

Initial immunological changes as predictors for house dust mite immunotherapy response

E. Gómez^{1,*}, T. D. Fernández^{1,*}, I. Doña², C. Rondon², P. Campo², F. Gomez², M. Salas², M. Gonzalez¹, J. R. Perkins¹, F. Palomares¹, M. Blanca², M. J. Torres^{2,*} and C. Mayorga^{1,*}

¹Research Laboratory–Allergy Unit, IBIMA–Regional University Hospital of Málaga, UMA, Malaga, Spain and ²Allergy Service, IBIMA–Regional University Hospital of Málaga, UMA, Malaga, Spain

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Summary

Background Although specific immunotherapy is the only aetiological treatment for allergic disorders, the underlying mechanisms are not fully understood. Specific immunotherapy induces changes in lymphocyte Th subsets from Th2 to Th1/Treg. Whether differences in immunological patterns underlie patient response to immunotherapy has not yet been established.

Objectives We studied the immunological changes occurring during a 1-year period of *Dermatophagoides pteronyssinus* (DP) immunotherapy and their relation with clinical outcome.

Methods We included 34 patients with DP allergy who received subcutaneous specific immunotherapy (SCIT) for 1 year. Following treatment, patients were classified as responders or non-responders. Fourteen allergic subjects who did not receive SCIT were included as controls. Peripheral blood was obtained at 0, 1, 3, 6 and 12 months and cultured with nDer p 1. Phenotypic changes, cytokine production and basophil response were analysed by flow cytometry; transcription factors were measured by mRNA quantification. Serum immunoglobulin levels were also measured.

Results After 1 year of SCIT, 82% of cases showed improved symptoms (responders). Although increases in sIgG4 were observed, BAT reactivity was not modified in these patients. Increases in T-BET/FOXP3 as well as nDer p 1-specific Th1/Treg frequencies were also observed, along with a decrease in Th2, Th9 and Th17. These changes corresponded to changes in cytokine levels.

Conclusion Patients who respond well to DP-SCIT show immunological differences compared to non-responders. In responders, basal differences include a lower frequency of Th1 and higher frequencies of Th2, Th9 and Th17 cells. After 1 year of treatment, an increased production of sIgG4 was observed in responders, along with a change in Th2 response towards Th1/Treg.

Keywords allergic rhinitis, basophils, cytokines, *Dermatophagoides pteronyssinus*, immunoglobulins, specific immunotherapy, T cells

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Correspondence:

Cristobalina Mayorga, Research Laboratory–Allergy Service, Pavilion 5, Basement, University Hospital of Malaga (Pavilion C), Plaza del Hospital Civil, 29009 Malaga, Spain.

E-mail: lina.mayorga@ibima.eu

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Introduction

Allergic rhinitis (AR) is a common disorder that affects 500 million people world-wide [1]. It adversely impacts the social life, school performance and work productivity of patients [2, 3], and produces a large economic burden on governments [4]. Frequently, AR presents

comorbidities with allergic conjunctivitis as well as other respiratory disorders such as rhinosinusitis, and especially asthma [5–7]. Among inhaled allergens, *Dermatophagoides pteronyssinus* (DP) is considered one of the major aetiological factors world-wide [1, 8].

Current therapies include those based on the strict avoidance of causative allergens, which can be difficult, or the use of pharmacotherapy that allows a transient reduction of the symptoms, but not a cure. Specific

*The Authors contributed equally to this manuscript.

immunotherapy represents the only allergen-specific and disease-modifying treatment with long-lasting effects [9]. Although different routes of administration are available, the subcutaneous specific immunotherapy (SCIT) has been suggested as the first choice for adults and children with AR caused by DP [10, 11].

Subcutaneous specific immunotherapy aims to modify the allergic response underlying AR, which is characterized by a Th2 pattern with release of IL-4, IL-5 and IL-13 and low production of IFN- γ , provoking a skew towards Th1 patterns [9, 12]. This immunodeviation includes a switched production of IgE towards blocking IgG antibodies, inducing an allergen-specific suppression of basophil and mast cell activity [13, 14]. There is also an early generation of specific Treg cells [15] that may lead to the production of IL-10 and TGF- β , both important in the induction of tolerance [16–18].

Although much is known about the mechanisms involved and the changes induced by SCIT, less is known about the immunological differences between SCIT responder (RP) and non-responder patients (NRP). It has been estimated that NRP may encompass up to 30% of patients treated with SCIT [19]. Although some parameters have been postulated as good markers of SCIT efficacy (bronchial provocation [19], Ig variations [20], decreased BAT reactivity [21], T cell modulation/Treg appearance [22]), their predictive value is still under debate and contradictory results have been published [23, 24]. There is currently an intensive search for a single marker that can predict SCIT efficacy, and to improve the early recognition of those patients most likely to benefit from SCIT [25].

We studied the immunological changes occurring during DP-SCIT in a group of patients with AR to DP and compared them with those observed in a control group (CG) of AR to DP patients not receiving DP-SCIT, during a 1-year period. We studied changes in immunoglobulin levels, basophil reactivity, transcription factor (TF) expression, frequencies of T cell subsets and cytokine production. Furthermore, we compared the immunological changes with the clinical outcome for RP and NRP. We found that the initial T cell population frequencies were related with the clinical response to DP-SCIT, as well as initial changes in cell subpopulations and Ig levels, pointing to their potential utility at predicting which patients are likely to respond well to this treatment.

