

Short-Term Preseasonal Immunotherapy: Is Early Clinical Efficacy Related to the Basophil Response?

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Key Words

Allergic rhinitis · Allergoids · Basophil activation ·
Grass pollen allergy · Short-term immunotherapy

Abstract

Background: An aluminum hydroxide-adsorbed depot allergoid preparation of six-grass pollen allergens has been developed for short-term preseasonal immunotherapy in pollinosis. However, only limited knowledge exists about its immunological and clinical effects. The aim of this study was to evaluate the basophil response, which can explain early clinical findings of short-term preseasonal allergoid immunotherapy in allergic rhinitis. **Methods:** In a double-blind, placebo-controlled study, 31 patients allergic to grass pollens received one course of short-term preseasonal allergoid immunotherapy or placebo. Immunogenicity was assessed by the levels of specific IgG4, IgE antibodies and an allergen-induced CD203c basophil activation test. The primary clinical end point was the combined symptom and medication score/average combined score (ACS). **Results:** There was a 52.9% difference in ACS between the treatment and placebo groups in favor of immunotherapy ($p = 0.01$). Active treatment induced *Phleum pratense*-specific IgG4 and IgE antibodies ($p < 0.05$). A decrease in allergen-induced basophil activation at submaximal allergen concentrations was dem-

onstrated at the end of immunotherapy and at the peak of the grass pollen season after immunotherapy. **Conclusions:** This study shows that grass pollen-allergic patients treated with one course of short-term preseasonal allergoid immunotherapy exhibit a decrease in allergen-induced basophil activation, an increase in allergen-specific IgG4 antibodies and early clinical improvement.

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Introduction

The efficacy of allergen-specific immunotherapy has been reported with different allergens including grass pollens [1, 2], which are a major cause of pollinosis in many parts of the world [3, 4]. Despite the benefits of immunotherapy, there is still a need for safer and more effective allergen-specific immunotherapy strategies [5]. The use of chemically altered allergens, allergoids, recombinant allergens and relevant T-cell epitope peptides are all approaches that have yielded positive results [5]. Most of the information on the immunological mechanisms of immunotherapy has been derived from conventional immunotherapy studies, but the data on the effects of allergoid immunotherapy are insufficient [6]. Recently, the basophil activation test has proved to be a useful tool for

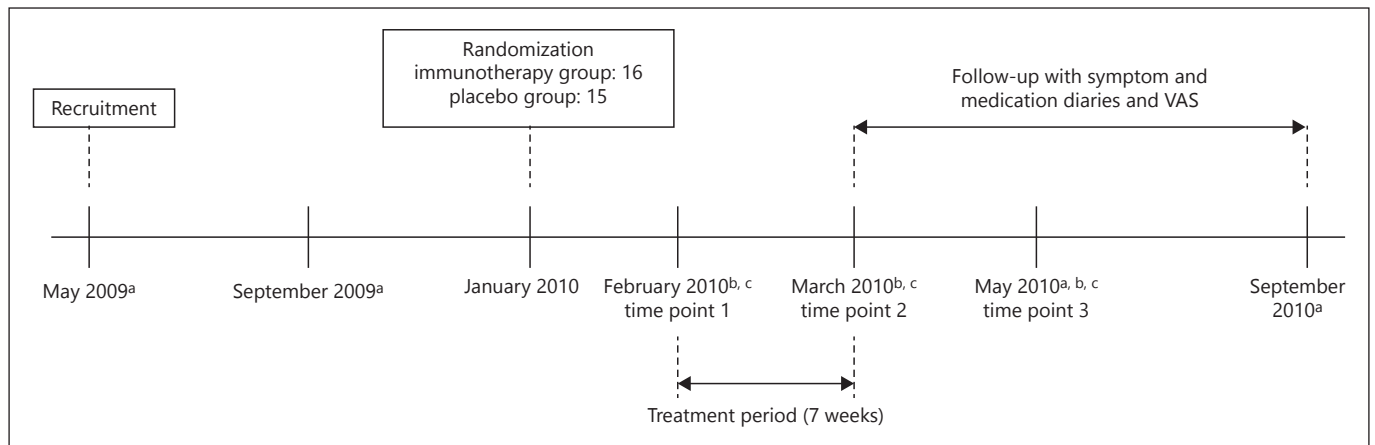


Fig. 1. The design and time course of the study. ^a RQLQ; ^b Basophil activation test utilizing CD203c; ^c *P. pratense*-specific IgG4 and IgE.

allergy diagnosis and several studies also support its usefulness in monitoring immunotherapy [7, 8]. High sensitivity and specificity values have been obtained with this test in the diagnosis of grass pollen allergy [9, 10].

