

Three-Year Follow-Up Study of Allergen-Induced *in vitro* Cytokine and Signalling Lymphocytic Activation Molecule mRNA Responses in Peripheral Blood Mononuclear Cells of Allergic Rhinitis Patients Undergoing Specific Immunotherapy

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Key Words

Pollen allergy • Allergic rhinitis • Specific immunotherapy • Cytokines • Signalling lymphocytic activation molecule • Th1 cells • Th2 cells • T regulatory cells

Abstract

Background: Allergen-specific immunotherapy (SIT) is known to affect the allergen-specific T helper cell (Th2/Th1) balance and to induce T regulatory (Treg) cells. These observations have usually been made during the first treatment year and often without symptom monitoring. This study was performed to investigate allergen-induced Th2 (IL-4, IL-5)-, Th1 [IFN- γ , IL-18, signalling lymphocytic activation molecule (SLAM)]- and Treg (IL-10)-type immune responses in peripheral blood mononuclear cells (PBMC) and their association with symptom improvement in allergic rhinitis patients after 3 years of SIT. **Methods:** Twenty patients were treated with SIT and 8 patients were studied as untreated controls. PBMC were collected before and after 1 and 3 years of SIT and stimulated with specific allergen. Cytokine and SLAM mRNA expression was determined by TaqMan[®] RT-PCR. Symptoms were recorded yearly using visual analogue scale (VAS) scoring. **Results:** IL-18, SLAM and IL-10 mRNA expression increased after 3 years of SIT, with a peak at 1 year, whereas IL-5 mRNA expression transiently decreased and IFN- γ mRNA

expression transiently increased after 1 year of SIT. The increases in IL-18 and SLAM expression were not associated with symptom improvement, whereas decreases in both IL-4 expression and the IL-4/IFN- γ ratio after 1 year of SIT were found in patients with a good therapeutic outcome (>40 percentage unit reduction in VAS). **Conclusions:** SIT has long-term effects on allergen-specific immune responses. The induced Treg- and Th1-type responses persist over 3 years of SIT, whereas Th2-type responses are transiently decreased only during early therapy.

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Introduction

Allergen-specific immunotherapy (SIT) represents an effective treatment for type I, IgE-mediated hypersensitivities, including pollen allergies. It induces long-term remission in allergy symptoms, reduces medication usage and may even prevent the development of new sensitizations and asthma in allergic rhinitis patients [1]. Understanding of the immunological mechanisms behind SIT has significantly increased during the past couple of decades, especially since the discovery of Th2 and Th1 subsets and, more recently, CD4+CD25+ T regulatory (Treg) cells [2, 3]. During SIT, there is a shift from the

Table 1. Study patients

No.	Gender	Age years	SIT allergen	SIT regimen
<i>Allergic rhinitis patients undergoing SIT</i>				
1	male	37	birch	clustered rush
2	male	40	timothy	clustered rush
3	female	28	birch	conventional
4	female	36	birch	conventional
5	female	31	timothy	conventional
6	male	29	birch, timothy	conventional
7	female	32	birch	clustered rush
8	male	32	birch	conventional
9	female	19	birch	conventional
10	female	18	birch, timothy	conventional
11	male	29	birch	clustered rush
12	female	25	birch	clustered rush
13	female	30	birch	conventional
14	female	35	birch	clustered rush
15	male	39	birch	clustered rush
16	male	30	birch	conventional
17	male	30	birch, timothy	conventional
18	male	33	birch, timothy	conventional
19	male	25	timothy	clustered rush
20	male	33	birch, timothy	conventional
Mean		30.6		
SD		5.8		
<i>Allergic rhinitis controls</i>				
1	male	44		
2	female	33		
3	female	25		
4	female	28		
5	male	28		
6	female	27		
7	female	27		
8	female	26		
Mean		29.8		
SD		6.2		

predominant allergen-induced Th2-type cytokine responses (IL-4, IL-5) towards a more Th1-type pattern (IFN- γ , IL-12), as well as the induction of Treg cells that produce IL-10 [4–8].

IL-10 is an anti-inflammatory cytokine that has several anti-allergic immune effects. It inhibits cytokine synthesis and proliferation by Th1 and Th2 cells and induces a long-term antigen-specific nonresponsive (anergic) state in CD4+ T cells [9, 10]. IL-10 also inhibits IL-4-mediated IgE synthesis in B cells, while inducing IgG4 production [11, 12] and preventing degranulation of mast cells and basophils [13, 14].