Methods

Thirty-four patients with AR to DP who began treatment with SCIT (Acaroid®; Allergopharma KG, Reinbek, Germany) using a conventional schedule (Table 1) were enrolled in the study. Acaroid® is an aluminium hydroxide-adsorbed depot allergoid preparation of

Table 1. Subcutaneous specific immunotherapy protocol

Vial	Interval time of dose administration	Doses administered (cc)
A	7 days	0.1
		0.2
		0.4
		0.6
B	7 days	0.1
		0.2
		0.4
		0.6
	14 days	0.6
	1 month	0.6
		0.6

The patients received eight subcutaneous injections at interval of 1 week at progressively increasing doses (0.1, 0.2, 0.4 and 0.6 cc firstly from vial A and then the same protocol from vial B). They received another dose of 0.6 cc from vial B 2 weeks later, and then, they were administered maintenance injections of 0.6 cc from vial B every 4 weeks. If necessary, the dose and timing were modified by a physician according to the manufacturer's recommendations. Patients were asked to stay under the supervision of site personnel for at least 30 min after each injection. Rescue medication (oral, nasal and ocular antihistamines, nasal corticosteroids, nasal vasoconstrictors, beta2-agonist, inhaled corticosteroid) was administered if patients had nasal, ocular or bronchial symptoms.

standardized (in therapeutic units) high concentrations of powdered diafiltered dust mite allergens of 11.66 g/mL Der p 1, and 10 g/mL Der p 2 prior to allergoidization. The evaluation criteria included an unequivocal history of persistent AR to DP, with a positive nasal provocation test (NPT) and positive skin prick test (SPT), plus high levels of serum specific IgE (sIgE) against DP (> 0.35 Ku/L). Patients were excluded if they were sensitized to other inhaled allergens, if they presented other immunological diseases, severe psychological upset and severe atopic dermatitis or if they had followed another SIT immunotherapy during the 5 previous years to this study. As a CG, 14 patients with AR to DP of the same severity who did not receive SCIT were included.

All the cases were prospectively evaluated over a 1-year period [before SCIT (basal) and 1, 3, 6 and 12 months after starting SCIT]. In every evaluation, total rhinoconjunctivitis symptom score (RCSS) and medication scores were obtained [26], intradermal (ID) skin tests and NPT were carried out, and samples were collected for *in vitro* determinations, always before SCIT administration.

Patients were defined as RP or NRP based on the evolution of symptoms and consumption of rescue medication, being classed as responders when both scores decreased by more than 20%.

The study was conducted according to the declaration of Helsinki and was approved by the Ethics Committee

of Malaga. All the subjects signed informed consent forms before carrying out the study.

Total rhinoconjunctivitis symptom and medication scores

The clinical response was evaluated, following the indications of a recent EAACI (European Academy of Allergy and Clinical Immunology) position paper, as the average daily RCSS and medication score [26]. Patients were instructed to keep a diary during the previous month of all visits (basal, 1, 3, 6 and 12 months), for a daily evaluation of the use of rescue medication and five rhinoconjunctivitis symptoms (sneezing, rhinorrhea, itchy nose, nasal obstruction and ocular) according to a 4-point scoring system: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. The maximum daily total symptom score was 15. The medication score was as follows: 1 point for oral antihistamines, 2 points if intranasal corticosteroids were also required, and 3 points if oral corticosteroids were also needed. The change in the mean daily RCSS and medication scores obtained during the previous month of each visit were used to assess the clinical improvement in response to SCIT.

Skin tests

For the initial diagnosis, SPT was performed with a battery of 17 common inhalant allergens, including pollens, house dust mites, moulds and animal danders (ALK, Madrid, Spain). For the follow-up, ID testing was carried out with freshly reconstituted freeze-dried DP (0.4 mg/mL) (ALK, Madrid, Spain) as previously described [27]. The results were expressed as mm².

Nasal provocation test

Nasal provocation test was carried out as described previously [28, 29]. Briefly, symptom-free patients [total visual analogue scale (VAS), < 60 mm] were intranasally challenged with two puffs (100 mL) of saline in each nostril to exclude nasal hyperreactivity. If this result was negative, 15 min later we began administering reconstituted freeze-dried allergen solutions of DP (0.04, 0.4 and 4 mg/mL) at 15-min intervals. Two puffs (100 mL) of the solution at room temperature were applied in each nostril. Responses were monitored by means of acoustic rhinometry (SRE 2000 rhinometer; Rhinometrics, Lynge, Denmark) and symptoms' score. Acoustic rhinometry was performed following the guidelines of the Standardization Committee on Acoustic Rhinometry (E4), measuring the volume of the nasal cavity that corresponds to the lower turbinate (VOL 2–6 cm) in each nostril. Symptoms of nasal obstruction,

rhinorrhea, itching, sneezing and ocular were monitored at each time-point by placing a vertical mark on a horizontal VAS of 100 mm. The total range of the VAS during NPT was 0–500 mm. The response to nasal challenge was evaluated based on subjective (VAS of nasal–ocular symptoms) and objective (VOL 2–6 cm) parameters. A positive NPT response was considered when an increase greater than 30% in the total VAS score and a decrease of 30% or greater in nasal cavity volume were observed, compared with the baseline test.

Sample collection and storing

Whole peripheral blood was obtained from patients by venopuncture using different container tubes according the requirements for each technique (silica gel-coated tubes for total and allergen-specific IgE and IgG4 determinations, acid–citrate–dextrose additive solution tubes for basophil activation test, and heparin- and EDTA-coated tubes for flow cytometry and gene expression studies, respectively; all from Becton-Dickinson, San Jose, CA, USA). Blood sample was processed following standard procedures and, where appropriate, frozen immediately after their reception by the Málaga Hospital-IBIMA Biobank, Andalusian Public Health System Biobank.

Peripheral blood mononuclear cells from subjects before and at different time intervals during SCIT were isolated by Ficoll-Paque density gradient (Pharmacia Biotech, Barcelona, Spain). Cells were frozen for future experiments in RPMI 1640 medium supplemented with 2 mM of L-glutamine (BioWhittaker, Pittsburgh, PA, USA), gentamicin (5 mg/mL) (Normon, Madrid, Spain), streptomycin (50 ng/mL), penicillin (100 IU/mL), 40% of foetal bovine serum (BioWhittaker) and DMSO (10%) (Sigma, St. Louis, MO, USA) and stored in liquid nitrogen, while serum samples were stored at –20°C, in all cases at the BIOBANK of University Hospital of Malaga, for future studies.

Total and specific IgE and IgG4 assays

Total IgE, sIgE and sIgG4 to DP (d1) were determined by ImmunoCAP-FEIA in serum samples, according to the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden).

