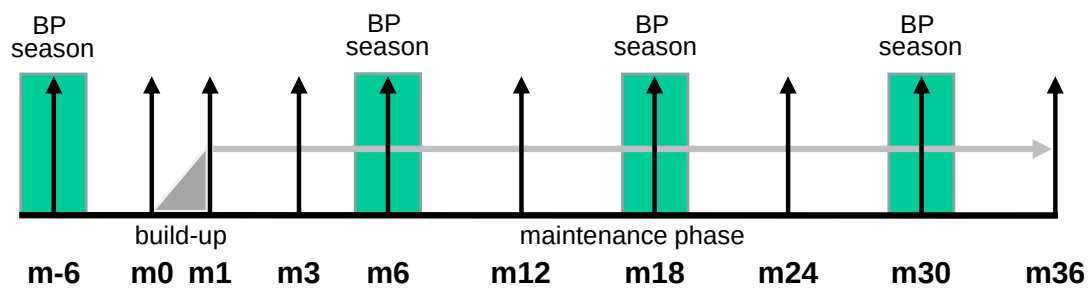


Figure S1. Timeline representing the study time-points before and during allergoid-IT. During the build-up phase of treatment patients received weekly increasing injections of glutaraldehyde-modified birch pollen (BP) extract followed by monthly applications of the maintenance dose. Periods of seasonal BP exposure are highlighted in green.

