

Allergen immunotherapy with the hypoallergenic B-cell epitope-based vaccine BM32 modifies IL-10- and IL-5-secreting T cells

To the Editor,

Allergen immunotherapy (AIT) is the only curative treatment for immediate-type allergies. However, adverse reactions occur and patient adherence is poor. Therefore, new approaches focus on vaccines with increased immunogenicity but ameliorated allergenicity.¹ With this strategy, therapeutic efficacy should be increased while minimizing the number of administrations and reducing side effects.

Recently, the hypoallergenic grass pollen (GP) allergy vaccine BM32 has been examined in a double-blind placebo-controlled trial and proven both safe and effective in diminishing allergic symptoms of patients with GP allergy.² BM32 consists of non-IgE-binding peptides from the B-cell epitopes of GP allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) fused to an immunogenic carrier protein.³ In contrast to other novel AIT approaches, in this vaccine allergen-specific

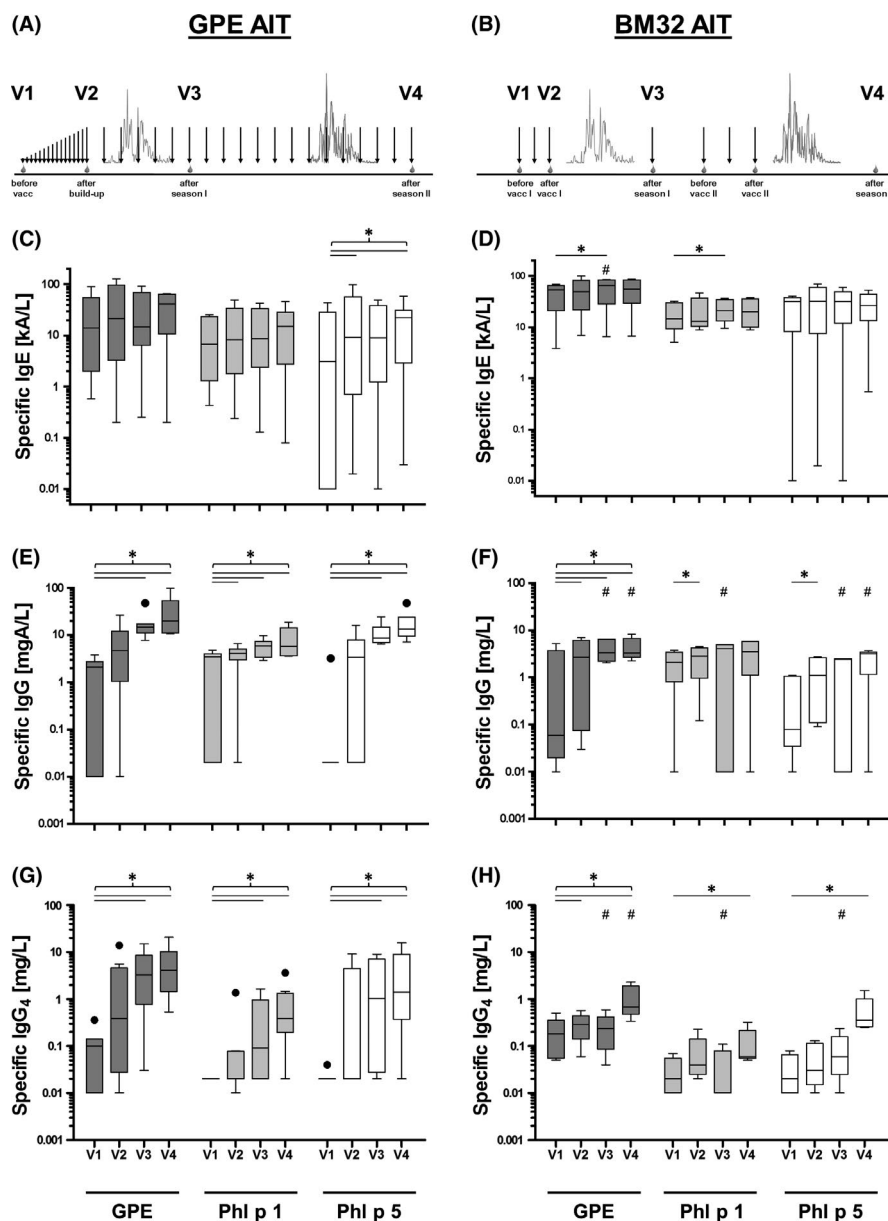


FIGURE 1 Timeline representing grass pollen seasons, points of subcutaneous injections (arrows), and blood sampling before and during GPE-AIT (A) and AIT with BM32 (B), respectively. Concentrations of specific IgE (C, D), IgG (E, F), and IgG₄ (G, H) to grass pollen extract (GPE), Phl p 1 and Phl p 5 in patients treated with either GPE-AIT or BM32-AIT. Differences between baseline (V1) and time-points during AIT (V2, V3, and V4) were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$). Differences between the two cohorts (GPE-AIT vs BM32-AIT) were calculated using the nonparametric Mann-Whitney U test ($^{\#}P < 0.05$). Vacc, vaccination; V, visit