Basophil activation test during SCIT

BAT was performed as previously described [30] with three different concentrations of nDer p 1 (0.1, 1 and 10 µg/mL) according to the dose–response curve (not shown). Cells were stained with anti-human IgE-FITC and anti-human CD63-PE monoclonal antibodies (Caltag Laboratories, Burlingame, CA, USA) as described [31]. Results are represented as percentage of activation

(IgE⁺CD63^{high} cells). Results were analysed by CellQuest Pro software (BDTM Biosciences, Milpitas CA, USA). Patients who produced a negative BAT result to an anti-IgE-positive control were excluded from further study [31].

Gene expression studies

One million cells were incubated with nDer p 1 at a final concentration of 10 µg/mL for 6 days (5% CO₂ and 37°C). Cells were then harvested and stored at -80°C in RLT buffer (Qiagen, Hilden, Germany) with 10 µL of β-mercaptoethanol until use. Total mRNA was isolated with RNeasy (Qiagen) according to the manufacturer's instructions and following the MIQE guidelines [32]. A total of 0.5 µg of mRNA was reverse-transcribed into cDNA with SuperScript II enzyme (Invitrogen, Barcelona, Spain) and oligo (dT) 12-18 primer (Invitrogen) in a final volume of 20 µL. The expression of GATA3, T-BET, FoxP3 and RORγt was determined in triplicate by qPCR (LightCycler480; Roche, Mannheim, Germany) using SYBR[®] Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). β-Actin was used as a reference gene. Primer sequences (5'-3') (Sigma-Aldrich, Madrid, Spain) are shown in Table S1. Relative quantification was carried out according to the mathematical model proposed by Pfaffl [33]. Briefly, the relative expression ratio of the target genes vs. the reference gene was calculated considering efficiencies of amplifications and crossing point deviations, using an internal control (calibrator) for normalization [33].

Flow cytometry phenotype

Peripheral blood mononuclear cells were cultured with nDer p 1 (10 µg/mL) for 6 days. Brefeldin A (10 µg/mL) (Sigma-Aldrich) was added during the last 6 h of stimulation, and then, supernatants were collected and stored at -20°C for future determinations of cytokine production. Thereafter, surface characterization and intracellular characterization were carried out with specific MoAbs and proper buffers following the manufacturer's instructions. For intracellular staining, cells were fixed with BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BDTM Biosciences). CD3⁺ cell subpopulations were defined as follows: CD4⁺IFN-γ⁺ (Th1); CD4⁺CD27⁺CD294⁺ (Th2 cells); CD4⁺CD294⁺IL-9⁺ (Th9); CD4⁺RORγt⁺IL-17a⁺ (Th17); and CD4⁺CD127⁺CD25^{high} (Treg). For Treg cell staining, human FoxP3 buffer (BDTM PharMingen) was used, following the manufacturer's instructions. Cells were washed and immediately phenotyped with a BDTM FACSCanto II flow cytometer (BD Biosciences) and analysed with FlowJo[®] software (Tree Star, Inc., Ashland, OR, USA) (Fig. S1). Results were expressed as percentage of CD3⁺CD4⁺ cells.

Human Th1/Th2/Th19/Th17/Th22 cytokine levels were determined by flow cytometry with 13-Plex Flow-CytomixTM kit (eBioscience[®], Bender MedSystems[®], San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Demographic, clinical and other quantitative variables were analysed using the non-related Mann-Whitney *U*-test and chi-square test. Comparisons of related samples were carried out by Wilcoxon test. All reported *P*-values represent two-tailed tests, with values *P* ≤ 0.05 considered statistically significant. The Bonferroni correction was applied for multiple comparison testing.

Results

We evaluated a group of patients receiving SCIT (*n* = 34) and a control group (*n* = 14) not receiving SCIT. There were no significant differences in the clinical characteristics (age, sex, asthma symptoms, symptoms score, treatment score, prick, ID, NPT, sensitization to non-DP allergens) between groups at baseline.

All patients received SCIT for 12 months without any systemic adverse effects. Following this period, they were classified into RP (*n* = 28) and NRP (*n* = 6). Although not significant, the NRP group was more likely to be female (4; 66.7%) compared to the RP group (10; 35.7%) and CG (6; 42.8%). The median age was 28.5 (22–33) years, and no differences were found between groups. The NRP group had a longer time of evolution of rhinitis symptomatology compared to the RP group [180 (180–240) months vs. 36 (24–120) months, *P* < 0.0001]. At basal level, there were no significant differences in asthma, symptoms and treatment score, prick, ID, NPT and sensitization to the other allergens tested in both RP and NRP.

Comparisons of the ID test area before and after 12 months of SCIT showed a decrease for both RP and NRP, although this was not significant. We did not find any differences in NPT between these time-points. On the contrary, CG showed an increase in the ID test area (although not significant) and a significant decrease in the concentration of DP solution inducing positivity in NPT (*P* = 0.008) (Table 2).

Evolution of serum immunoglobulins

To understand the effect of SCIT in the immunoglobulin production, sera were collected at different time-points during the treatment to measure total IgE, sIgE and sIgG4 by ImmunoCAP-FEIA. All the determinations were performed at the same time and before the