In this placebo-controlled, randomized study, we investigated the influence of short-term, preseasonal grass pollen allergoid immunotherapy on allergen-induced basophil activation and *Phleum pratense*-specific IgG4 and IgE levels in patients with allergic rhinitis (AR). We also examined the clinical efficacy of this immunotherapy strategy.

Material and Methods

Subjects

Thirty-one patients were recruited in 2009 according to the following criteria: a history of seasonal AR caused by grass pollens, a positive result for the skin-prick test (i.e. a wheal diameter of at least 3 mm or greater than that of the negative control) to grass pollen extracts and no sensitization to inhaled allergens other than pollens. All of the participants underwent skin-prick tests for a standard panel of common aeroallergens including grass mix (*Dactylis glomerata*, *Lolium perenne*, *P. pratense*, *Poa pratensis* and *Festuca pratensis*), cereal mix (*Hordeum vulgare*, *Avena sativa* and *Triticum sativa*), *Secale cereale*, weeds (*Artemisia vulgaris*, *Urtica dioica*, *Taraxacum vulgare*, *Plantago lanceolata* and *Chenopodium album*), trees (*Betula verrucosa*, *Fagus silvatica*, *Quercus robur*, *Platanus orientalis*, *Salix caprea*, *Populus alba*, *Ulmus scabra*, *Alnus glutinosa* and *Coryllus avellana*), mold mix 1 (*Alternaria alternata*, *Cladosporium herbarum*, *Botrytis cinerea*, *Curvularia lunata*, *Fusarium moniliforme* and *Helminthosporium halodes*), mold mix 2 (*Aspergillus fumigatus*, *Mucor mucedo*, *Penicillium notatum*, *Pullularia pullulans*, *Rhizopus nigricans* and *Serpula lacrymans*), feather mix, cat and dog dander, house-dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) and *Blatella germanica* (Allergopharma, Reinbek, Germany). According to the skin-prick test

results, 3 patients were sensitized to grass pollens only and the remaining 28 to weed and/or tree pollens in addition to grass pollens. The clinical classification of AR was 'moderate-to-severe persistent' in all subjects. All of the patients had a methacholine PC₂₀ level of >8 mg/ml, and no symptoms suggestive of asthma. All subjects gave their written informed consent and the Local Ethics Committee at the Ankara University, Ankara, Turkey, approved the protocol.

Study Design

The study was designed as a double-blind, randomized, placebo-controlled study. The trial was registered at the Australian New Zealand Clinical Trials Registry (registration No. ACTRN12613000484741). The immunotherapy product was a preparation of extracts of grass pollens (*Holcus lanatus*, *D. glomerata*, *L. perenne*, *P. pratense*, *P. pratensis* and *F. pratensis*) treated with formaldehyde to produce an allergoid and then adsorbed onto aluminium hydroxide (Allergovit®, Allergopharma). It was supplied in two concentrations, strength-A, at 1,000 therapeutic units (TU)/ml and strength-B, at 10,000 TU/ml. The placebo solution contained physiological saline. Subcutaneous injections commenced with 0.1 ml of strength-A in February 2010, followed by an approximate doubling of the dose weekly up to 0.6 ml of strength-B. The standard regimen comprised seven injections. Dose adjustments were made according to individual tolerance. The time course of the study and the clinical and laboratory investigations performed are outlined in figure 1.