IL-18 and signalling lymphocytic activation molecule (SLAM; CD150) are 2 molecules suggested to play a role

in the Th2/Th1 shift during SIT [15, 16]. SLAM is a self-ligand, transmembrane, cell surface glycoprotein belonging to the CD2 subset of the immunoglobulin superfamily and is preferentially expressed on Th1 over Th2 cells [17–19]. It has been shown to induce proliferation and IFN- γ production in T cells and reverse the cytokine production profile of Th2 clones to a Th0/Th1 type [20, 21]. IL-18, in turn, is a macrophage-derived cytokine originally defined as a factor capable of inducing IFN- γ production in T cells [22]. Its effects in vitro, however, have been shown to depend on co-stimulatory factors, in particular IL-12. In the presence of IL-12, IL-18 has been shown to enhance IFN- γ production in T cells and B cells, inhibit IgE production and prevent antigen-specific Th2-like cell development [23–25], but in the absence of IL-12, it induces IL-18 receptor- α expression on mast cells and basophils and stimulates IL-4 and IL-13 production, resulting in increased IgE levels [26]. IL-12 mRNA expression increases in macrophages during SIT [27, 28].

While there is strong evidence that all these cytokines and SLAM play a role in SIT, the evidence is from short-term studies, with a maximum duration of 1 or 2 years [15, 16, 29–34]. In other words, studies on these immunological mechanisms extended to cover the whole treatment period are currently lacking. This study was undertaken to analyze the expression of IL-4, IL-5, IL-10, IL-18, IFN- γ and SLAM mRNAs in in vitro allergen-stimulated peripheral blood mononuclear cells (PBMC) during the complete 3-year course of SIT in pollen-allergic rhinitis patients. The expression of these mRNAs was compared with the clinical efficacy of SIT.

Patients and Methods

Patients

Twenty-eight patients with skin prick test-verified pollen allergy, elevated allergen-specific serum IgE levels and a history of allergic symptoms were included in the study. The patient characteristics are presented in table 1. The patients were enrolled from the outpatient clinic of the Department of Pulmonary Diseases and Clinical Allergology of the Turku University Central Hospital. Twenty patients were treated with SIT and 8 were studied as untreated allergic controls. The study was performed with the approval of the local ethics committee and with the patients' written informed consent.

Specific Immunotherapy

Twelve patients were treated with Alutard SQ (ALK-Abelló, Hørsholm, Denmark) birch (*Betula verrucosa*), 3 with timothy (*Phleum pratense*) and 5 with a combination of birch and timothy. The maintenance dose was 100,000 SQ-U given at 6-week intervals over 3 years of therapy. The up-dosing was performed by increasing

the dose of Aquagen SQ (ALK-Abelló) over 5 weeks using the clustered rush schedule ($n = 8$) or Alutard SQ at weekly intervals for 15 weeks using the conventional schedule ($n = 12$). Clustered rush SIT was performed in patients who preferred this option.

Blood Samples

Blood samples were collected before SIT and 1 and 3 years after the start of therapy, each time out of pollen season. The blood samples from untreated allergic controls were collected at equal intervals.

Pollen Counts

Pollen data originates from the monitoring site in Turku, Finland. Sampling and analysis was carried out according to the European standards [35]. Pollen samples were taken with a Burkard spore trap (Burkard Manufacturing Ltd., Rickmansworth, Hertfordshire, UK). The trap was situated on the roof of a university building, 18 metres above the ground. All pollen counts were analysed daily. The pollen season was 90% of the pollen total. Pollen counts were expressed as the number of pollen grains per cubic meter of air and the mean seasonal count was used as a representative value for the season. A high sampling site is representative for pollen types with a high source, for instance birch trees, but grass pollen counts are often underestimated, because their source is at a low level and most pollen grains settle close to the source area (counts 4.4 times higher at the ground level than on the roof) [36].