Table 2. Clinical data of patients included

	Basal	1 month	3 month	6 month	12 month	P
Patients <i>n</i> = 34						
RCSS	2.78 (1.35–4.95)	3.57 (0.57–4.39)	4.46 (0.95–5.66)	2.8 (1.55–7.98)	2.21 (1.32–9.43)	0.255
Medication score	0.25 (0.07–2.92)	0 (0–0.52)	0.25 (0.12–1.41)	0.30 (0.17–2.08)	0.67 (0.07–1.21)	0.220
ID DP (mm ²)	147 (90–176)	147 (120–238)	150 (81–256)	162.5 (120–192)	100 (88.5–148)	0.022
Concentration DP at positive NPT (mg/ml)	0.04 (0.04–4)	0.4 (0.04–4)	0.4 (0.04–4)	0.04 (0.04–4)	0.04 (0.04–4)	0.153
Responders to immunotherapy (RP) <i>n</i> = 28						
RCSS	2.17 (1.35–2.92)	2.10 (0.57–4.33)	1.57 (0.95–4.55)	2.80 (1.55–4.15)	1.56 (1.32–2.85)	0.002
Medication score	0.54 (0.07–1.20)	0.03 (0–0.52)	0.67 (0.19–1.41)	0.30 (1.17–2.08)	0.25 (0.07–1.21)	0.065
ID DP (mm ²)	149 (90–168)	138 (120–154)	150 (101–194.25)	169 (144–180)	100 (88.5–148)	0.007
Concentration DP at positive NPT (mg/mL)	0.004 (0.004–4)	0.4 (0.04–4)	0.4 (0.04–4)	0.4 (0.04–4)	0.04 (0.04–0.4)	0.389
Non-responders to immunotherapy (NRP) <i>n</i> = 6						
RCSS	3.85 (1.58–4.95)	3.98 (3.57–4.39)	4.46 (3.26–5.66)	6.78 (4.98–7.98)	8.32 (6.52–9.43)	0.063
Medication score	0.25 (0.12–0.42)	0.17 (0.12–0.22)	0.3 (0.12–0.43)	0.54 (0.32–0.67)	0.67 (0.57–0.88)	0.458
ID DP (mm ²)	172 (168–176)	182 (120–238)	168.5 (81–256)	121 (120–192)	109.5 (99–120)	0.245
Concentration DP at positive NPT (mg/mL)	0.04 (0.04–0.4)	4 (0.04–4)	4 (0.4–4)	0.04 (0.04–0.04)	0.04 (0.04–0.04)	0.002
Control group not receiving immunotherapy (CG) <i>n</i> = 14						
RCSS	4.75 (1.46–5.89)	4.56 (1.23–5.78)	4.05 (0.92–5.75)	0.67 (0.42–0.78)	5.53 (0.92–7.60)	0.122
Medication score	0.07 (0–0.41)	0.12 (0–0.38)	0.16 (0–0.74)	0.26 (0–0.85)	0.46 (0–2.03)	0.458
ID DP (mm ²)	132 (118.25–189)	145 (126–210)	168 (120–323)	165 (87.75–334.5)	143 (83.5–191.25)	0.669
Concentration DP at positive NPT (mg/mL)	0.4 (0.04–0.4)	0.4 (0.04–0.4)	0.04 (0.04–4)	0.4 (0.04–4)	0.04 (0.04–0.04)	0.040

RCSS, total rhinoconjunctivitis symptom score; ID, intradermal test; DP, *Dermatophagoides pteronyssinus*; NPT, nasal provocation test. Data are presented as medians and range. Statistical analysis for related samples was carried out by Wilcoxon test, with values $P \leq 0.05$ considered statistically significant. *P*-value corresponds to the differences between baseline and 12 months.

Table 3. Summary of immunological differences found for different groups

Group	slg		TF		Phenotype		Cytokines	
	Basal	12 M	Basal	12 M	Basal	12 M	Basal	12 M
RP		↑ sIgG4 ↓ sIgE/IgG4		↓ GATA3 ↑ T-BET ↑ FOXP3	↓ Th1 ↑ Th2 ↑ Th9 ↑ Th17	↑ Th1 ↑ Treg ↓ Th2 ↓ Th9 ↓ Th17	↑ IL-5 ↑ IL-9 ↑ IL-13 ↑ TNF-α	↑ IL-10 ↑ IL-12 ↑ IFN-γ ↓ IL-5 ↓ IL-9 ↓ IL-13
NRP	–	–	–	–	↑ Th1 ↓ Th2 ↓ Th9 ↓ Th17	–	–	–
CG				↑ GATA3 ↑ FOXP3				↑ IL-13 ↑ TNF-α ↓ IFN-γ

RP, responders to immunotherapy; NRP, non-responders to immunotherapy; CG, control group not receiving immunotherapy; M, months following start of immunotherapy; slg, specific immunoglobulin; TF, transcription factors.

classification of patients as RP or NRP. At the basal evaluation, total IgE, Der p 1-sIgE and Der p 1-sIgG4 levels did not show significant differences among NRP, RP and controls. For total IgE, time-series analysis showed that differences were found for RP only increasing at 1 and 3 months ($P = 0.021$ and $P = 0.047$, respectively) and returning to baseline in the

following months (data not shown). Regarding the specific humoral response, we found an increase in sIgE after 1 month, and this was sustained until 3 months ($P = 0.001$, $P = 0.013$), with values returning to baseline (Fig. 1a). The biggest differences between RP and NRP were detected for sIgG4, the expression of which increased at 1 month ($P = 0.0061$) and stayed elevated

throughout the study ($P = 0.0052$ at 3 months, $P = 0.0017$ at 6 months and $P = 0.0002$ at 12 months) (Fig. 1b). Analysing the results as the ratio sIgE/sIgG4, we found a significant decrease very early after starting the SCIT that was maintained throughout the evaluation period ($P = 0.0105$ at 1 month, $P = 0.0046$ at 3 months, $P = 0.0005$ at 6 and $P = 0.0002$ at 12 months) (Fig. 1c).

Basophil activation test

As the reactivity of basophils is linked to the production of sIgE, we wanted to analyse its evolution during the treatment using the classic basophil activation test. The analysis of basophil reactivity showed that there were no differences between CG, RP and NRP at basal levels. However, RP subjects showed a constant increase in the percentage of activated basophils during the 12 months, although no significant differences with the basal level were found (Fig. 2).

Transcription factors mRNA expression

The shift from a Th2 towards a Th1 pattern begins with the expression of different TF, which lead to different gene expression patterns and consequently cell phenotypes. The analysis of mRNA expression was carried out in cryopreserved cells, from the different blood extractions, after 12 months of treatment and before classification of patients as RP and NRP. We studied the mRNA expression of different Th cell-lineage TFs, T-BET, GATA3, ROR γ t and FOXP3 genes and their variation after 12 months. No differences in gene expression were found between the groups (Fig. 3). Related sample analysis comparing basal and 12 months showed a decrease in GATA3 expression ($P = 0.012$) together with an increase in T-BET and FOXP3 genes ($P = 0.002$; $P = 0.023$, respectively) in the RP group; no changes were found for NRP (Fig. 3a–d). Regarding the CG, significant increases in GATA3 and FOXP3 after 12 months ($P = 0.012$; $P = 0.005$, respectively) were