Assessment of Clinical Efficacy

All patients kept a symptoms and medication diary during the pollen season, i.e. 1 March to 1 September 2010; this period was determined according to a previous study performed in our study center [11]. Nasal (itching, sneezing, discharge and obstruction) and ocular (itching or eyes watering) symptoms were recorded separately on a scale of 4: 0 = no symptoms and 3 = severe symptoms. The average rhinoconjunctivitis total symptom score (ARTSS) was calculated as the mean of the daily total symptom score [12]. For rescue medication, patients were instructed to use a stepwise regimen (step 1: 5 mg of oral desloratadine, step 2: fluti-

Table 1. Characteristics of the study population

	Immunotherapy (n = 16)	Placebo (n = 15)	p value
Gender			0.36
Female	7	9	
Male	9	6	
Age, years (mean $\pm$ SD)	28.0 $\pm$ 9.3	31.9 $\pm$ 10.0	0.27
Median duration of rhinitis, years (min–max)	4.0 (2.0–30.0)	7.0 (3.0–30.0)	0.10
Results of skin-prick testing, mm ^a	9.8 $\pm$ 3.5	10.7 $\pm$ 4.3	0.53

^a Values are the mean $\pm$ SD wheal diameter (to a mixture of six grasses).

casone furoate nasal spray and step 3: 4 mg of oral metilprednisolone). Medication scores (MS) were assigned as follows: 0 = no medication, 1 = desloratadine, 2 = nasal fluticasone furoate and 3 = oral metilprednisolone. The highest score for a given day was recorded as the MS. The average combined score (ACS) was calculated as the mean of the ARTSS and the MS [12]. In addition, patients assessed the severity of their allergic symptoms for each month during the pollen period on a visual analog scale (VAS, consisting of a 10-cm line: 0 = no symptoms and 10 = highest level of symptoms). The primary clinical end point was the area under the curve (AUC) of the daily ACS over the pollen period. Secondary clinical end points were the quality of life [assessed with the Turkish version of the Juniper rhinoconjunctivitis quality of life questionnaire (RQLQ)] [13, 14], the AUCs of the daily ARTSS and MS over the pollen period and the VAS scores. The immunotherapy group was compared to the placebo group with respect to ACS, ARTSS, MS and VAS scores. The ratings from the Juniper RQLQ performed in the last grass pollen season before the treatment were compared with the RQLQ performed after the treatment (i.e. May 2009 vs. May 2010 and September 2009 vs. September 2010).

Determination of Specific IgE and IgG4 Levels

P. pratense-specific IgE and IgG4 antibody concentrations were measured before and after treatment and at the peak of the pollen season. The levels of specific IgE and IgG4 were quantified using the CAP fluoroenzyme immunoassay system according to the recommendations of the manufacturer (Phadia, Uppsala, Sweden).

Evaluation of Basophil Activation via Measurement of CD203c Expression

Basophil activation tests were performed before and after treatment and at the peak of the pollen season. Whole venous blood samples (4 ml) from all subjects were drawn into EDTA-containing tubes (Vacutainer, Becton Dickinson) at the same time of day. Tests were performed within 2 h of blood sampling. CD203c expression as a marker was evaluated using the Allergenkit (Beckman Coulter, Marseille, France) according to the manufacturer's instructions (and our previous study) [15]. Briefly, 100 μ l venous blood was stimulated with 4 different concentrations of grass pollen extract (Allergopharma) in polypropylene tubes. The extract comprised *P. pratense*, *L. perenne*, *H. lanatus*, *D. glomerata*, *P. pratensis* and *F. pratensis*, prepared from raw material. The concentrations were expressed as the content of the major allergen Phl p 5. The

extract was diluted to final concentrations of 45.1, 4.5, 4.5×10^{-4} and 4.5×10^{-5} μ g/ml of Phl p 5, in phosphate-buffered saline (Sigma Diagnostics, St. Louis, Mo., USA); these concentrations, which induce maximal and submaximal cell stimulation, were determined in our previous study [9]. In order to evaluate background basal values with no stimulation (negative control), phosphate-buffered saline was added to whole blood. Monoclonal anti-IgE antibody was used as a positive control. Flow cytometric analysis (FC500 Cytomics, Beckman Coulter, Miami, Fla., USA) was performed using a three-color staining method (CRTH-2-FITC/CD203c-PE/CD3-PC7) and the data were analyzed with CXP2.2 software. Cells expressing both CRTH-2-FITC and CD203c-PE were identified as basophils. At least 500 basophils were counted in each assay. Results were expressed as the percentage of activated basophils according to the upregulation of CD203c expression.