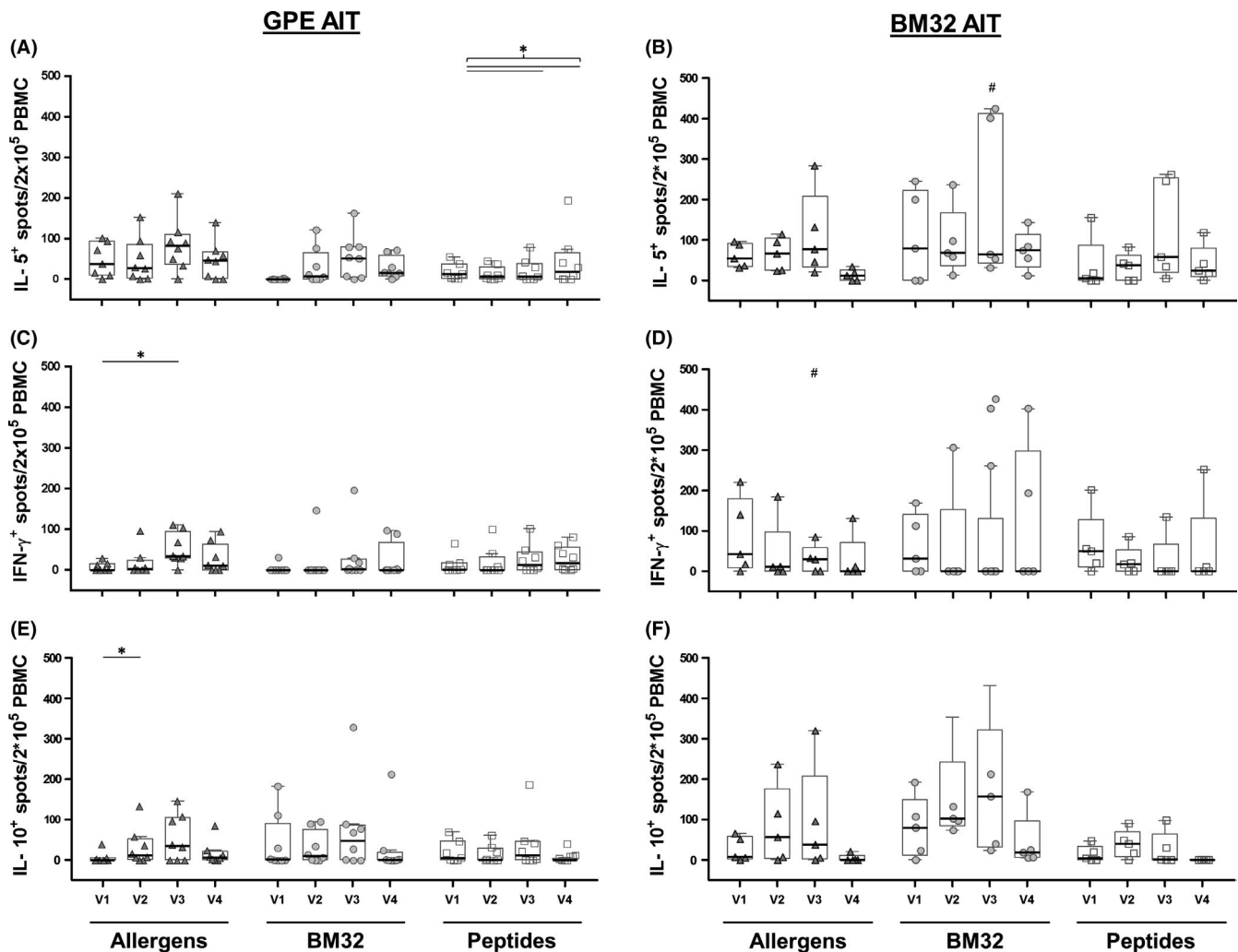


FIGURE 2 Specific frequencies of IL-5- (A, B), IFN- γ - (C, D), and IL-10-secreting T cells (E, F) after stimulation with either GP allergen mix (rPhl p 1, 2, 5, 6), BM32 or hypoallergenic peptides without immunogenic carrier in grass pollen-allergic patients treated with GPE-AIT and BM32-AIT, respectively. Differences between baseline (V1) and time-points during AIT (V2, V3, and V4) were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$). Differences between the two cohorts (GPE-AIT vs BM32-AIT) were calculated using the nonparametric Mann-Whitney U test (# $P < 0.05$)