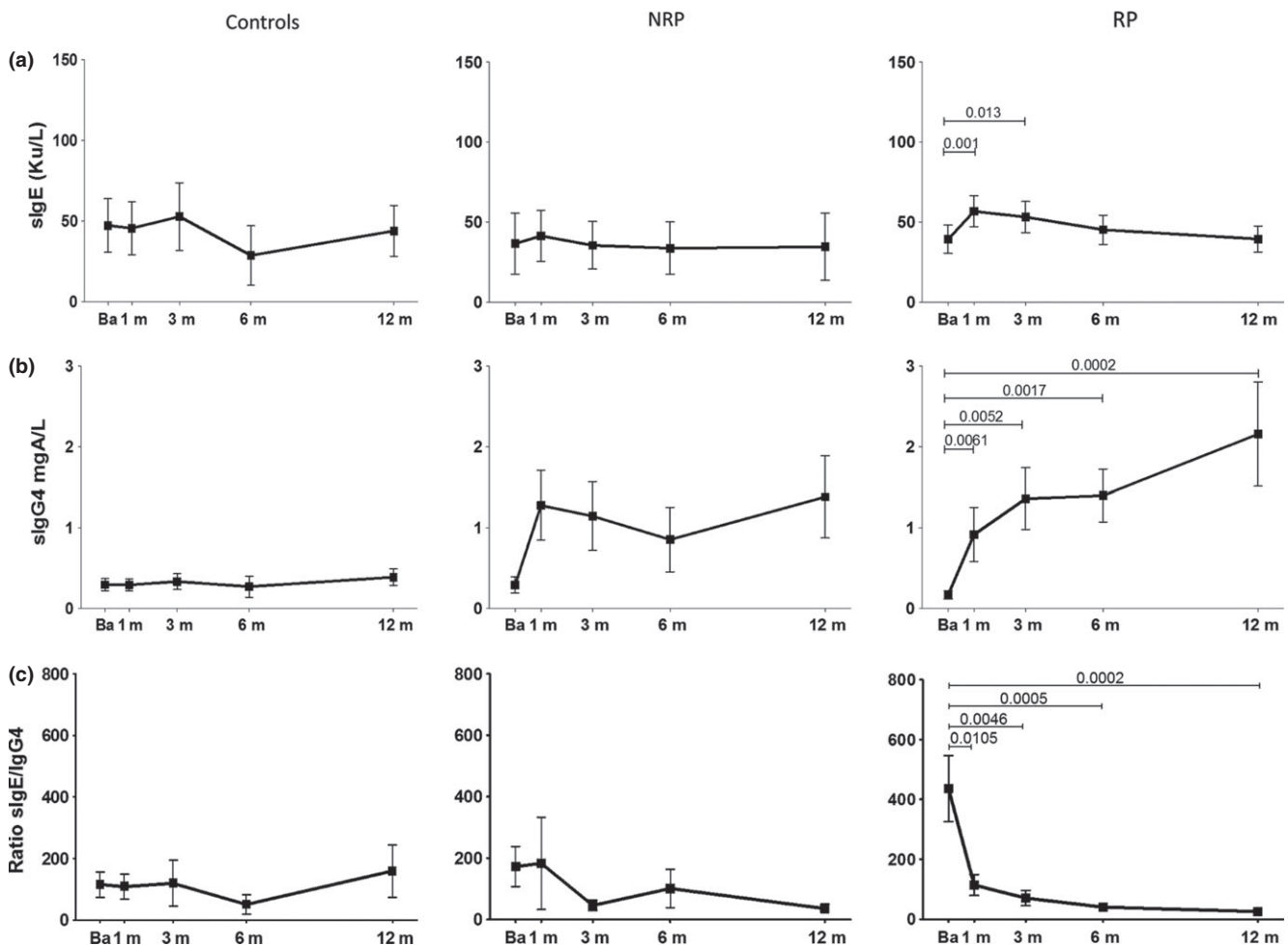


Fig. 1. Immunoglobulins determination. Comparison of (a) specific IgE (sIgE), (b) sIgG4 to DP and (c) ratio sIgE/sIgG4 during 1-year follow-up of SCIT in CG ($n = 14$), NRP ($n = 6$) and RP ($n = 28$). sIgE (Ku/L) and sIgG4 (mgA/L) to DP were determined by ImmunoCAP-FEIA. Results are presented as mean $\pm$ SEM. Significant changes were considered when $P < 0.0125$, applying Bonferroni correction.