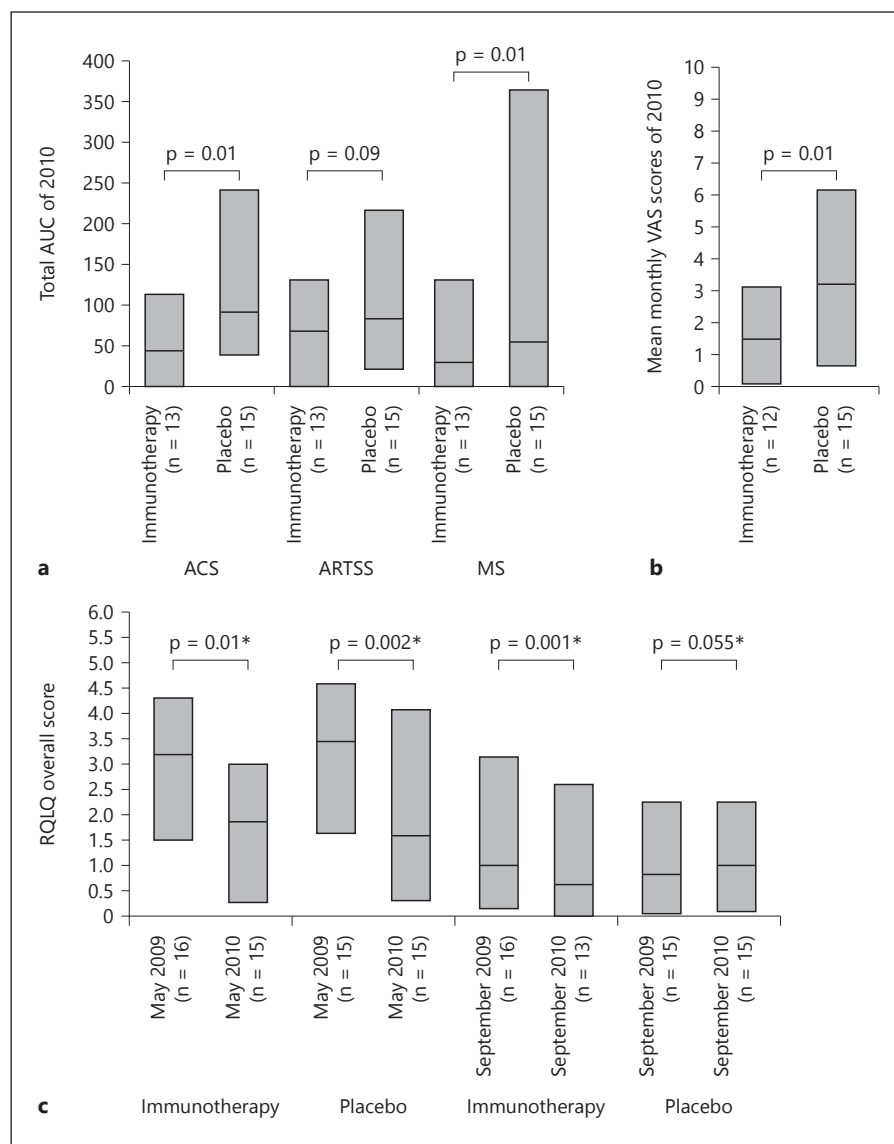
Statistical Analysis

Comparisons between groups were performed using the Student t test or the Mann-Whitney U test. Evaluations of group-time comparisons of specific IgE, IgG4 and basophil CD203c expression were performed using a macro developed for F1-LD-F1 design with the SAS9.0 program [16]. ANOVA-type statistics were used to evaluate the significance of the factors. Percentage changes were analyzed using the OWL method, the nonparametric counterpart of one way analysis of variance (Kruskal-Wallis method). Pairwise comparisons within the groups were made with the Wilcoxon signed-rank test. The Bonferroni correction was applied for the statistical significance of pairwise comparisons. The Spearman correlation test was used for correlation studies.

Results

Demographic and baseline characteristics showed no relevant differences between the 2 groups (table 1). All patients concluded the treatment period. Reactions to injections were all local and mild. Three patients in the immunotherapy group withdrew before the end point of the study; 2 of them were lost to follow-up and 1 withdrew for reasons not related to the treatment or the study.