Clinical Evaluation

Allergy symptoms and the use of medication were scored post-seasonally prior to commencement of SIT and thereafter after 1, 2 and 3 years of therapy and adjusted to pollen counts as described earlier [32]. Symptoms were recorded using the visual analogue scale (VAS) of 9 different symptoms (conjunctival itching, erythema and discharge; nasal itching, blockage and discharge; nocturnal and daytime cough induced by exercise). Medication was scored on a scale of 0–5 [not at all (0), occasionally seldom (1), occasionally often (2), almost daily (3), continuously (4), continuously with the maximal dose (5)] separately for peroral antihistamines, topical nasal steroids and eye drops (cromones or antihistamine). The mean VAS of the 9 different symptoms was calculated as percentage units of the maximal VAS score to represent seasonal symptoms. The natural logarithms of the mean seasonal pollen counts were calculated to represent seasonal pollen exposure [37]. The percentage unit score of VAS symptoms was adjusted to pollen exposure by multiplication with the ratio of the natural logarithms of the long-term and seasonal mean pollen counts.

Antigens

Whole allergen extracts of birch (*B. verrucosa*) and timothy grass (*P. pratense*) pollen were kindly provided by ALK-Abelló.

Lymphocyte Cultures

PBMC were isolated from heparinized blood samples by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. The PBMC were washed twice with Hank's balanced salt solution and suspended in RPMI (Gibco Life Technologies, Paisley, Scotland, UK), supplemented with 5% autologous serum, 2 mM L-glutamine (Gibco Life Technologies), 100 U/ml penicillin and 100 µg/ml gentamicin (Nordvacc Media,

Skärholmen, Sweden). The PBMC were then applied to 48-well culture plates, at a density of 2×10^5 cells per well, and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 3 days. Stimulation was performed in the presence of 50 µg/ml relevant whole-allergen extract. After 72 h of stimulation, the PBMC were collected by centrifugation, suspended in 500 µl of Trizol reagent (Gibco Life Technologies) and stored at –70°C.

Cytokine mRNA Detection by TaqMan® RT-PCR

Total RNA was isolated from PBMC according to the Trizol instructions (Gibco Life Technologies) with the exception that prior to isopropanol precipitation, 1 µl of glycogen was added to enhance RNA precipitation. The extracted RNA was stored in 200 µl of 75% ethanol at –20°C, and prior to RT reaction, suspended in 20 µl of diethylpyrocarbonate-treated water. The RT reaction was performed with a First Strand cDNA Synthesis Kit (Pharmacia, Sweden) using oligo(dT) primers, and cDNA was stored at –70°C. The amplification of β-actin, IL-4, IL-5, IL-10, IL-18, SLAM and IFN-γ cDNAs was performed in MicroAmp® optical 96-well reaction plates (Applied Biosystems, Foster City, Calif., USA). The 25-µl reaction in each well contained 1 µl of total cDNA, 300 nM sequence-specific primers and 200 nM dual-labelled fluorogenic probe in 1× TaqMan Universal PCR master mix (Applied Biosystems). The primer and probe sequences for the analysis of β-actin, SLAM, IL-4 and IFN-γ expression were as previously published [19, 32], and the analysis of IL-5, IL-10 and IL-18 expression was performed with cDNA-specific Pre-Developed Assay Reagents kits purchased from Applied Biosystems. A negative PCR control without template and a positive PCR control with a template of known amplification were included in each assay. The reaction was carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems), and after completion, the Ct value for each amplification product was determined using a threshold value of 0.03. The cytokine- and SLAM-specific signals were normalized to the constitutively expressed β-actin signals, and the stimulation indexes, indicating the fold changes in expression in allergen-stimulated versus non-stimulated cultures, were thereafter calculated according to the comparative Ct ($2^{-\Delta\Delta C_t}$) method (User Bulletin #2, P/N 4303849, Applied Biosystems). The obtained means of duplicates were used for statistical analysis. All analyses of all genes were performed in the linear range of the amplification, verified by dilution series of each cDNA. β-Actin was not regulated during allergen stimulation [unstimulated Ct: mean (SD) 18.20 (0.97); stimulated Ct 18.19 (1.02); $n = 180$].

Statistics

The Mann-Whitney U test was used for unpaired comparisons between the groups, and the Wilcoxon signed rank test was used for paired comparisons within the groups. All data are given as mean (SEM) of the study groups.