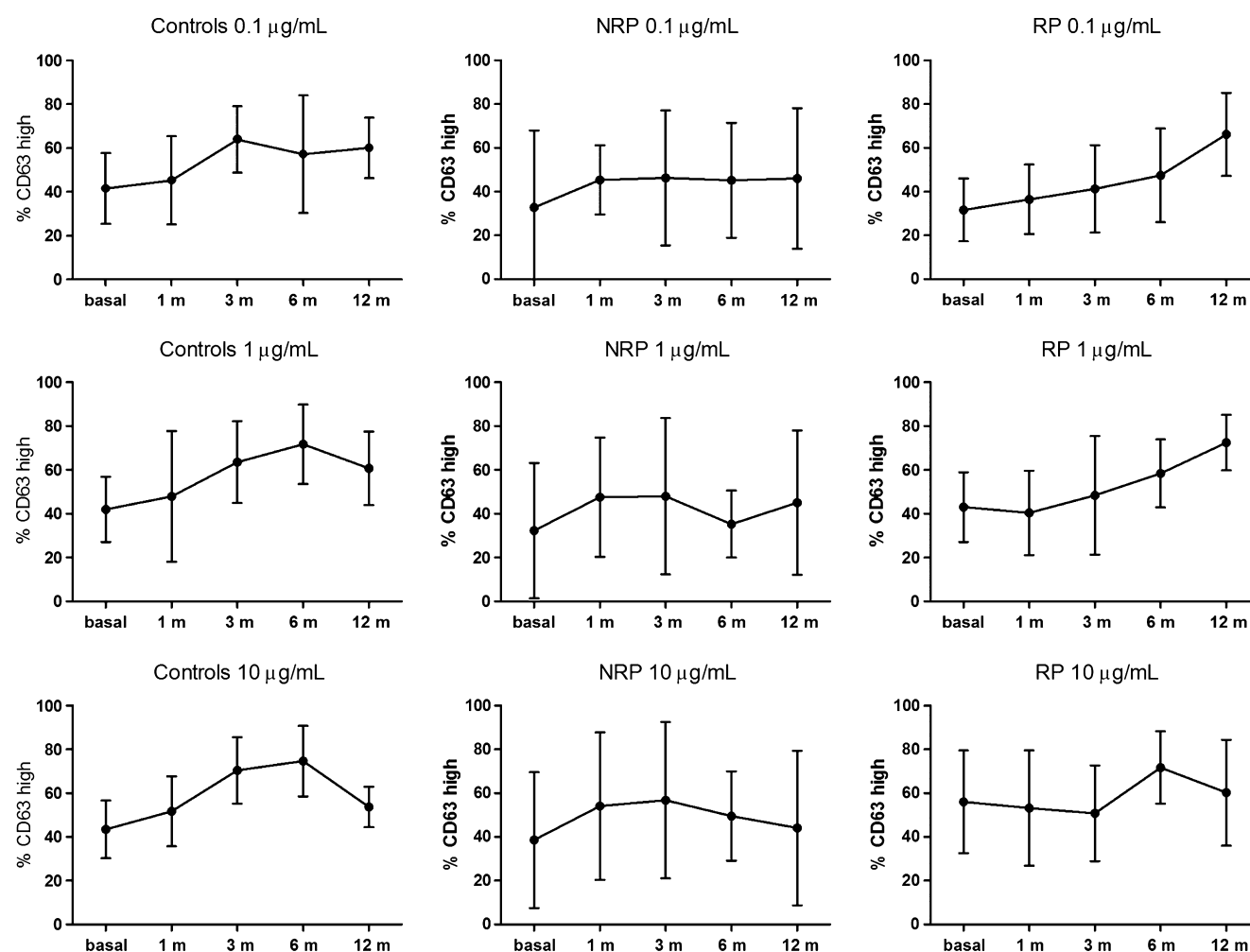


Fig. 2. Basophil activation test to DP. Comparison of basophil activation throughout the first year of SCIT in CG ($n = 14$), NRP ($n = 6$) and RP ($n = 28$), at three different concentrations of DP (0.1, 1 and 10 µg/mL). Results represent the mean $\pm$ SEM of percentage of CD63+ basophils. Significant differences were considered when $P < 0.0125$, applying Bonferroni correction.

observed. Notably, ROR γ t expression did not show significant changes in any group (Fig. 3d).

Evolution of T cell subpopulations

Once the transcription factors were analysed, we wanted to know whether the changes observed lead to the phenotype of the main cellular subtypes involved in the allergic reaction. For this, DP-specific Th1, Th2, Th9, Th17 and Treg were evaluated by flow cytometry in cryopreserved cells (Fig. S1). Th2 cells were characterized as expressing CD294 (CRTH2) [34], and not expressing CD27, as it has been shown that a selective elimination of CD27 in peripheral blood is associated with successful immunotherapy [35]. Th9 cells were characterized as expressing IL-9 [35] and not expressing CRTH2 to exclude those Th2-IL-9-producing cells from our analysis [36]. At baseline, significant differences were detected in Th1 cells, being lower in RP when

compared to CG and NRP ($P = 0.023$ in both) (Fig. 1a), and in Tregs, being lower in NRP compared to CG ($P = 0.004$) (Fig. 1e). Comparisons between basal and 12 months showed differences in RP only, with an increase in Th1 and Treg frequencies ($P = 0.005$ in both) and a decrease in Th2, Th9 and Th17 ($P = 0.012$; $P = 0.005$; $P = 0.005$, respectively) (Fig. 4a–e).

We compared cytokine profiles after specific-DP stimulation at basal and 12 months using a cytometric method that allows us to quantify all the cytokines at the same time in the same sample. Using this technique in the frozen supernatants from the cell cultures, we found differences at basal levels with significantly higher values of IL-5 and IL-9 ($P = 0.007$; $P = 0.049$, respectively) in RP compared to CG, and of IL-5 in RP compared to NRP ($P = 0.036$) (Fig. 5e,g). Comparison between basal and 12 month results showed a significant increase in IL-12p70, IFN- γ , IL-2 and IL-10 ($P = 0.005$; $P = 0.005$; $P = 0.017$; $P = 0.026$, respec-

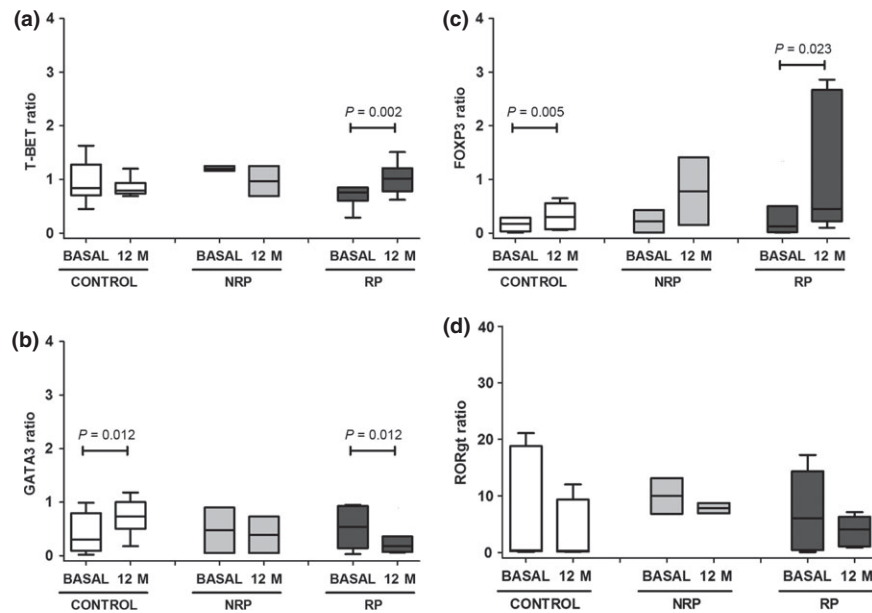


Fig. 3. Expression of T-BET, GATA3, ROR γ t and FOXP3 transcription factors. Comparison of gene levels, before (basal) and after 12 months (12 m) of SCIT in CG ($n = 14$), NRP ($n = 6$) and RP ($n = 28$). The relative expression ratio (R) of the target genes vs. the reference gene, β -actin, was calculated considering efficiencies of amplifications and crossing point deviations, using an internal control to normalize [33]. Significant differences when $P < 0.05$.

tively) (Fig. 5a–d), and a significant decrease in IL-5, IL-13 and IL-9 ($P = 0.004$; $P = 0.006$; $P = 0.049$, respectively) in RP (Fig. 5e–g). On the contrary, the CG showed significant decreases in IFN- γ ($P = 0.026$) (Fig. 5b), and significant increases in IL-13 and TNF- α ($P = 0.035$, $P = 0.033$, respectively) (Fig. 5f,h). No differences were observed for the NRP group (Fig. 5). The findings obtained for the different markers in each group are summarized in Table 3.