T-cell epitopes have been reduced and T-cell help is provided by the nonallergenic but immunogenic carrier, PreS from the Hepatitis B virus. To better understand the immune mechanisms induced by AIT with this recombinant allergen vaccine, we investigated humoral and cellular immune responses, in particular focusing on different T-cell subsets, in a single center subgroup of patients with GP allergy treated in the abovementioned trial with BM32 and compared these with the immune alterations caused by subcutaneous AIT with a conventional GP extract (GPE) for which safety and efficacy has been demonstrated in a large double-blind, placebo-controlled clinical study.⁴

Five patients with GP allergy received seven subcutaneous injections of BM32 (Biomay AG) over a time period of 15 months according to the recently published protocol.² Furthermore, eight GP-allergic patients were treated with weekly increasing concentrations of GPE (Alutard SQ grass, ALK-Abelló), as recommended by the manufacturer, until the maintenance dose of 100 000 SQ-U was

reached, followed by monthly subcutaneous injections. Thus, within the same observation period of two years the latter cohort received five times more, that is, a total of 35, injections. Blood was drawn before starting and at different time-points during AIT (Figure 1A,B). Further details on patient characteristics, blood sampling, and preparation of PBMC as well as on evaluation of both allergen-specific antibody responses and T-cell frequencies are provided in the Methods section in this article's Data S1.

By the end of the 2-year observation period, median allergen-specific IgE levels had increased twofold to sevenfold in patients receiving GPE-AIT but not in individuals treated with BM32 (Figure 1C,D, and see Table S1 in the Data S1). On the other hand, serum concentrations of GPE-, Phl p 1-, and Phl p 5-specific IgG (Figure 1E,F) as well as IgG4 antibodies (Figure 1G,H) were enhanced during both AIT types although to a lower amount in the BM32 treatment group. Similar results were seen for Phl p 2- and Phl p 6-specific IgG antibodies, while higher peptide-specific IgG values were detected in

the BM32-AIT group, indicating that BM32 induces an IgG response targeted toward the IgE-binding sites of the allergens (see Figure S2-S5 in the Data S1).

We were specifically interested in potential alterations of T-cell responses and thus closely investigated different allergen-specific T-cell subsets by utilizing a very sensitive ELISPOT assay, stimulating patients' PBMC with either GP allergen (ie, a mixture of equal amounts of recombinant Phl p 1, Phl p 2, Phl p 5, and Phl p 6), BM32, or hypoallergenic GP peptides (ie, the nonallergenic peptides derived from the major IgE-binding sites of the four GP allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6). In the first year of AIT, in both treatment cohorts IL-5-secreting T cells tended to increase (Figure 2A,B), while eventually allergen-specific Th2 cell reactivity in the form of IL-5 production was lost as determined at the end of the second GP season (Figure S1). Furthermore, numbers of IFN- γ -secreting cells stimulated with all three different allergen preparations remained unaltered (Figure 2C,D). However, in contrast to the other T-cell subsets, allergen-specific IL-10-secreting T cells temporarily increased at the beginning of AIT in both groups (Figure 2E,F), returning to pretreatment levels during the first year. Interestingly, there was a strong induction of IL-10-secreting cells noticed in PBMC samples of BM32-treated patients after stimulation with BM32 containing the hepatitis B-derived PreS protein which was also observed for a similar PreS-based birch pollen vaccine.⁵

It has been shown that AIT with conventional allergen extracts results in distinct changes of allergen-specific T cells, namely transient induction of regulatory T cells and loss of Th2 cell reactivity.^{6,7} We here show that AIT with BM32 similarly modifies allergen-specific T-cell responses, thus confirming the immunogenicity of this B-cell epitope-based vaccine. Interestingly, although the BM32 vaccine does not contain T-cell epitopes specific for GP allergens, not only peptide-specific but also allergen-specific T cellular changes were detectable in BM32-treated patients. The mechanistic aspects of BM32 on B cells and how PreS affects T-cell responses are demonstrated in detail in the study by Ziegelmayer et al.⁸ Of note, while GPE-AIT resulted in an induction of allergen-specific IgE, their serum concentrations remained rather stable in BM32-treated patients pointing toward a lower allergenicity of the recombinant vaccine.

In summary, this study, although limited by low patient numbers, gives first evidence for comparable immune mechanisms by which AIT with the hypoallergenic BM32 vaccine establishes allergen tolerance in relation to a conventional allergen extract. This entails both the induction of IL-10-secreting T cells and the loss of IL-5-producing Th2 cells as important hallmarks of tolerance induction,^{7,8} while IFN- γ -releasing cells were merely unaltered. Remarkably, in addition to these immune alterations also caused by mere T-cell epitope-based peptide therapies,⁹ this new approach utilizing allergen-derived B-cell epitopes also results in a lasting allergen-specific IgG antibody response. Thus, while AIT with BM32 implies substantially fewer injections, the short, safe, and simple treatment protocol represents an interesting therapeutic alternative for patients suffering from GP allergy.