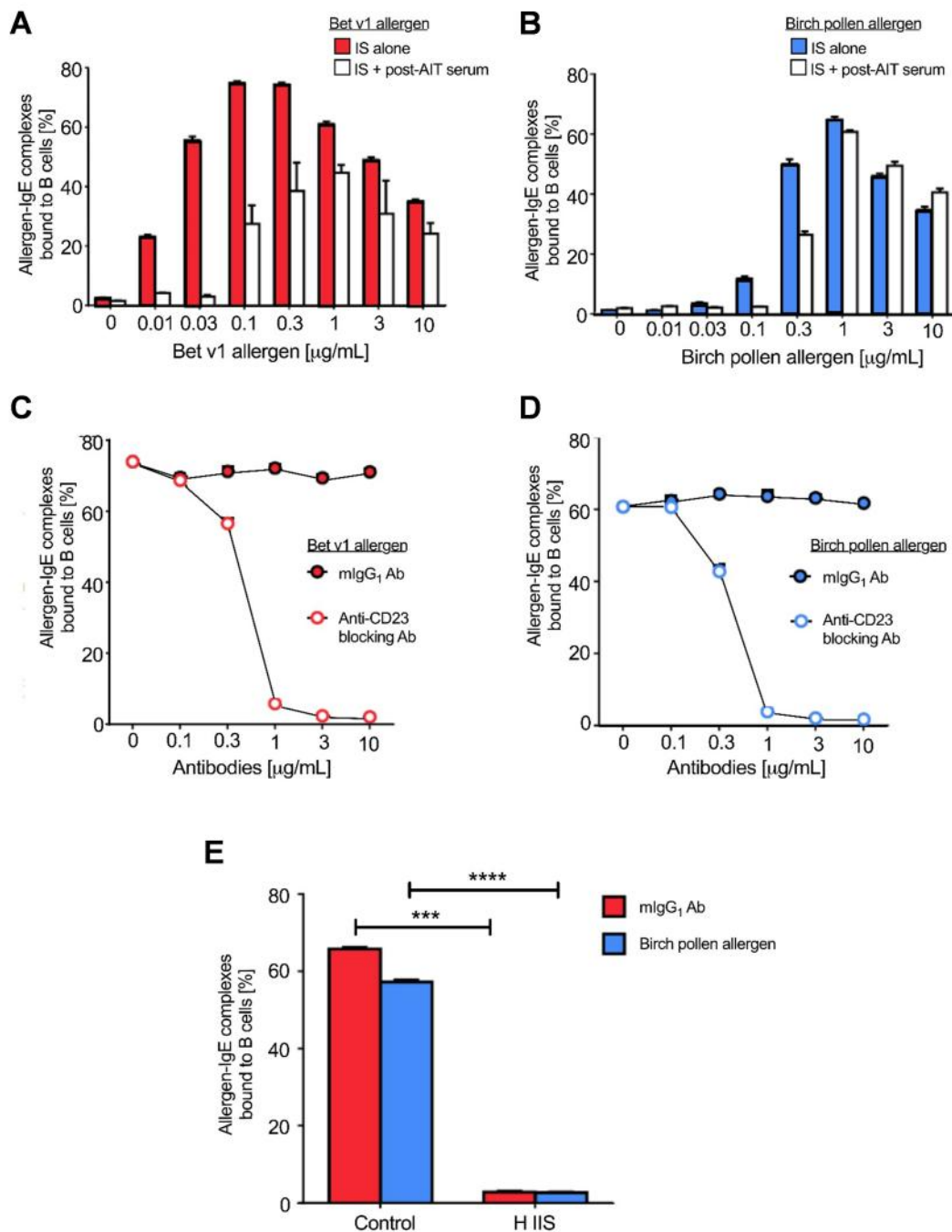


Figure S2. Allergen-, CD23- and IgE-dependency of the IgE-facilitated allergen binding (IgE-FAB) assay. Indicator serum was incubated with various concentrations of (A) Bet v 1 and (B) birch pollen (BP) allergen in the absence (closed bars) or presence (open bars) of post-allergoid-IT serum. (C, D) Dependency of the IgE-FAB on CD23. The percentage of B cells bound to allergen-IgE complexes after pre-treatment with various concentrations of either isotype control (mIgG₁) or anti-CD23 blocking antibodies at 4°C for 1 hour (n=3). Allergen concentrations used: 0.03 $\mu\text{g/mL}$ Bet v 1 and 0.3 $\mu\text{g/mL}$ BP allergen, respectively. (E) Dependency of the IgE-FAB on IgE. Heated indicator serum was compared with non-treated serum in the presence of either Bet v 1 or BP allergen (n=9). *** P <0.001, **** P <0.0001. Results are expressed as mean $\pm$ SEM (error bars). AIT, allergoid immunotherapy; HIIS, heat-inactivated indicator serum; IS, indicator.