Fig. 2. a–c Primary and secondary end points of clinical efficacy. The boxes represent the values from the minimum to the maximum and the line within the box indicates the median. * Wilcoxon signed-rank test with the Bonferroni correction.



Clinical Efficacy

The ACS scores over the pollen period were significantly lower in the immunotherapy group than the placebo group ($p = 0.01$). The difference between the 2 groups described by the median AUC of the ACS was 52.9% in favor of immunotherapy. No significant difference was found between groups in the ARTSS ($p = 0.09$). MS values were significantly lower in the immunotherapy group ($p = 0.01$). The mean monthly VAS scores during the pollen season of the active group were also significantly lower ($p = 0.01$; fig. 2).

There was a significant improvement in overall RQLQ scores at the peak of the grass pollen period between 2009 and 2010 in both groups ($p = 0.01$, $p = 0.002$). At the end

of the grass pollen season ($p = 0.001$), these scores had reduced significantly in the immunotherapy group, but no significant change was detected in the placebo group ($p = 0.055$; fig. 2).

Basophil Activation

The basophil CD203c expression responses to the positive and negative controls determined before treatment (i.e. at time point 1), at the end of treatment (time point 2) and at the peak of the pollen period (time point 3) were similar in the immunotherapy and placebo groups ($p = 0.23$ and $p = 0.54$, respectively; fig. 3). Basophil activation responses to the 4.5×10^{-4} and 4.5×10^{-5} $\mu\text{g/ml}$ allergen concentrations decreased significantly in the immuno-

Fig. 3. The basophil CD203c response to the positive and negative controls and to 45.1, 4.5, 4.5×10^{-4} and 4.5×10^{-5} $\mu\text{g}/\text{ml}$ of allergen extract. The central box represents the values from the lower to the upper quartile (25th and 75th percentile), the line within the box indicates the median and the whiskers show the 5th and 95th percentile.

