

ZAP70 expression in regulatory T cells in allergic rhinitis: effect of immunotherapy

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Summary

Background Some allergic diseases may be accompanied by inappropriate number or malfunction of regulatory T cells, which seems to be modified by specific immunotherapy (SIT).

Objectives To assess if immunotherapy affects regulatory T lymphocytes (Tregs) and their expression of zeta chain associated protein kinase (Zap70), which is essential for T cell activation and intracellular signal downstream transduction.

Methods A 3-year prospective, placebo-controlled, double-blind trial of grass SIT was conducted. Forty-one patients sensitized to grass pollen with intermittent allergic rhinitis were randomized to receive SIT ($n = 21$) or placebo ($n = 20$) and 15 healthy were included as a control. Concentration of exhaled nitric oxide (ENO), lung function, symptom scores, the subsets of regulatory T cells (CD4⁺ CD25hiCD127low) which express ZAP70 and the level of ZAP70 expression in this subset were assessed at baseline and during the treatment period: before the onset, at the height of the pollen season and after the end of the pollen season.

Results The concentration of nitric oxide and the symptom score were significantly higher in allergic rhinitis patients as compared with the control group. Natural allergen stimulation diminished both the numbers of regulatory T cells that express ZAP70 and the expression of Zap70 within these cells. In the second year of treatment, immunotherapy reduced significantly the symptom scores, concentrations of ENO ($P < 0.01$), intensively increased expression of ZAP70 in regulatory T cells ($P < 0.001$) and the percentage of cells that express ZAP70 ($P < 0.05$) at the height of the pollen season. Placebo treatment did not reduce scores, ENO ($P > 0.05$) nor had influence on Zap70 expression ($P > 0.05$).

Conclusions SIT with grass pollen effectively reduces rhinitis severity and affects allergic airway inflammation reflected by reduction of ENO. Beneficial role of immunotherapy may result not only from the induction of Treg numbers but especially from cell activation and restitution of Treg intracellular signal transduction.

Keywords allergic rhinitis, immunotherapy, inflammation, regulatory lymphocytes, Zap 70
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Introduction

Specific immunotherapy (SIT) represents potentially curative and specific method of treatment for intermittent allergic rhinitis [1]. The effectiveness of SIT depends on the induction of peripheral tolerance to antigens which is mediated mainly by regulatory T cells (Tregs) [2]. Naturally occurring Tregs represent about 5% of the CD4 T cell subset in peripheral blood [3] and constitutively express high levels of the high-affinity

IL-2 receptor (CD25hi) [4], receptor for glucocorticoid induced tumour necrosis factor (GITR), cytotoxic T lymphocyte associated antigen (CTLA-4) and Forkhead Box Protein P3 (Foxp3) as well as display low expression of α chain of the IL-7 receptor (CD127) [5, 6]. Regulatory T cells induced in periphery comprise IL-10 producing type 1 regulatory T cells (Tr1) and regulatory T cells subset arising *in vitro* from CD4⁺ CD25[−] and *in vivo* from peripheral memory T cells [7, 8]. Clinical improvement seen after specific allergen immunotherapy in

patients with allergic rhinitis is associated with the increase in Tregs number, induction of IL-10 and TGF β and decrease in Th2 cytokine profile in the inflammatory milieu and subsequent decrease in airway hyperresponsiveness [3, 9].

Although clinical and immunological outcomes of SIT were profoundly studied, little is known about the molecular mechanisms of Tregs activation and intracellular signal transduction which are required for the suppression in the course of allergen specific immunotherapy.

In effector T cells engagement of T cell receptor (TCR) together with costimulatory signals results in phosphorylation of tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR-CD3 complex by the p56lck kinase [10]. Next, phosphorylated zeta chain of CD3 binds to zeta-associated protein (ZAP70) enabling phosphorylation and activation of the latter protein by p56lck kinase [10]. Activated ZAP70 initiates intracellular signal transduction resulting in activation of protein kinase C (PKC), calcium mobilization and up-regulation of genes crucial for effector T cell function [10].

Since expression of ZAP70 in peripheral lymphocytes reflects TCR ligation and lymphocytes activation [10, 11], we hypothesize that both natural allergen stimulation and allergen specific immunotherapy may affect TCR-associated downstream signal transduction in regulatory T cells reflected by ZAP70 expression.

In this study, we have assessed the subsets of regulatory T cells (CD4CD25hiCd127low) before the start, at the height of the pollen season and after the termination of pollen season both in untreated patients, during the treatment with specific allergen immunotherapy or placebo and healthy control. Furthermore, we have checked if natural allergen-stimulation affects ZAP70 expression in Tregs and whether specific SIT is able to modulate ZAP70 expression in regulatory T cells.

Methods

This was a 3-year, prospective, double-blind, randomized, placebo-controlled single-centre study in which the second, 2-year phase was blinded. Forty-one patients suffering from moderate-to-severe intermittent allergic rhinitis (AR) caused by sensitization to grass pollen were included. The diagnosis of AR was based on Allergic Rhinitis and its Impact on Asthma guideline [12] with a positive history, a skin prick test, specific serum IgE and positive results of conjunctival provocation test (CPT) with grass pollen allergens extracts. Persistent allergic rhinitis, sensitization to seasonal allergens that could interfere with grass pollen, asthma, ciliary dysmotility syndrome, cystic fibrosis, bronchiectasis, smoking, tuberculosis, neoplastic disease,

chronic sinusitis, nasal polyps, systemic glucocorticosteroids treatment, treatment with angiotensin converting enzyme inhibitors or beta-blockers as well as chronic obstructive pulmonary disease were excluding criteria. Fifteen healthy, non-allergic patients were included as a control.