Discussion

We have evaluated the initial immunological changes that occur in response to SCIT and their association with clinical outcomes. Based on the clinical symptoms and treatment scores, we found that 82.3% of cases receiving SCIT were RP and 17.7% NRP, a lower percentage than previously reported [19].

Differences in ID area and NPT were found between treated and untreated patients with AR. However, there were no differences between RP and NRP. This suggests that these changes are due to SCIT itself, and not related to clinical response. Whether these variables will change with a longer period of SCIT is unknown.

Variation in sIgE/sIgG4 ratio has been considered a biomarker for immunotherapy evaluation in AR [37, 38]. In our study, we have detected changes in IgE, sIgE and sIgG4 levels over time in RP only. There was an increase in total and specific IgE during the first 6 months of SCIT that then returned to basal levels, and steady increase in sIgG4 throughout the 12-month

period of evaluation. Increases in sIgG4 have been associated with SCIT improvement [39–42] due to the IgE-blocking activity [43, 44] of mast cells or basophil activation as well as a shift from Th2 to Th1 patterns [39].

Regarding the cellular response, basophils are key players in type I reactions, and the question arises whether the changes in Ig levels found in our study could affect their reactivity, as reported previously [45–47]. Contrary to expected, we did not observe a reduction in basophil activity over time – in fact, we noted a slight increase in RP. Other studies have also been unable to demonstrate such a reduction in basophil reactivity with SIT [48–51]. This could be related to the study time period as it has been recently reported that basophils from DP-sublingual immunotherapy patients did not show reduced activation until 24 months of treatment [52]. Our data raise the question of whether the BAT can be used as a biomarker test for DP-SCIT efficacy; however, further study is needed using a wider range of DP concentrations to fully answer this question. Nevertheless, in this study we determined the concentrations to apply using the dose–response curve and found that lower concentrations did not affect the results of the test.

Subcutaneous specific immunotherapy induces changes in the late-phase response that are associated with cellular markers, with a shift from Th2 responses towards the less inflammatory Th1/Treg pattern and a reduction in Th17 [53, 54]. The differentiation of these Th subsets is regulated by specific TF such as T-BET, GATA3, ROR γ t and FOXP3 [55]. The mRNA levels expressed after 1 year revealed that in RP, there was a reduction in

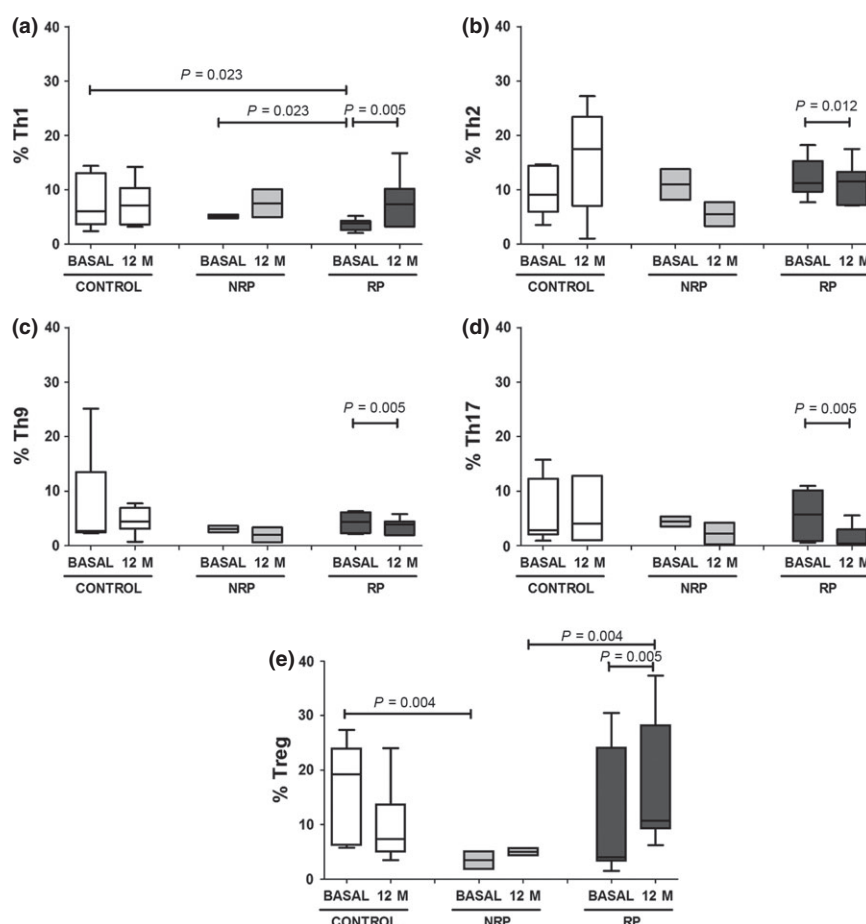


Fig. 4. Changes in T cell populations due to SCIT. Comparative analysis for CG ($n = 14$), NRP ($n = 6$) and RP ($n = 28$) before (basal) and after 12 months (12 m) of SCIT. T cell subpopulations, expressed as percentages, were defined as follows: (a) $CD4^+IFN-\gamma^+$ (Th1); (b) $CD3^+CD4^+CD27^-CD294^+$ (Th2 cells); (c) $CD3^+CD4^+ROR\gamma^+IL-17a^+$ (Th17); (d) $CD3^+CD4^+CD294^-IL-9^+$ (Th9); (e) $CD3^+CD4^+CD127^-CD25^{high}$ (Treg). Significant differences when $P < 0.05$.