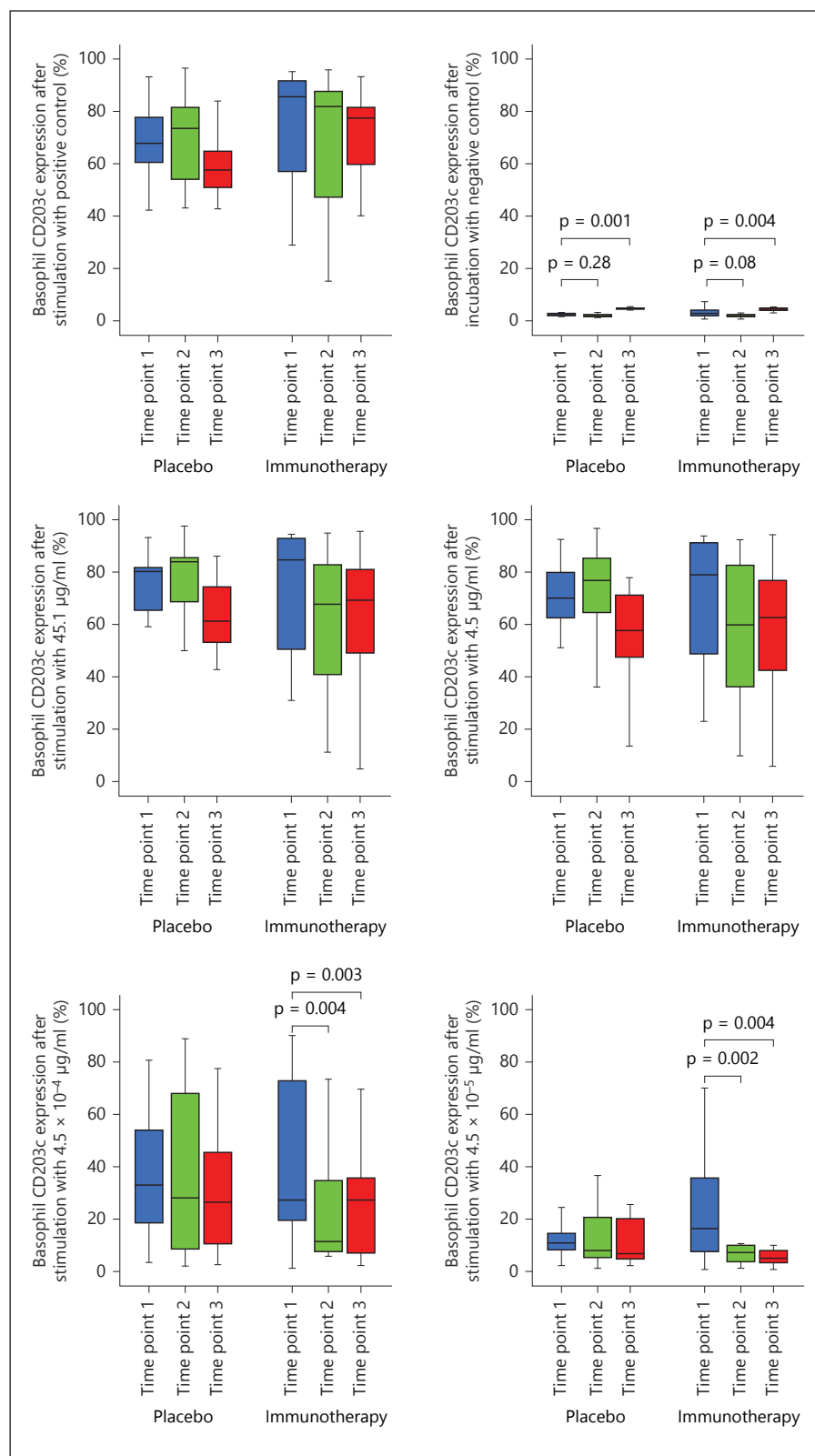


Table 2. Basophil CD203c responses (%) after in vitro stimulation with 2 allergen extract concentrations measured at time points 1–3

Allergen extract concentration	Time point 1	Time point 2	Time point 3	Δ between time points 1 and 2 (%)	Δ between time points 1 and 3 (%)
<i>4.5 × 10⁻⁴ μg/ml</i>					
Immunotherapy					
Median	27.3	11.7	27.6	-1.9	-3.5
Min-max	1.6–90.2	6.0–73.5	2.8–69.8	-61.9–693.7	-76.1–256.2
Placebo					
Median	33.3	28.4	26.5	72.7	50.2
Min-max	3.7–80.8	2.3–89.0	2.9–77.9	-44.5–172.7	-22.4–438.2
p value		0.68 [†] 0.001 [‡] 0.03 [§]		0.006 [†]	0.057 [†]
<i>4.5 × 10⁻⁵ μg/ml</i>					
Immunotherapy					
Median	16.6	7.5	5.0	-57.9	-72.1
Min-max	0.7–80.5	1.4–31.0	0.8–23.8	-96.7–1,442.9	-98.9–571.4
Placebo					
Median	11.1	8.1	6.9	25.2	-9.5
Min-max	2.3–28.4	1.2–78.3	2.4–69.7	-87.5–175.7	-68.0–464.6
p value		0.46 [†] 0.003 [‡] 0.02 [§]		0.002 [†]	0.01 [†]

[†] Immunotherapy vs. placebo. [‡] Comparison of the 3 time points. [§] Group-time interaction.

therapy group at the end of the treatment ($p = 0.004$ and $p = 0.002$, respectively), and stayed similarly low at the peak of the pollen period after immunotherapy ($p = 0.003$ and $p = 0.004$, respectively) compared to at baseline (fig. 3). However, basophil activation after stimulation with 45.1 and 4.5 μg/ml of allergen extracts did not significantly change at any of the 3 time points in the immunotherapy group (fig. 3). In the placebo group, there were no significant changes at the 3 time points in the basophil CD203c response to 45.1, 4.5, 4.5×10^{-4} and 4.5×10^{-5} μg/ml of allergen extract compared to at baseline (fig. 3). The changes in basophil CD203c expressions from the values at baseline (Δ CD203c) are shown in table 2. The difference between the immunotherapy and placebo groups in terms of Δ CD203c at 4.5×10^{-5} μg/ml concentration was statistically significant (table 2).