Nasal corticosteroids were excluded 6 weeks before and during the study. Oral antihistamines were excluded 2 weeks before the screening visit; however, topical (emedastine, olopatadine) or oral antihistamines (cetirizine 10 mg tablets, levocetirizine 5 mg tablets, fexofenadine 120 mg tablets or desloratadine 5 mg tablets) were allowed as a rescue medication during the pollen season.

After the first year of evaluation, eligible patients were randomized to receive either SIT with Allergovit grass pollen 100% ($n = 21$) (Allergopharma, Ganzer KG, Reinbeck, Germany) or placebo ($n = 20$) for 2 years.

Both during the baseline year and during the treatment period, 6 weeks before (A), at the height of the pollen season (B) and 6 weeks after the end of the pollen season (C) the patients underwent clinical assessment and physical evaluation by the physician, spirometry, measurement of exhaled nitric oxide and blood sampling. All participants evaluated nasal symptoms score and eye symptoms daily in the evening and documented symptoms and adverse events in diaries. Tolerability was evaluated by adverse event reporting.

The local ethical committee approved the study (approval number KE RNN/89/07/KG) and a written informed consent was obtained from each participant before entering the trial. The study was registered in www.clinicaltrials.gov, number NCT01475188.

Patients' compliance

Compliance was assessed by the patients' adherence to the visit schedule, return of diaries and compliance with their completing.

Clinical efficacy

Clinical efficacy was assessed using scales on symptoms and medication use and lung function tests. Severity of allergic rhinitis was monitored daily using 0–3 point scale (0 lack of symptoms, 1 mild symptom with minimal inconvenience, 2 moderate symptoms, 3 severe symptoms that interfere with everyday activity and/or sleep). Patients reported the presence of eye symptoms (itching, redness, lacrimation), nasal symptoms (itching, sneezing, discharge from the nose, blockage) as well as dyspnoea, cough, wheezes and medicaments. The results for nasal symptoms were extracted from the patients' diaries for further analysis.

Spirometry

Spirometry was performed according to the American Thoracic Society guidelines (ATS) [13] with a Lung Test 1000 spirometer (MES, Krakow, Poland). The best forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) out of a minimum three acceptable and comparable forced manoeuvres were used. Values were expressed as a percentage of the predicted values. Provocation tests were performed according to the method described by Cockcroft *et al.* [14] with successively increased concentrations of methacholine inhaled from jet nebulizer (DeVilbiss 646, DeVilbiss Healthcare, Sommerset, PA, USA). The concentration of methacholine (PC20) that provoked a 20% fall of FEV₁ was calculated. Patients with PC20 < 8 mg/mL were considered to have bronchial hyperresponsiveness and were excluded from the study.

Concentration of exhaled nitric oxide (ENO)

The concentration of exhaled nitric oxide (NO) was measured according to the current ATS/ERS guidelines [15] with recommended single-breath method at the expiratory flow rate of 50 mL/s (Hyp'Air FeNO, Medisoft, Belgium). During a single slow exhalation from total lung capacity, the initial part of the NO was discarded and the NO was analysed during end-expiratory, high repeatable phase. The measurement was repeated three times and the mean value was collected for statistical analysis. The results were presented as a number of parts of NO per billion (ppb).

IgE, skin prick tests and conjunctival provocation test

IgE kit (UniCap, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) was used for the quantitative determination of specific serum IgE levels. The sensitivity for detection was 0.15 kU/L. Patients were included in the study if the concentration of specific IgE for grass was higher than 1.5 kU/L and predominated over concentrations for other interfering allergens. Skin prick tests with commercial set of 11 common allergens (Allergopharma J. Ganzer KG) were performed in each patient at the screening visit with histamine (10 mg/mL) as a positive, and diluent as a negative control. The results were regarded as positive when the mean weal diameter (assessed as a sum of the largest diameter and its perpendicular measurement) was 3 mm or greater.

CPT was performed outside the pollen season according to conjunctival provocation test recommendations [16]. Standardized pollen allergen extract of six grass species together with diluent (Allergopharma J. Ganzer KG) were used in the test. The concentration of allergen extract was increased every 10 min in half logarithmic

steps with the initial concentration 5 BU/mL and subsequent allergen concentrations: 50, 160, 500, 1600 and 5000 BU/mL. The test result was determined positive if the redness exceeded more than half of the conjunctiva and the eye was itching and/or there was general irritation.