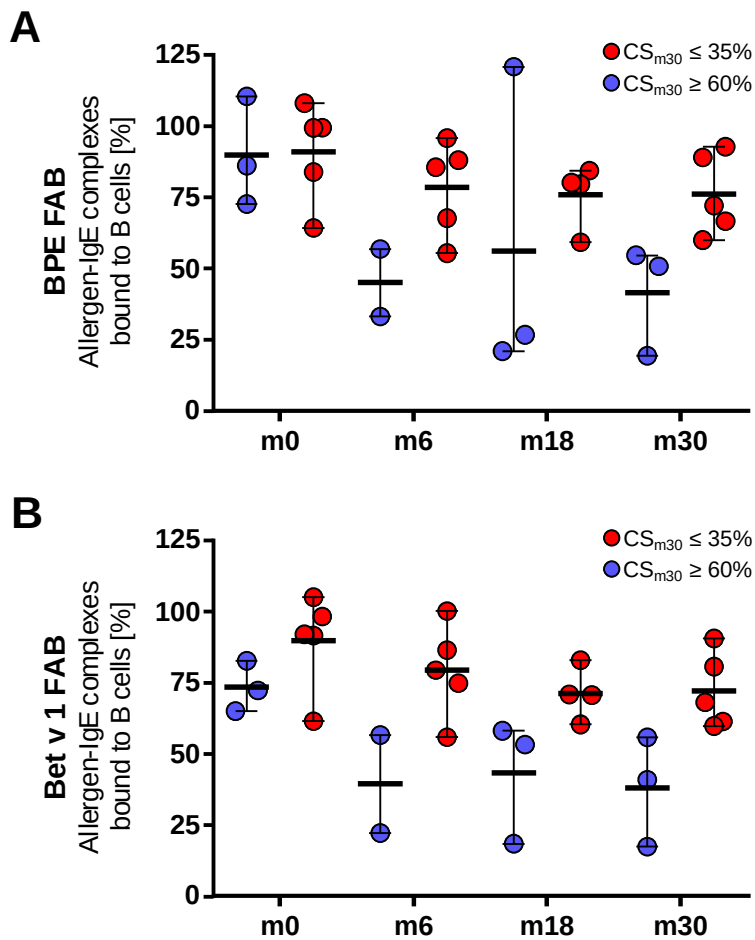


Figure S3. IgE-facilitated allergen binding (IgE-FAB) using either (A) BP extract (BPE) or (B) Bet v 1 for allergen-IgE complex formation in relation to clinical improvement during allergoid-IT. Eight patients completing the treatment period of 3 years were stratified according to their symptom scores during the last birch pollen season (m30) in comparison to baseline values, and were thus subdivided into either individuals with an improvement in clinical symptoms of $\geq 60\%$ ($CS_{m30} \geq 60\%$) or subjects with an amelioration of $\leq 35\%$ ($CS_{m30} \leq 35\%$).

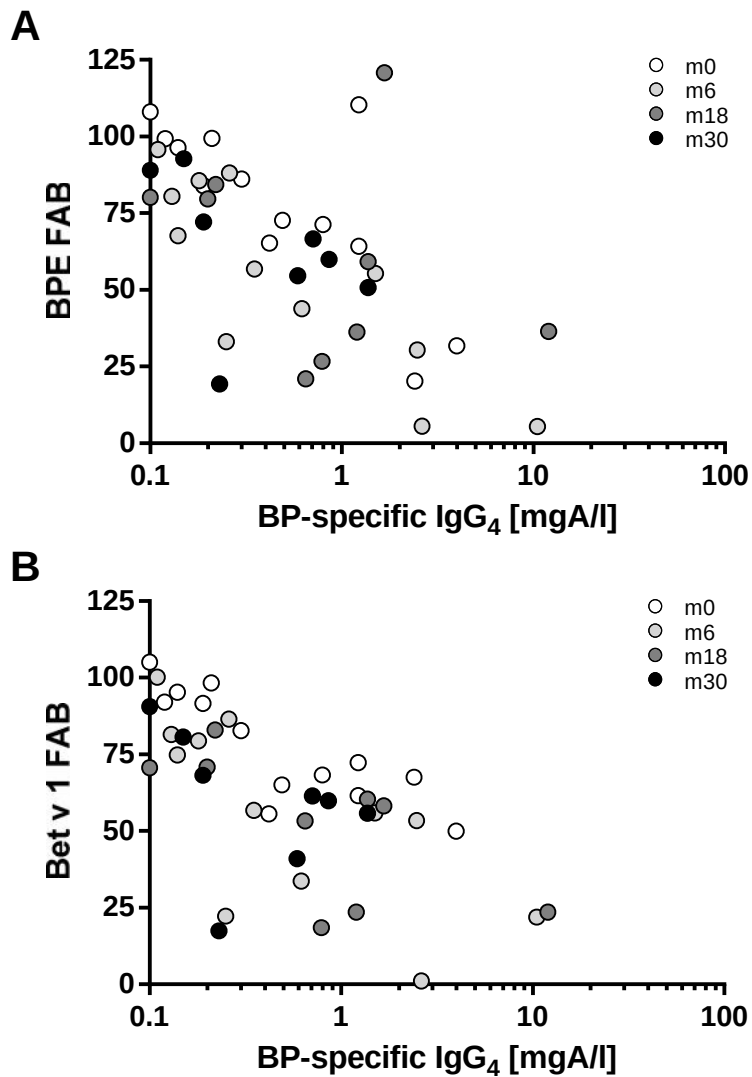


Figure S4. Correlation between birch pollen (BP)-specific IgG4 concentrations and IgE-facilitated allergen binding (IgE-FAB) during allergoid-IT. (A) IgE-FAB with BP extract (BPE; m0: $r_s = -0.66$, $P=0.014$; m6: $r_s = -0.839$, $P<0.001$). (B) IgE-FAB with Bet v 1 (m0: $r_s = -0.82$, $P<0.001$; m6: $r_s = -0.797$, $P=0.002$); as evaluated by the two-tailed Spearman's rank correlation coefficient.