P. Pratense-Specific IgG4 and IgE Levels

After treatment, *P. pratense*-specific IgG4 levels had a median 216.6% increase in the active group whereas a median 5.4% decrease was observed in the placebo group. The difference between the 2 groups was significant ($p <$

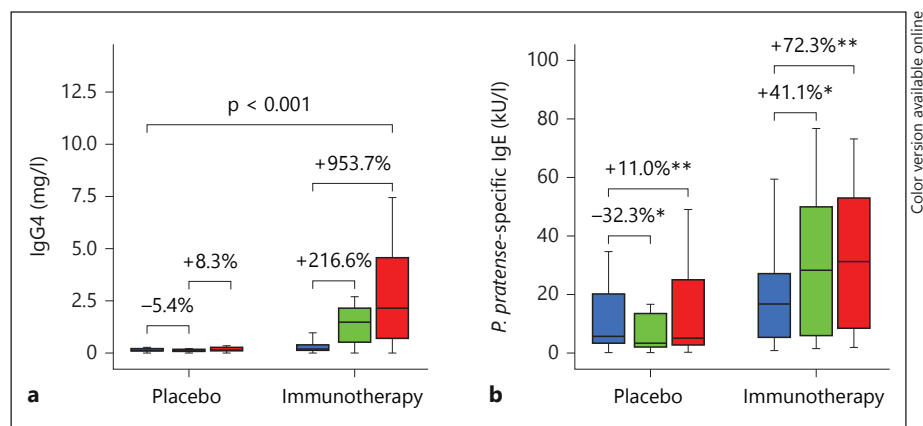
0.001). An even more pronounced increase of *P. pratense*-specific IgG4 was detected for the active group at the peak of the grass pollen period after treatment (953.7% in the immunotherapy group vs. 8.3% in the placebo group). The difference between the 2 groups here was also significant ($p < 0.001$; fig. 4).

After treatment, the change in *P. pratense*-specific IgE with respect to baseline was a median 41.1% (min-max: -31.0–325.2%) in the immunotherapy group and -32.3% (-51.9–31.7%) in the placebo group ($p < 0.001$ for the difference between the 2 groups). In the immunotherapy group, allergen-specific IgE values increased by 72.3% (min-max: -48.7–355.0%) at the peak of the pollen period. In the placebo group, allergen-specific IgE values increased by 11.0% (-36.0–212.2%) at the peak of the pollen period ($p = 0.05$ for the difference between the 2 groups; fig. 4).

Correlations

There was no correlation between the specific IgG4 levels and activated basophil percentages, except for a weak negative correlation at a 4.5 μg/ml allergen concen-

Fig. 4. **a** Levels of *P. pratense*-specific IgG4 (median percentage changes of antibody levels are shown in the diagram). **b** Levels of *P. pratense*-specific IgE. Median percentage changes in antibody levels are shown in the diagram. * $p < 0.001$, ** $p = 0.05$, comparison between 2 groups. The central box represents the values from the lower to the upper quartile (25th and 75th percentiles), the line within the box indicates the median and the whiskers show the 5th and 95th percentiles.



tration at the end of treatment ($r = -0.387$, $p = 0.03$). There was a weak negative correlation between the specific IgG4 levels and the MS values at the end of treatment as well as at the peak of the pollen season ($r = -0.406$, $r = -0.392$ and $p = 0.03$, $p = 0.03$, respectively). There were no correlations between the specific IgG4 levels and ACS or ARTSS ($p > 0.05$). Activated basophil percentages were not correlated with ACS, MS, ARTSS or specific IgE levels ($p > 0.05$).

Discussion

In our study, one course of short-term preseasonal grass pollen allergoid immunotherapy was shown to be effective in AR, as there was a 52.9% difference in ACS between the active and placebo groups in favor of immunotherapy. Quality of life scores were also improved with allergen immunotherapy. This result is in agreement with previous studies [17, 18], and suggests that short-term preseasonal allergoid immunotherapy is a convenient alternative approach to traditional immunotherapy for pollen allergy. We also demonstrated an early decrease in allergen-induced basophil activation at the end of the immunotherapy period (the 7th week after the initiation of injections) at submaximal allergen concentrations. Importantly, it remained low at the peak of the grass pollen season after immunotherapy. However, no decrease was observed for basophil activation with maximal allergen concentrations. This observation parallels the findings of Ebo et al. [19] and Lalek et al. [20], showing that decrement of basophil activation was restricted to experiments in which basophils were stimulated submaximally. Ebo et al. [19] demonstrated a decrease in CD63 expression with submaximal allergen stimulation after 6 months of