Separation of CD4⁺ cells

Within 30 min peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated peripheral blood by Ficoll Paque (GE Healthcare, Warsaw, Poland) density gradient centrifugation, which gives a high yield of mononuclear leucocytes with low contamination by other cells. At the end of PBMCs preparation cells were re-suspended at a concentration of 5×10^7 cells/mL in dilution buffer. CD4⁺ cells were isolated by negative magnetic cell separation system (EasySep, Stemcell, Wroclaw, Poland). PBMCs were incubated for 10 min at room temperature with human T cell enrichment cocktail (EasySep), which contains tetrameric antibody complexes (TAC) directed against cell surface antigens on the human blood cells and dextran (Stemcell, Wroclaw, Poland). Next, the cells were incubated with magnetic beads coupled to dextran (EasySep D Magnetic Particles) according to the manufacturer's instructions and CD4⁺ cells were sorted negatively using magnet (EasySep Magnet). Selected CD4⁺ cell fractions were stained with fluorochrome-conjugated anti-CD4 antibodies and analysed by flow cytometry. The CD4⁺ population was sorted to a purity of 95%.

Flow cytometry

For Zap70 staining 2 tubes on each cells suspensions were initially stained using peridinin chlorophyll protein complex conjugated anti-CD4 (BioLegend, San Diego, CA, USA), FITC-labelled anti-CD25 (Stemcell Technologies, Grenoble, France) and Alexa Fluor® 647 conjugated anti-CD127 (BioLegend) for 15 min at room temperature in the dark. Subsequently, these cells were fixed in 10% Cell FIX™ solution (Becton Dickinson, Franklin Lakes, NJ, USA) for 20 min at room temperature. Then, cells were washed and phycoerythrin conjugated anti-Zap70 antibody (BioLegend) was added to one tube (test sample) and mouse isotype antibodies IgG1 (BioLegend) to the other (negative control). Both tubes were incubated and mixed for 30 min than cells were washed and resuspended in PBS for flow cytometric analysis. Cells were analysed on FACS Canto™ II, a LSR (3 laser, 8 colours benchtop flow cytometer, Becton Dickinson) and were determined by comparison with cells stained with isotype control. ZAP70 expression was calculated as the percentage of regulatory T cells (CD4⁺ CD25hiCD127low)

expressing ZAP70 above the baseline threshold set by the isotype control. The data acquisition threshold was set on a forward channel to exclude dead cells and the low size debris. The results were analysed using FACS Diva software (Becton Dickinson). The ZAP70 expression within regulatory T cells was presented as median fluorescence intensity (MFI).

Pollen season

The course of grass pollen season (the onset, the height, the end and intensity) was predicted and assessed on the basis of analysis of local airborne pollen count reports prepared annually.

Administration of immunotherapy

SIT was performed according to the European Academy of Allergy and Clinical Immunology recommendations [17] with commercial grass pollen extracts (Allergovit, grass pollen, Allergopharma). The treatment was initiated in November and included a 9-week up-dosing phase with the initial dose of 0.1 ml (concentration A) which was increased every 7–14 days, until the highest tolerated dose was reached (0.6 mL of concentration B) and SIT was continued for 2 years with injection intervals of 4–6 weeks. Each injection was given subcutaneously in the hospital by well-trained medical staff and the patients were monitored for 30 min after injection. Administered doses were recorded in patients' source data.

Statistical analysis

All statistical procedures were performed with Statistica 5.1 PL (StatSoft, Krakow Poland). Normal distribution of the data was confirmed with Kolmogorov–Smirnov test. For normally distributed data, values were expressed as the mean with the standard error of mean (SEM) and for not normally distributed data values were additionally presented as median with 25th and 75th percentiles. To compare the data of patients from the one group at the different visits (intergroup analysis) paired *t*-test was used for normally distributed values, whereas Wilcoxon test was used for not normally distributed data. Correlations were tested using Spearman test. One-way analysis of variance (ANOVA) was carried out with *post hoc* analysis to compare results between the groups. The study was designed to detect significant changes from baseline (intragroup) and changes between the healthy control and SIT or placebo-treated group in subsets of Treg Zap70 positive cells. An increase in the regulatory T cells subset of 10% or greater from the baseline and between SIT and placebo were considered efficacious. Thus, with a power of 80%

and $\alpha = 0.05$ a sample size of 40 (SIT = 20 and placebo = 20) was required to yield a statistically significant result.

Results

Of 126 screened patients 46 were randomized and 41 completed the study (16 women and 25 men aged 21–57). The control group comprised 15 subjects: 7 men and 8 women aged 19–36. Nine dropouts occurred during the study. Of 46 patients with AR included initially, five patients discontinued the trial due to the consent withdrawal ($n = 1$), change in residence ($n = 2$) and two patients were excluded due to the low compliance, whereas in the control group out of 19 patients included initially two patients withdrew their consent and two patients were excluded due to the low compliance. There were no differences in sex, age, and duration of AR between patients from the placebo and allergen treated groups. Detailed characteristics of patients participating in the study are presented in Table 1.

The conjunctival provocation test

The CPT was positive in all patients with AR. The majority of patients developed symptoms at medium concentrations of allergen extracts (1600 and 500 BU/mL), while two subjects had positive results at low concentration (50 BU/mL).

In 32 patients, itching and eye irritation occurred simultaneously (Table 2).

All data (with the exception of the subsets of regulatory T cells and MFI of ZAP70 in regulatory T cells) are presented for the baseline year and for the second year of treatment.