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CONFLICTS OF INTEREST

R. Henning is employed by and has received stock/stock options from Biomay. R. Valenta has received research grants from Biomay AG; has consultant arrangements with Biomay AG and Viravaxx, Vienna, Austria; and has received payment for development of educational presentations from Thermo Fisher. M. Focke-Tejkl has received a grant from the Austrian Science Fund (FWF P29991). W. Pfützner has received grants from Biomay AG and ALK-Abelló and has consultant arrangements with ALK-Abelló, Novartis, and Thermo Fisher. The rest of the authors declare that they have no relevant conflicts of interest.

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SUPPORTING INFORMATION

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

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Effect of *Lactobacillus plantarum* YIT 0132 on Japanese cedar pollinosis and regulatory T cells in adults

To the editor,

Japanese cedar pollinosis (JCPsis) is a major health problem that has increased over recent decades and impairs daily activities.¹ Besides the standard treatments specified in the guidelines,² some strains of lactic acid bacteria (LAB), such as *Bifidobacterium* and *Lactobacillus*, have been shown to improve pollinosis symptoms.^{3,4} Although LAB are believed to induce regulatory T (Treg) cells, thereby alleviating allergic symptoms,⁴ whether LAB augment Treg cell abundance, together with clinical symptoms, remains unclear.

Lactobacillus plantarum YIT 0132 (LP0132), derived from fermented food, induces a high level of IL-10 secretion from murine macrophages.⁵ Previously, LP0132-fermented citrus juice, which contained approximately 8.0×10^{10} heat-killed LP0132 cells/100 mL, showed therapeutic potential to improve JCPsis-associated symptoms.⁵ In this trial, we further evaluated the efficacy of this beverage for the treatment of JCPsis, in association with immunological parameters including peripheral Treg cells in the pollen season.

We randomly assigned 100 adults with JCPsis to consume 125 mL of fermented citrus juice containing heat-killed LP0132 (LP0132, n = 50) or unfermented citrus juice (placebo, n = 50) for 8 weeks from mid-February to mid-April 2017, from the beginning to the end of the pollen season (Figure S1). Throughout the trial period, the subjects were asked to avoid taking LAB-containing food and keep daily records of their consumption of beverages, medications, and medical visits, and to perform a weekly self-assessment of JCPsis symptoms based on the Japanese Rhino-conjunctivitis Quality of Life Questionnaire.² The primary outcome was total symptom score (TSS) in March (predicted peak season). Peripheral blood samples were obtained at enrollment, in late March, and during follow-up to evaluate the percentage of CD4 + CD25 + Foxp3 + Treg cells. Detailed methods and statistical analyses are described in supporting information. P-values <.05 were considered statistically significant. The trial protocol was approved by the Ethics Committee of Shimoshizu National Hospital, in accordance with the Declaration of Helsinki, and was registered at the University Hospital Medical Information Network (UMIN000025924). Written-informed consent was obtained from all subjects.

Online Repository

METHODS

Patients

Subjects receiving AIT with BM32 (n=5; 26 (22-33 years); 2 female) were part of a multicenter, double-blind, placebo-controlled, parallel-group, prospective study to investigate the safety and efficacy of 2 years of treatment with BM32 in patients with grass pollen (GP) allergy with allergic rhinitis, mild asthma, or both (1). The trial has been registered under EudraCT no. 2012-000442-35 and ClinicalTrial.gov Identifier NCT01538979. BM32-treated patients received in the first year three pre-seasonal injections, followed by one injection in autumn. In the next year, patients again received three pre-seasonal injections. Blood was drawn before and after the three-injection interval, and after the two subsequent grass pollen seasons.

In addition, eight patients with GP allergy (n=8; 30 (21-55 years); 5 female) were treated with subcutaneous AIT with a conventional GP extract (GPE; Alutard SQ grass, ALK-Abelló, Hørsholm, Denmark) and served as a comparator cohort. These patients received weekly injections of grass pollen extract until the maintenance dose of 100,000 SQ-U was reached followed by monthly injections of the maintenance dose. Inclusion criteria for the patients treated with GPE-AIT were a history of GP allergy with moderate-to-severe allergic rhinoconjunctivitis and/or mild-to-moderate allergic asthma, positive skin prick test reactivity to GPE (ALK Prick SQ, ALK-Abelló, Hørsholm, Denmark), and specific IgE to GPE, Phl p 1 and/or Phl p 5 (Phadia ImmunoCAP System, Thermo Fisher Scientific, Uppsala, Sweden; Table E1). Patients with perennial symptoms and chronic and/or non-allergic asthma were not included. 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.