venom immunotherapy. Lalek et al. [20] showed a decrease in the allergen threshold sensitivity of basophils after 2, 3 and 5 months of subcutaneous birch pollen allergoid immunotherapy on a perennial scheme. In another study, on rush immunotherapy with Japanese cedar pollen, allergen-induced CD203c expression had decreased 1 month after treatment and continued to decrease for up to a year [21]. To our knowledge, our study is the first to report on the suppressive effect of short-term preseasonal allergoid immunotherapy on basophil activation in a double-blind, placebo-controlled setting. As the therapy involved only seven injections on a weekly basis, it would be interesting to follow up whether the suppressive effect on basophil activation would be sustained over a longer period. However, we did not evaluate basophil activation beyond the first grass pollen season after treatment. There was a finding of a sustained decrease of basophil activation compared to baseline after 8 months of short-term, preseasonal, olive pollen allergoid immunotherapy in 1 uncontrolled study [22], but some studies have not demonstrated a change in basophil activation with immunotherapy [23, 24]. A placebo-controlled trial with five-grass pollen sublingual immunotherapy did not find any alteration in allergen-induced basophil CD203c expression after 4 months of treatment, despite a symptomatic improvement of the subjects' rhinoconjunctivitis [23]. Similarly, Erdmann et al. [24] did not observe a decrease in basophil activation after 6 months of venom immunotherapy in most of their study subjects. Differences in the basophil activation markers used and the duration of the immunotherapy schedules may account for contradicting results across different studies.

In basophil activation tests, natural exposure in vivo to the allergen tested may cause high basal activation [25].

In accordance with this, basophil CD203c expression responses to the negative control increased significantly at the peak of the pollen season in both groups in our study; this may have contributed to the decrement of basophil activation responses at this time. However, the decrease in allergen-induced basophil activation at submaximal allergen concentrations during the peak pollen period was detected only in the immunotherapy group. In addition, at the end of the treatment, basophil CD203c expression responses to the negative control had not changed significantly, but a decrease was detected in basophil activation responses at submaximal allergen concentrations in the active group. Therefore, the effect of in vivo allergen stimulation does not explain the decrease in allergen-induced basophil activation obtained with immunotherapy.

One concern about the allergoids is that the low allergenicity in comparison with standard extracts might actually be associated with reduced immunogenicity [5]. However, there are several studies showing the increment of specific IgG antibodies by allergoid immunotherapy [22, 26, 27]. As shown in our study, the suppressive effect of the therapy on basophil activation at the end of treatment and at the peak of the grass pollen season after treatment further supports the powerful immunogenicity of allergoids. Some studies have shown that sera containing therapy-induced allergen-specific IgG can suppress basophil activation [20, 26–28]. In this study, we reported a pronounced increase of allergen-specific IgG4 after immunotherapy. However, no correlation was observed between the allergen-specific IgG4 levels and the basophil CD203c response, except for a weak negative correlation

at only one allergen concentration. The heterogeneous induction of IgG antibodies to different components in a therapeutic allergen extract and the fact that the protective activity of IgG antibodies may vary according to other variables (e.g. epitope specificity and affinity/avidity) [26] could be the factors responsible for preventing a simple correlation of allergen-specific IgG levels and basophil allergen responsiveness or clinical efficacy. It would be more relevant to measure the blocking activity and affinity of specific IgG and its subsets instead of their levels in the sera [29].

In conclusion, we have demonstrated both the cellular and humoral immune responses after one course of short-term preseasonal grass pollen allergoid immunotherapy. These changes include reduced basophil sensitivity to the allergen at the effector cell level and increased allergen-specific IgG4 antibodies at the B cell level. The elevation of blocking antibodies and blunting of basophil activation were accompanied by significant early clinical efficacy. The basophil CD203c response appears to be a promising tool for early monitoring of the protective immune responses induced by immunotherapy.

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