Expression of ZAP 70 in regulatory T cells

Numbers of regulatory T cells (CD4⁺ CD25⁺ CD127^{low}) that express ZAP70 as well as the superficial expression

Table 1. Participants' baseline characteristics. Values are presented as mean $\pm$ SD

	Placebo group	SIT group	Healthy control
Number of subjects	20	21	15
Age	32.3 $\pm$ 6.8	34.1 $\pm$ 9.7	26.4 $\pm$ 4.2
Sex F : M	7 : 13	9 : 12	8 : 7
History of allergic rhinoconjunctivitis (years)	11.9 $\pm$ 7.4	13.8 $\pm$ 8.5	0
slgE for grass pollen	8.55 $\pm$ 5.3	7.98 $\pm$ 6.1	0.15 $\pm$ 0.14
FEV1 (L/s)	93.6 $\pm$ 1.74	92.38 $\pm$ 1.54	95.8 $\pm$ 2.07

Table 2. The conjunctival provocation test in patients with AR. Results are presented as number of patients with the positive reaction in CPT produced by fixed concentration of allergen extract

Concentration of grass pollen in allergen extract (BU/mL)	Placebo group	SIT group
5	0	0
50	1	1
160	2	3
500	9	7
1600	8	9
5000	0	1
Total	20	21

of ZAP70 in Treg cells were assessed at baseline and in patients undergoing grass pollen immunotherapy, treated with placebo and in control subjects. Immunotherapy did not increase the numbers of CD4CD25hiCD127low cells both during the first and second year of treatment (Table 3). Fractions of CD4CD25hiCD127low T cells expressing ZAP70 were not significantly lower in the healthy group when compared to AR (except for the height of the baseline pollen season, Table 3). In the latter, T regulatory ZAP70 positive cells decreased at the height of the baseline pollen season compared with pre- and post-season values ($P < 0.001$ vs. pre- and post-pollen evaluation in SIT group, $P < 0.001$ vs. pre- and $P < 0.05$ vs. post-pollen season in the placebo group). Active treatment increased the subsets of ZAP 70 positive regulatory T cells at the height of the pollen season both during the first as well as the second year of SIT; however, only in the last year of the immunotherapy the increase was significant ($P < 0.05$), whereas the subsets of these cells at pre- and post-pollen season evaluation remained unchanged ($P > 0.05$, Table 3, Fig. 2). The numbers of regulatory T cells that express ZAP 70 generally remained unchanged both in the placebo and the healthy group (Table 3, Fig. 2).

Furthermore, numbers of ZAP70 positive regulatory T cells correlated negatively with the concentration of ENO and correlation was significant especially at the height of the pollen season in patients treated with SIT and placebo (Table 4).

Median fluorescence intensity for ZAP 70 in regulatory T cells at baseline year and during the first and the second year of the study, both before the start and after the termination of the pollen season, did not differ from MFI observed in the healthy control; however, at baseline it decreased significantly at the height of the pollen season in AR patients (Table 3, Fig. 3). Specific immunotherapy resulted in up-regulation of ZAP70 in regulatory T cells in the second year of SIT ($P < 0.001$) when placebo was ineffective ($P > 0.05$) (Table 3, Fig. 3).

Table 3. CD4⁺ CD25hiCD127lowZAP70 T cell subsets, median fluorescence intensity of ZAP70 within regulatory T cells and subpopulation of CD4⁺ CD25hiCD127low cells of total peripheral blood CD4⁺ in patients treated with SIT, placebo and healthy group before (A), at the height (B) and after the termination (C) of the pollen season during the baseline year and before, at the height and after the termination of the pollen season during the first (A1, B1, C1) and second (A2, B2, C2) year of treatment respectively. Values are presented as mean $\pm$ SEM (normally distributed) and median [25th and 75th percentiles] (not normally distributed data)