Blood sampling and preparation of PBMC

Peripheral blood was drawn in citrate-phosphate-dextrose-adenine (CPDA)-containing tubes at defined time points before and during AIT. PBMC were isolated by Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. Initial gradual freezing of 10^7 PBMC/mL in 90% heat-inactivated fetal calf serum (FCS, PAA

Laboratories, Pasching, Austria) and 10% dimethyl sulfoxide (Sigma, Schnellendorf, 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.

Determination of allergen-specific antibody concentrations

Concentrations of specific IgE, IgG and IgG4 to GPE, Phl p 1 and Phl p 5 were quantified in patients' sera by the Phadia ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

Measurement of specific IgG reactivity

ELISA plates (Greiner, Kremsmünster, Austria) were coated with rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6 or their respective peptides dissolved in carbonate buffer, pH 9.6 (5µg/mL) for 1h at 37°C and 2h at room temperature. Plates were washed three times with PBS-T (PBS 0.05% Tween 20) and blocked with 2% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) in PBS-T at 4°C overnight. ELISA plate-bound allergens were exposed to each patients' serum diluted 1:100 in PBS-T with 0.5% BSA for 2h at 37°C and 1h at room temperature. After washing five times with PBS-T, bound human IgGs were detected with rabbit anti-human Pan IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Plates were incubated overnight at 4°C, washed 5 times with PBS-T and bound human IgG antibodies were detected with an anti-rabbit IgG HRP linked whole antibodies from donkey (GE Healthcare, UK Limited) for 1h at 37°C and 4°C. Plates were washed four times with PBS-T and the color reaction was determined by addition of staining solution ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, St.Louis, Missouri, USA; 100µl/well). The optical density was measured using an ELISA Reader (Dynatech, Denkendorf, Germany) at 405nm. Results are displayed as mean OD values of duplicate determinations with an SD of less than 5% for IgG responses. Plate-to-plate normalization was obtained by including a positive serum control (serum from a grass pollen allergic patient sensitized to Phl p 1, Phl p 2, Phl p 5 and Phl p 6) and a negative control (buffer without serum) on each of the ELISA plates. The mean optical density (OD) for the positive control of each plate is determined as well as the mean OD of the positive control on all plates. By dividing the individual OD of the positive control by the mean OD for all plates, the correction

factor for the individual plate is estimated. All wells of this individual plate are divided by the individual correction factor to ensure optimal comparability.

ELISpot assay

The ELISpot assay was performed as described previously (2). Briefly, frozen PBMC were thawed and cultured at a concentration of 10^6 PBMC/mL with either recombinant allergens (rPhl p 1, 2, 5, and 6; $1\mu\text{g/mL}$ each), BM32 ($1\mu\text{g/mL}$) or hypoallergenic peptides without immunogenic carrier ($1\mu\text{g/mL}$ each; all Biomay) or left unstimulated. After 48h of culture, IL-2 (10U/mL ; Roche, Mannheim, Germany) and IL-7 (10ng/mL ; Miltenyi Biotech, Bergisch Gladbach, Germany) were added to expand allergen-specific T cells. On day 8, PBMC were restimulated with $1\mu\text{g/mL}$ of the respective allergen(s) on an 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.

Pollen analysis

As part of the Network of the Foundation German Pollen Information Service (Stiftung Deutscher Polleninformationsdienst – PID) local pollen (Marburg: 50.81442°N , 8.80794°E) was collected by using the Burkard volumetric spore trap (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK). The airborne pollen was trapped on an adhesive tape at a constant rate of 10L/min . The tape was mounted on a rotating drum (2mm/h) driven by a clockwork mechanism which enabled continuous sampling for up to seven days. For pollen analysis the exposed tape was mounted on slides, and the trapped pollen grains were classified and counted by light microscopy. The amount of pollen was expressed as number of pollen grains/ m^3 air. The begin of the local grass pollen season was defined as the first day of 5 days (out of 7 consecutive days) each of these 5 days with ≥ 3 pollen/ m^3 and with the a sum of these 5 days of ≥ 30 pollen/ m^3 according to Pfaar et al. (3). Accordingly, the end of the local grass pollen season was specified as the last day of a series of 5 days (out

of 7 consecutive days) with ≥ 3 pollen/m³ and with the a sum of these 5 days of ≥ 30 pollen/m³.

Statistical analysis

Because of the nonparametric distribution of data, median values $\pm$ range were depicted as box-and-whisker plots. Differences between baseline (V1) and time-points during AIT (V2, V3 and V4) were determined by the nonparametric Wilcoxon signed-rank test. Differences between the two cohorts (GPE-AIT vs. BM32-AIT) at each follow-up time-point (V2, V3 and V4) were calculated using the nonparametric Mann–Whitney U-test after subtracting the individual baseline values from the respective follow-up. 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).