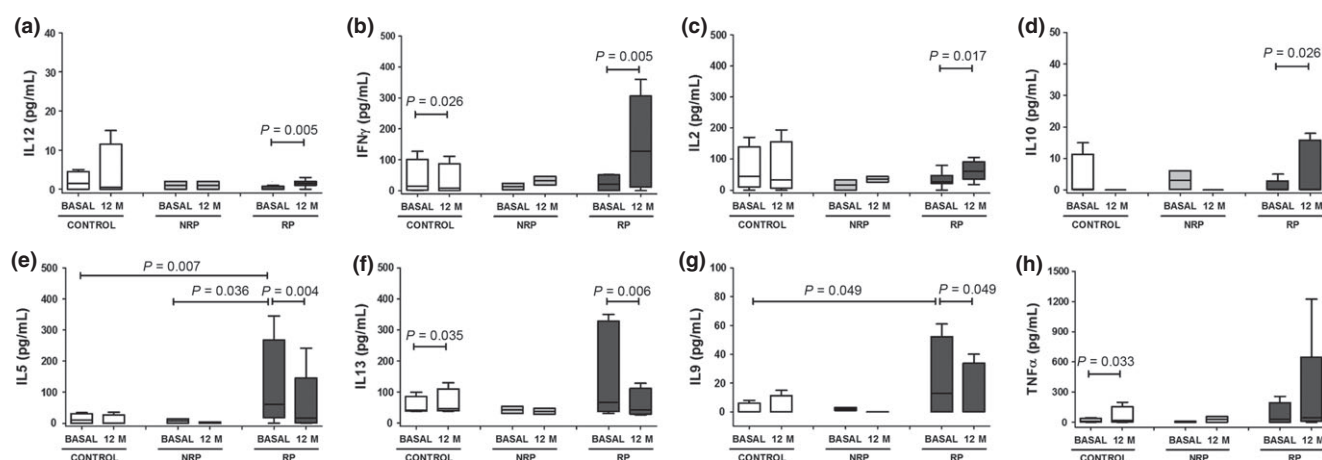


Fig. 5. Cytokine determination. The cytokine concentrations measured in three groups [CG ($n = 14$), NRP ($n = 6$) and RP ($n = 28$)], before (basal) and 12 months (12 m) after SCIT. Levels of cytokines are presented as pg/mL. Significant differences when $P < 0.05$.

GATA3, which is involved in effector Th2 responses [56], and an increase in T-BET and FOXP3, which are considered key regulators of Th1 and Treg cell differentiation,

respectively [57, 58]. Therefore, our data are consistent with a shift from Th2 towards Th1 and Treg patterns due to SCIT, as previously reported [59, 60]. Although the

transcription factor studies have not been performed in isolated cell subpopulations, they showed a similar pattern to that observed with cell phenotype and cytokine production. Moreover, this study shows for the first time that these changes are related to clinical improvement. This is particularly important when compared with the evolution of the CG, where a Th2 pattern, with significant increases in GATA3 and FOXP3, was detected. Interestingly, NRP did not show any Th1 or Th2 shift.

Analysis of the DP-specific *in vitro* response before starting the treatment showed two distinct patterns: (1) the *high reactive state*, mainly observed in RP, which is defined by lower frequencies of Th1 and high values for Th2, Th9 and Th17 cells, and higher levels of pro-inflammatory cytokines like IL-5, IL-9, IL-13 and TNF- α ; and (2) the *low reactive state*, defined by high frequencies of Th1 and lower frequencies of Th2, Th9 and Th17, which was largely detected in patients who did not improve after 1-year SCIT (NRP group). The CG presented an intermediate phenotype in terms of reactivity, probably because this group potentially included both RP and NRP. We speculate that the *high reactive state* could be a predisposition for a favourable clinical outcome and could therefore be of use when deciding whether a patient should undergo SCIT. To our knowledge, no previous evidence about this initial reactive state related with the clinical improvement has been reported; however, further studies are needed to confirm this observation.

After 1 year of SCIT, data indicated that in RP, there was a skew from Th2, Th9 and Th17 cells towards Th1 and Treg cells with changes in their corresponding cytokines (increase in Th1 and immunosuppressive cytokines and decrease in Th2 cytokines). These variations are in line with previous reports. No changes were observed in terms of a shift towards a Th1 pattern for NRP. However, it is important to point out that only a small number of patients did not respond to the treatment, limiting our power to detect changes between basal and 12 months for this group. Despite this limitation, the statistical differences between NRP and RP before starting the treatment suggest that immunological differences exist between these groups. This is the first time that variations in T cell subpopulations were related to clinical improvement. It is interesting to note

the role of Th9 cells, as they have been proposed to have a pathogenic role in inflammatory diseases [61, 62] although other authors propose an immunosuppressive function [63, 64]. Results from our study suggest an effector function, as after 1 year of SCIT there was a decrease in both Th9 cells and IL-9 production in RP. No changes were detected in either cellular or cytokine patterns in NRP. Regarding CG, there was a trend towards an increase in the Th2 pattern.

In conclusion, our results revealed that those patients with clinical improvement after receiving DP-SCIT were characterized by an increased production of sIgG4, and a skewed pattern from Th2 responses towards less inflammatory Th1 and Treg responses at the molecular, cellular and cytokine production levels. Moreover, basal differences in T cell populations and cytokine production after DP stimulation could be useful for predicting SCIT efficacy. This may be due to a state of high reactivity in those patients that will improve by taking SCIT, which is characterized by a low frequency of Th1 and high values for Th2, Th9 and Th17 cells. More studies in this direction are required to determine whether some of these changes could be used as predictive markers of SCIT efficacy.

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Conflict of interest

None of the authors have any conflict of interest. All authors have read and approved the manuscript.

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Supporting Information

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

Figure S1. Flow cytometry gating strategy.

Figure S2. Positive control in basophil activation test.

Table S1. Primer sequences for qPCR.