	SIT	Placebo	Control
CD4 ⁺ CD25hiCD127lowZAP70 (%)			
A	83.24 $\pm$ 2.3	82.51 $\pm$ 2.4 85.7 [78.95–88.55]	77.73 $\pm$ 3.4
B	60.01 $\pm$ 1.9	62.23 $\pm$ 2.9	74.36 $\pm$ 3.6*
C	88.49 $\pm$ 4.2 93.5 [81.3–98.8]	89.64 $\pm$ 2.9	85.72 $\pm$ 2.5 89.4 [79.6–90.83]
A1	80.82 $\pm$ 1.76	81.25 $\pm$ 1.97	83.11 $\pm$ 2.9
B1	69.71 $\pm$ 1.19 [63.0–75.9]	61.95 $\pm$ 2.21	80.05 $\pm$ 3.2 [†]
C1	82.6 $\pm$ 1.9	83.17 $\pm$ 2.02	79.91 $\pm$ 3.12
A2	90.12 $\pm$ 1.6	89.83 $\pm$ 2.0	80.35 $\pm$ 3.4
B2	76.48 $\pm$ 2.7 [‡] 74.9 [65.23–89.23]	64.76 $\pm$ 3.8	79.16 $\pm$ 3.1
C2	83.35 $\pm$ 2.0	83.78 $\pm$ 3.1	78.59 $\pm$ 3.0
MFI of ZAP70 in regulatory T cells			
A	2382 $\pm$ 396 2311 [2047–2701]	2426 $\pm$ 261	2190 $\pm$ 131 2134 [1765–2474]
B	1869 $\pm$ 188 [§]	1985 $\pm$ 302 [§] 1871 [1733–2228]	2440 $\pm$ 184
C	2115 $\pm$ 393 2094 [1913–2221]	2114 $\pm$ 392	2092 $\pm$ 172
A1	2146 $\pm$ 434	2259 $\pm$ 326	2329 $\pm$ 430
B1	2122 $\pm$ 439	1845 $\pm$ 210	2330 $\pm$ 332
C1	1972 $\pm$ 307	2087 $\pm$ 374 1996 [1804–2510]	2344 $\pm$ 456
A2	2036 $\pm$ 388 1940 [1795–2164]	2409 $\pm$ 361	2299 $\pm$ 160
B2	2573 $\pm$ 389 2616 [2367–2638]	1868 $\pm$ 192 [¶]	2556 $\pm$ 141
C2	1954 $\pm$ 305 1912 [1682–2306]	2016 $\pm$ 331 1897 [1755–2184]	2312 $\pm$ 152
CD4 ⁺ CD25hiCD127low cells of total peripheral CD4 ⁺ cells (%)			
A	4.25 $\pm$ 0.54	3.89 $\pm$ 0.56	3.78 $\pm$ 0.61
B	4.76 $\pm$ 0.45	4.36 $\pm$ 0.55	4.11 $\pm$ 0.61
C	4.55 $\pm$ 0.53	4.24 $\pm$ 0.49	4.31 $\pm$ 0.56
A1	4.34 $\pm$ 0.59	4.64 $\pm$ 0.48	4.19 $\pm$ 0.62
B1	4.29 $\pm$ 0.47	4.73 $\pm$ 0.52	3.96 $\pm$ 0.62
C1	3.98 $\pm$ 0.56	4.28 $\pm$ 0.53	3.78 $\pm$ 0.63
A2	4.35 $\pm$ 0.59	4.11 $\pm$ 0.5	4.96 $\pm$ 0.64
B2	4.24 $\pm$ 0.4	4.44 $\pm$ 0.47	4.05 $\pm$ 0.57
C2	4.38 $\pm$ 0.49	3.89 $\pm$ 0.59	4.25 $\pm$ 0.63

* $P < 0.05$ vs. B2 in patients treated with SIT and placebo.

[†] $P < 0.05$ vs. B1 in patients treated with placebo.

[‡] $P < 0.05$ for B2 SIT vs. B2 placebo.

[§] $P < 0.01$ vs. B2 control.

[¶] $P < 0.001$ for B2 placebo vs. B2 SIT and vs. B2 control.

MFI, median fluorescence intensity.

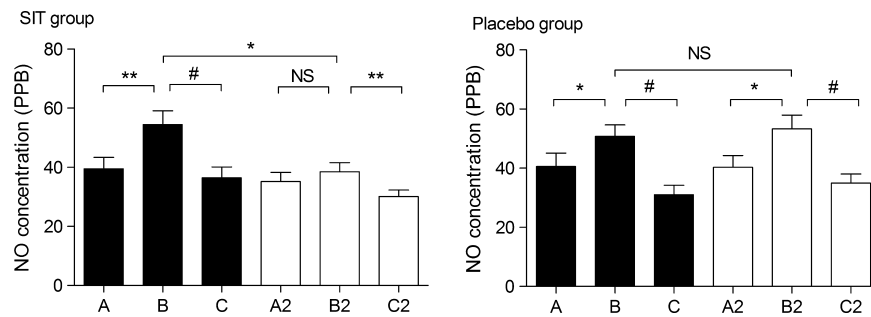


Fig. 1. Exhaled nitric oxide in patients treated with SIT or placebo before (A), at the height (B) and after the termination (C) of the pollen season during the baseline year and before (A2), at the height (B2) and after the termination (C2) of the pollen season during the 2nd year of treatment. Results presented as mean $\pm$ SEM, * P < 0.05, ** P < 0.01, # P < 0.001, NS, not significant.

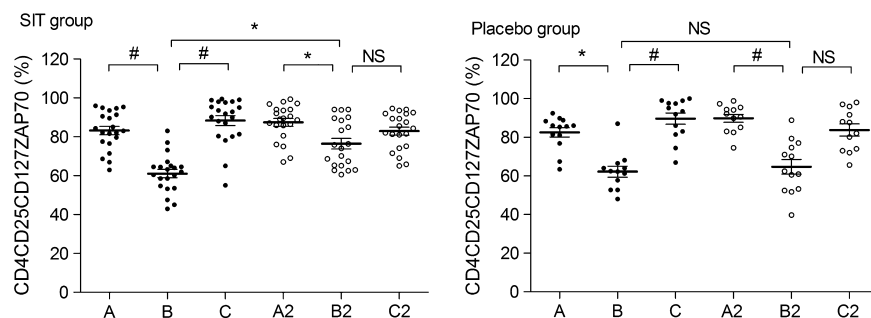


Fig. 2. Subsets of CD4⁺ CD25hiCD127low cells with ZAP70 expression in patients treated with SIT or placebo before (A), at the height (B) and after the termination (C) of the pollen season during the baseline year and before (A2), at the height (B2) and after the termination (C2) of the pollen season during the 2nd year of treatment. Values as mean $\pm$ SEM. Results presented as mean $\pm$ SEM, * P < 0.05, ** P < 0.01, # P < 0.001, NS, not significant.