1 **TABLE E1.** IgE antibody concentrations

		GPE AIT		BM32 AIT	
		V1	V4	V1	V4
IgE (kUA/l)	GPE	13.9 (0.58-89.4)	41.4 (0.2-64.7)	53.4 (3.83-68.2)	55.7 (6.66-85.9)
	Phl p 1	6.84 (0.43-25.6)	15.1 (0.08-45.6)	14.4 (5.08-32)	19.8 (8.93-37.4)
	Phl p 5	3.11 (0.01-43.1)	22.2 (0.03-58.9)	31.8 (0.01-40.5)	26.7 (0.55-52.4)

2

3 Data is represented as median with range.

Supplementary figure legends

Supplementary Figure 1. Specific frequencies of IL-5- secreting T cells after stimulation with either GP allergen mix (rPhl p 1, 2, 5, 6), BM32 or hypoallergenic peptides without immunogenic carrier in grass pollen-allergic patients treated with BM32-AIT.

Supplementary Figure 2. Comparison of IgG reactivity to recombinant Phl p 1 and Phl p 1-derived peptides (Phl p 1-1 to Phl p 1-5) in sera of patients with grass pollen (GP) allergy by means of ELISA. Patients received allergen immunotherapy (AIT) with either grass pollen extract (GPE) or BM32. Differences between baseline (V1) and time-points during AIT (V2, V3 and V4) were determined by the nonparametric Wilcoxon signed-rank test (** $P < 0.01$). Differences between the two cohorts (GPE-AIT vs. BM32-AIT) were calculated using the nonparametric Mann–Whitney U-test ($^{\#}P < 0.05$).

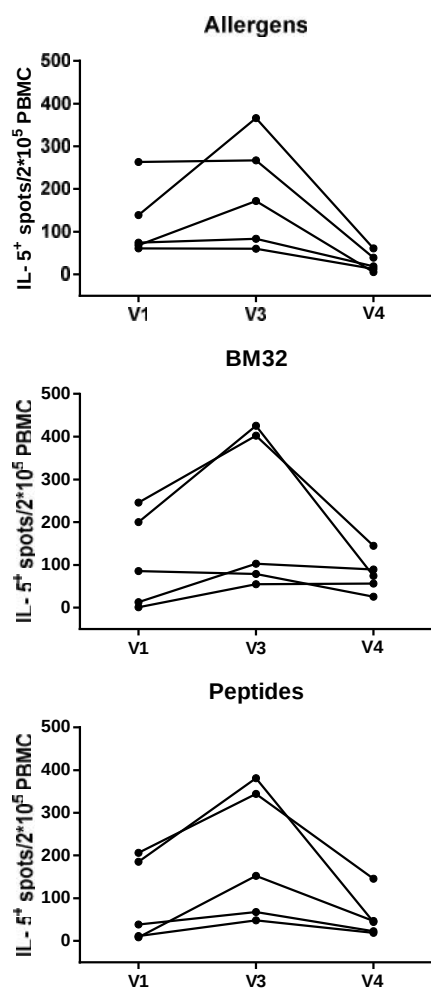
Supplementary Figure 3. Comparison of IgG reactivity to recombinant Phl p 2 and Phl p 2-derived peptides (Phl p 2-1 to Phl p 2-4) in sera of patients with grass pollen (GP) allergy by means of ELISA. Patients received allergen immunotherapy (AIT) with either grass pollen extract (GPE) or BM32. Differences between baseline (V1) and time-points during AIT (V2, V3 and V4) were determined by the nonparametric Wilcoxon signed-rank test (** $P < 0.01$). Differences between the two cohorts (GPE-AIT vs. BM32-AIT) were calculated using the nonparametric Mann–Whitney U-test ($^{\#}P < 0.05$).

Supplementary Figure 4. Comparison of IgG reactivity to recombinant Phl p 5 and Phl p 5-derived peptides (Phl p 5-1 to Phl p 5-7) in sera of patients with grass pollen (GP) allergy by means of ELISA. Patients received allergen immunotherapy (AIT) with either grass pollen extract (GPE) or BM32. Differences between baseline (V1) and time-points during AIT (V2, V3 and V4) were determined by the nonparametric Wilcoxon signed-rank test (* $P < 0.05$). Differences between the two cohorts (GPE-AIT vs. BM32-AIT) were calculated using the nonparametric Mann–Whitney U-test ($^{\#}P < 0.05$).