Clinical efficacy

Compared with baseline, we have observed statistically significant decrease in symptom scores for itching, sneezing, nasal discharge and congestion especially in the second year of SIT, whereas such improvement actually was not observed in the group treated with placebo (Table 4). Furthermore, the relief from itching, sneezing and nasal discharge was greater than observed in patients treated with placebo. In the control group, the scores were significantly lower throughout the study and no differences in rhinitis severity were observed in particular years (Table 4).

Fractional concentration of exhaled nitric oxide

At baseline, concentration of exhaled NO was significantly higher in AR patients when compared to healthy controls (P < 0.001). Both in the AR and the healthy group, ENO concentration increased at the height of the pollen season but only in the AR patients the increase was significant (P < 0.01 in SIT group, P < 0.05 in placebo group). Compared with the baseline, only the actively desensitized patients in the second year of treatment showed significant reduction in ENO concentration

at the height of the pollen season (P < 0.05, Fig. 1). The concentration of nitric oxide was reduced at the height of the pollen season as compared to the placebo treated group (P < 0.05). Placebo treatment did not diminish ENO concentration (P > 0.05, Fig. 1). When compared with the baseline, no significant differences were observed in ENO in the control group (Table 4, Fig. 1). Furthermore, ENO correlated positively with the majority of symptom scores both in patients treated with SIT, placebo and healthy controls; however, correlations were significant especially at the height of the pollen seasons.

Discussion

Our study shows that outside the pollen season atopic, asymptomatic patients with grass associated pollinosis are characterized by non-significantly higher fractions of CD4⁺ CD25hiCD127 low regulatory T cells with co-expression of ZAP70 in peripheral blood than the healthy controls. Furthermore, we have demonstrated that natural pollen exposure results in the prompt decrease in T regs ZAP70 expressing subset having no influence on these cells in healthy individuals. Our study also revealed strong restoration of regulatory T cells, which express ZAP70, especially at the height

Table 4. Exhaled nitric oxide, correlation between fractions of TregZAP70 cells and concentrations of exhaled nitric oxide and symptoms score in patients treated with SIT, placebo and healthy group before (A), at the height (B) and after the termination (C) of the pollen season during the baseline year and before (A2), at the height (B2) and after the termination (C2) of the pollen season during the 2nd treatment year. Values are presented as mean $\pm$ SEM. Values are presented as mean $\pm$ SEM (normally distributed) and median [25th and 75th percentiles] (not normally distributed data)

	A	B	C	A2	B2	C2
Exhaled nitric oxide (ppb)						
SIT	39.52 $\pm$ 3.9 27 [20–55]	54.46 $\pm$ 4.6 39 [25–80]	36.43 $\pm$ 3.7 25 [18–55]	35.16 $\pm$ 3.1 29 [18–44]	38.52 $\pm$ 3.1 32 [21–50]	30.08 $\pm$ 2.2 24 [16–43]
Placebo	40.57 $\pm$ 4.5 30 [22.3–40.6]	50.78 $\pm$ 3.9 40 [28.5–71]	31.03 $\pm$ 3.2 21.5 [14.3–36.5]	40.33 $\pm$ 3.9 33 [18.25–50]	53.30 $\pm$ 4.7 [‡] 39 [32–71]	34.95 $\pm$ 3.1 30 [16–44]
Control	15.44 $\pm$ 2.5 [†] 14 [7.5–16.5]	16.07 $\pm$ 3.2 [†] 9 [7–18]	15.62 $\pm$ 3.2 [†] 9 [8–14]	22.69 $\pm$ 2.9 [†] 16 [11–23.5]	18.62 $\pm$ 1.5 [†] 17 [11.5–22.5]	19.44 $\pm$ 2.6 [†] 16 [8–22]
Correlation of fractions of Treg ZAP70 cells and the concentrations of exhaled nitric oxide						
SIT	–0.55 $P < 0.05$	–0.46 $P < 0.05$	–0.21 $P > 0.05$	–0.07 $P > 0.05$	–0.53 $P < 0.05$	–0.36 $P > 0.05$
Placebo	–0.57 $P < 0.01$	–0.39 $P > 0.05$	–0.38 $P > 0.05$	–0.42 $P > 0.05$	–0.55 $P < 0.05$	–0.39 $P > 0.05$
Control	–0.15 $P > 0.05$	–0.22 $P > 0.05$	0.28 $P > 0.05$	–0.1 $P > 0.05$	–0.2 $P > 0.05$	–0.09 $P > 0.05$
Symptom scores						
SIT						
Nasal blockage	0.93 $\pm$ 0.1	1.12 $\pm$ 0.1	0.47 $\pm$ 0.1	0.3 $\pm$ 0.03 ^{‡,***}	0.9 $\pm$ 0.1 ^{§,††}	0.42 $\pm$ 0.04
Sneezing	1.04 $\pm$ 0.1	1.5 $\pm$ 0.1	0.9 $\pm$ 0.1	0.5 $\pm$ 0.04 ^{‡,***}	1.2 $\pm$ 0.1 ^{‡,}	0.81 $\pm$ 0.1
Nasal itching	0.78 $\pm$ 0.04	1.24 $\pm$ 0.1	0.55 $\pm$ 0.04	0.48 $\pm$ 0.04 ^{‡,}	0.87 $\pm$ 0.1 ^{‡,}	0.45 $\pm$ 0.1 ^{‡,}
Nasal discharge	1.23 $\pm$ 0.1	1.37 $\pm$ 0.1	0.83 $\pm$ 0.05	0.52 $\pm$ 0.04 ^{‡,}	1.15 $\pm$ 0.1 ^{§,}	0.68 $\pm$ 0.05 ^{§,}
Placebo						
Nasal blockage	0.61 $\pm$ 0.04	0.73 $\pm$ 0.05	0.61 $\pm$ 0.05	0.61 $\pm$ 0.05	1.28 $\pm$ 0.05 [‡]	0.61 $\pm$ 0.03
Sneezing	0.98 $\pm$ 0.04	1.54 $\pm$ 0.06	1.18 $\pm$ 0.06	0.65 $\pm$ 0.04 [‡]	1.6 $\pm$ 0.06	0.83 $\pm$ 0.04
Nasal itching	0.96 $\pm$ 0.04	1.25 $\pm$ 0.06	1.08 $\pm$ 0.06	0.84 $\pm$ 0.04 [¶]	1.5 $\pm$ 0.06 [‡]	0.96 $\pm$ 0.05
Nasal discharge	0.89 $\pm$ 0.05	1.33 $\pm$ 0.05	0.85 $\pm$ 0.04	0.65 $\pm$ 0.04 [‡]	1.5 $\pm$ 0.05 [§]	0.9 $\pm$ 0.05
Control						
Nasal blockage	0.35 $\pm$ 0.03	0.41 $\pm$ 0.04	0.37 $\pm$ 0.03	0.28 $\pm$ 0.03	0.42 $\pm$ 0.04	0.36 $\pm$ 0.03
Sneezing	0.36 $\pm$ 0.04	0.5 $\pm$ 0.04	0.5 $\pm$ 0.04	0.37 $\pm$ 0.04	0.53 $\pm$ 0.04	0.41 $\pm$ 0.03
Nasal itching	0.34 $\pm$ 0.04	0.38 $\pm$ 0.04	0.31 $\pm$ 0.03	0.27 $\pm$ 0.03	0.39 $\pm$ 0.04	0.39 $\pm$ 0.04
Nasal discharge	0.47 $\pm$ 0.04	0.52 $\pm$ 0.04	0.56 $\pm$ 0.04	0.35 $\pm$ 0.03 [§]	0.56 $\pm$ 0.04	0.46 $\pm$ 0.04 [¶]

* $P < 0.05$ vs. B2 SIT.

[†] $P < 0.001$ vs. placebo and SIT group.

[‡] $P < 0.001$, [§] $P < 0.01$, [¶] $P < 0.05$ for A vs. A2, B vs. B2, C vs. C2 for each symptom.

^{||} $P < 0.001$, ^{**} $P < 0.01$, ^{††} $P < 0.05$ for scores vs. placebo.

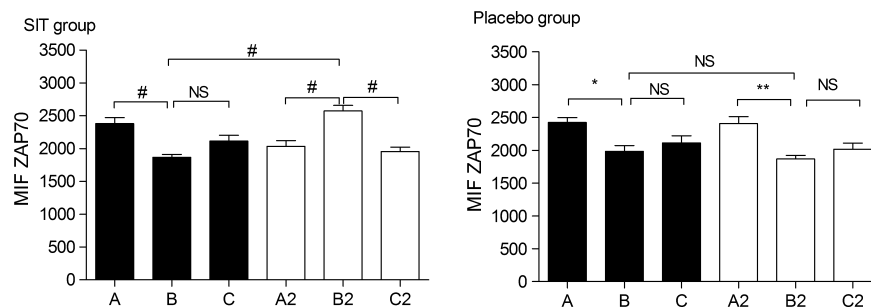


Fig. 3. Median fluorescence intensity (MFI) for ZAP70 within regulatory T cells in patients treated with SIT or placebo before (A), at the height (B) and after the termination (C) of the pollen season during the baseline year and before (A2), at the height (B2) and after the termination (C2) of the pollen season during the 2nd year of treatment. Values as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$, NS, not significant.

of the pollen season in patients treated with allergen SIT that was associated with clinical improvement in this population.