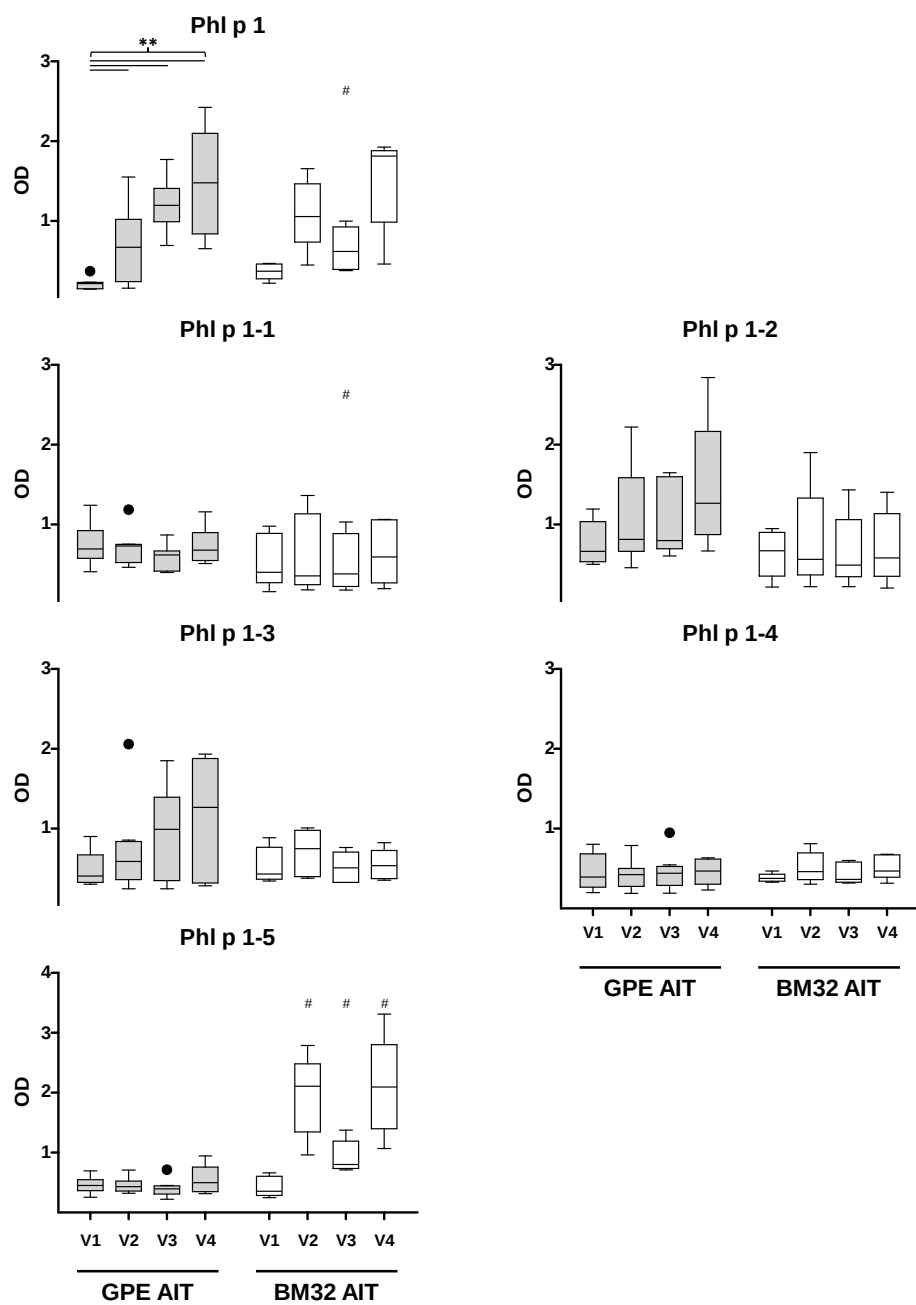
Supplementary Figure 5. Comparison of IgG reactivity to recombinant Phl p 6 and Phl p 6-derived peptides (Phl p 6-1 to Phl p 6-4) in sera of patients with grass pollen (GP) allergy by means of ELISA. Patients received allergen immunotherapy (AIT) with either grass pollen extract (GPE) or BM32. Differences between baseline (V1) and time-points during AIT (V2, V3 and V4) were determined by the nonparametric Wilcoxon signed-rank test (** $P < 0.01$). Differences between the two cohorts (GPE-AIT vs. BM32-AIT) were calculated using the nonparametric Mann–Whitney U-test ([#] $P < 0.05$).