In regulatory T cells engagement of TCR by the antigen is essential for later ZAP70 phosphorylation and intracellular signal transduction. Once specifically activated Tregs are able to inhibit the proliferation and cytokine production of co-cultured T effector cells in an antigen non-specific manner [18, 19]. In this study, we have demonstrated pronounced expression of ZAP70 in CD4⁺ CD25hiCD127 low T cells in patients with allergic rhinitis outside the pollen season. Subsets of ZAP70 expressing Tregs were higher than those in the healthy individuals. This remains in contrast to the results of Tsang study [18], who demonstrated that Tregs down-regulate ZAP70 after the TCR ligation *in vitro*. However, Shevach et al. opposes this statement showing that 'lifestyle' or behaviour of Tregs *in vivo* is different from seen *in vitro* and Tregs may respond to TCR ligation and proliferate similar to conventional effector T cells [20]. Increased ZAP70 expression in Tregs (CD4⁺ CD25hiCD127low) during the asymptomatic period in the AR patients with comparable numbers of Tregs between the healthy and AR patients, may result from malfunctioning of Tregs in the latter group. Both natural and adaptive Tregs are capable of suppressing Th2 responses to allergens in health, but this suppression may be attenuated in allergic conditions [21]. Dysfunction of regulatory T cells was reported in patients with diabetes [22], multiple sclerosis [23] and allergic diseases [4, 24]. Such a dysfunction is not fully understood and may result from alterations in internal signal transduction or rearrangement of TCR [25] which may modify antigen ligation and signal generation. However, Bacchetta et al. [4] revealed in diabetic patients that Tregs were not defective per se, and the inconsistency in the previous studies may result from low purity of Tregs fractions. Furthermore, higher fractions of Treg ZAP70 positive cells of AR patients outside the pollen season may result from minimal persistent inflammation. Bousquet et al. [12] observed chronic up-regulation of inflammatory cells and mediators in nasal fluids of patients with seasonal AR during symptom-free periods. Outside the pollen season, both increased numbers of eosinophils and neutrophils within the nasal mucosa and increased expression of adhesion molecules on epithelial cells were identified which contributes to chronic, minimal inflammation [26, 27]. Although seasonal AR is clinically silent, and there is no allergen stimulation, allergic inflammation is present and precedes the onset of clinical symptoms. Thus in AR patients, Tregs may be permanently engaged in modulation of chronic allergic inflammation; however, their suppressive functions diminish during excessive allergen exposure.

At the height of the pollen season, we have observed significant reduction in ZAP70 fraction within CD4⁺ CD25hiCD127low cells solely in the AR patients. Lower zeta-associated protein phosphorylation may result from continuous TCR engagement by high doses of allergens during the pollen season. Allergen-TCR ligation leads to TCR-CD3 complex internalization [10] that may limit antigenic signalling in T cells, with lower ZAP70 phosphorylation and T cell unresponsiveness [10, 28]. Furthermore, during the natural pollen exposure, Tregs migrate intensively to the sites of inflammation [23] resulting in the decrease in Tregs ZAP70 positive subsets in peripheral blood. Tregs were found in the nasal mucosa of symptomatic AR patients and the airway mucosa of asthmatics but none of these studies examined ZAP70 expression within Tregs [29]. What is more, histamine, released extensively by mast cells and basophils especially at the height of the pollen season, is capable of suppressing regulatory T cells function via H1 receptors and may augment inflammation resulting in aggravation of symptoms [30].

In addition, dysfunction of Tregs, which persists in asymptomatic patients with AR, may be escalated by the pollen exposure. Ling et al. [31] found that CD4⁺ CD25⁺ T cells were defective particularly when were isolated during symptomatic periods suggesting that increased allergen dose *in vivo* may provide a stimulus strong enough to override Treg-mediated suppression. Similarly, Grindebacke et al. demonstrated defective down-regulation of natural exposure-induced production of IL-5 and IL-13 by CD4CD25 regulatory T cells from atopic, but not non-atopic patients, during the birch pollen season [32].

Furthermore, we have demonstrated for the first time, that only specific allergen immunotherapy resulted in significant increase in Tregs subset expressing ZAP70 and up-regulated ZAP70 in T reg cells solely at the height of the pollen season, whereas entire numbers of regulatory T cells before, at the height and after the termination of pollen season remained unchanged. This is in agreement with Möbs' study who found no significant change in numbers of CD4⁺ CD25hiCD127 low regulatory T cells in patients treated with birch SIT [33]. Increased ZAP70 expression in Tregs corresponded to such an increase presented before only in patients with chronic lymphocytic leukaemia [34] and lymphoma [35] so that the imatinib, selective kinase inhibitor, was able to impair Treg immunosuppressive function via ZAP70 inhibition and Foxp3 expression thus enhanced anti-tumour immune responses [35]. However, the detailed mechanisms underlying such an increase in patients treated with allergen immunotherapy remain unclear. Reduction in IgE levels and increase in antigen sweeping specific IgG4 levels in the course of SIT, decreases mast cell activation and release

of histamine and abolishes the inhibitory effect of histamine on regulatory T cells. Furthermore, expansion of Tregs-ZAP70 positive fractions may result from very high dose of repeatable administered allergen and different route of administration. While during the natural pollen exposure airway antigen presenting cells (APC) are key presenters, subcutaneous administration of allergen may activate different APC subsets.

Conclusions

Our study suggests that regulatory T cells are permanently stimulated during the symptom-free periods and may modulate chronic minimal allergic inflammation. Their suppressive functions may be diminished during the pollen season, which contributes to the escalation of allergic inflammation and AR symptom occurrence. Specific allergen immunotherapy resulted in prompt restitution of ZAP70 expressing Tregs, especially at the height of the pollen season. Since the SIT solely may

enhance the immunosuppressive properties of Tregs and subsequently repress inflammation it is plausible that other supplementary stimuli which interact with the ZAP70 intracellular signal transduction in Tregs administered at the same time with SIT may bring additional benefits to patients. Therefore, further researches are needed regarding the clinical relevance of ZAP70 restitution within Treg cells.

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Conflicts of interest

The authors declare no conflict of interest.

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