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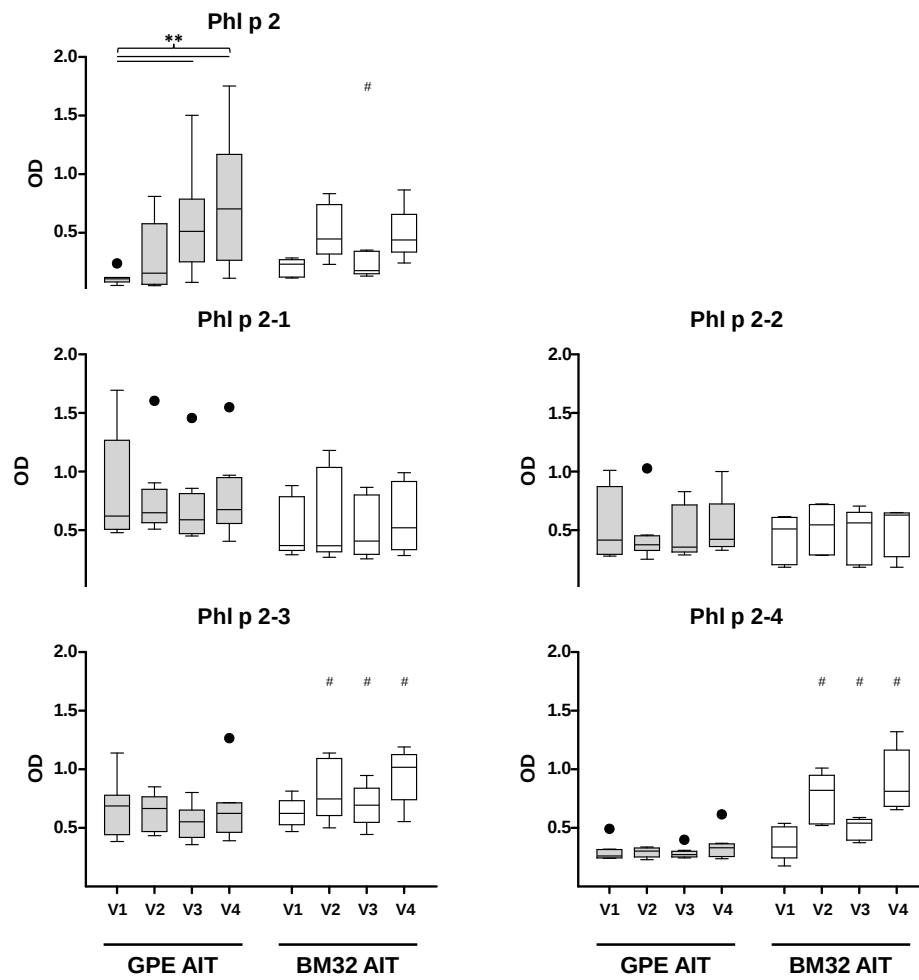
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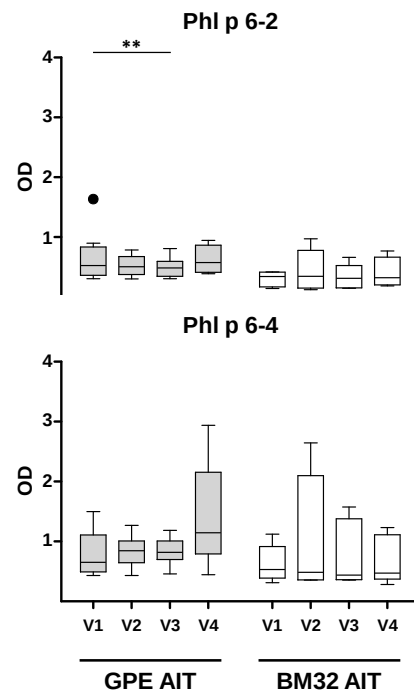
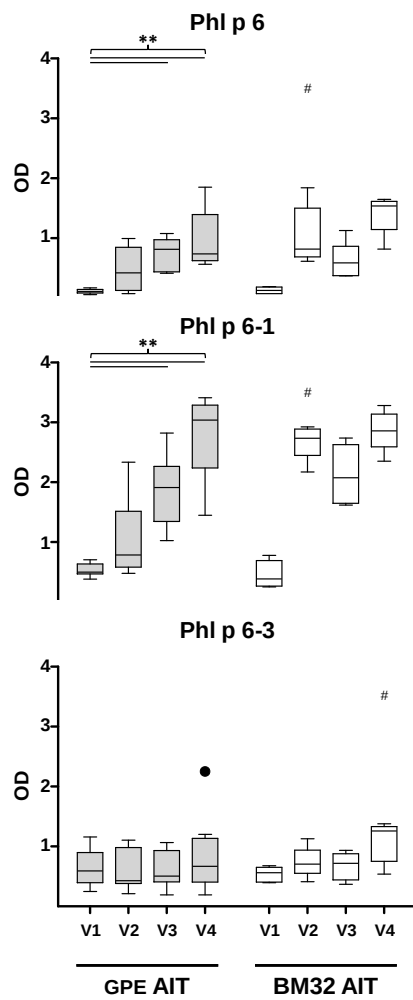
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 5