Results

The changes in VAS of symptoms, medication and combined symptoms medication scoring in patients treated with SIT and those included as untreated allergic

Table 2. Changes from baseline (year 0) in VAS, medication and combined symptom medication scoring in the 2 patient groups during the 3-year follow-up

Year	No SIT (n = 8)	SIT (n = 20)	SIT efficacy ¹	
			<40 (n = 8)	>40 (n = 12)
VAS				
1	-3.7 (7.5)	-22.6 (6.4)	-5.9 (10.2)	-33.8 (6.6)
2	-16.3 (6.6)	-44.1 (3.5)	-28.9 (2.0)	-53.8 (2.9)
3	-23.4 (6.1)	-37.6 (7.0)	-24.3 (10.4)	-47.0 (8.7)
MED				
1	+10.5 (4.5)	-24.0 (7.5)	-10.7 (10.5)	-32.9 (10.0)
2	-0.9 (4.2)	-36.8 (6.4)	-16.9 (8.1)	-49.5 (6.9)
3	+2.3 (2.7)	-35.3 (8.2)	-16.9 (12.9)	-48.2 (8.9)
SMS				
1	+6.7 (11.1)	-46.6 (13.0)	-16.5 (19.3)	-66.7 (15.5)
2	-17.2 (8.7)	-80.9 (8.7)	-45.8 (9.0)	-103.3 (7.3)
3	-21.0 (7.4)	-72.9 (13.3)	-41.2 (47.1)	-95.2 (16.0)

Results are shown as means (SEM). MED = Medication; SMS = symptom medication scoring.

¹ SIT efficacy: >40 percentage unit reduction or <40 percentage unit reduction in VAS symptom scores from year 0 to year 2.

controls during the 3-year follow-up are presented in table 2. SIT was highly effective in reducing allergy symptoms and the need for rescue medication during the subsequent pollen seasons. However, VAS scores also decreased slightly in the untreated allergic control patients, whereas medication scores decreased only in the SIT-treated group. Based on the changes in VAS scores after 2 years of treatment, the patients were divided into 2 groups: those who benefited well from SIT (>40 percentage unit reduction, n = 8) and those who had less symptom improvement (<40 percentage unit reduction, n = 12).

The allergen-induced in vitro expression of IL-4, IL-5, IFN- γ , IL-18, SLAM and IL-10 mRNAs and the IL-4/IFN- γ expression ratio in the PBMC of SIT-treated patients and those included as untreated allergic controls during the 3-year follow-up are presented in table 3. After 1 year of SIT, there was a significant decrease in the expression of IL-5 mRNA ($p < 0.05$), a tendency towards diminished IL-4 mRNA expression and a marked reduction in the IL-4/IFN- γ expression ratio ($p < 0.05$) in the allergen-stimulated PBMC of SIT-treated patients (n = 20) when compared to baseline. These changes were accompanied by significant increases in the expression of IFN- γ ($p < 0.02$), IL-18 ($p < 0.02$), SLAM ($p < 0.01$) and IL-10 ($p < 0.01$) mRNAs. IL-18 ($p < 0.01$), SLAM ($p < 0.01$)

Table 3. Allergen-induced in vitro expression of cytokine and SLAM mRNAs in the PBMC of the 2 patient groups during the 3-year follow-up

Year	No SIT (n = 8)	SIT (n = 20)	SIT efficacy ¹	
			<40 (n = 8)	>40 (n = 12)
IL-4				
0	1.7 (0.4)	3.3 (0.9)	1.5 (0.2)	4.4 (1.3)
1	1.6 (0.1)	1.9 (0.3)	2.4 (0.6)	1.6 (0.3)*
3	1.5 (0.3)	2.8 (0.6)	3.2 (1.4)	2.6 (0.5)
IL-5				
0	499 (333)	1,825 (1,557)	109 (59)	2,970 (2,585)
1	199 (164)	1,302 (374)*	1,413 (562)**	1,228 (518)
3	250 (178)	903 (335)	885 (596)**	915 (416)
IFN- γ				
0	2.6 (1.0)	1.4 (0.3)	1.0 (0.2)	1.6 (0.4)
1	2.7 (0.5)	3.1 (0.7)**	3.4 (0.9)**	3.0 (1.0)
3	2.4 (0.8)	2.5 (0.8)	3.2 (1.9)	2.1 (0.6)
IL-4/IFN- γ				
0	1.9 (0.7)	2.8 (0.5)	1.9 (0.5)	3.4 (0.7)
1	0.7 (0.2)	1.4 (0.3)*	1.3 (0.5)	1.4 (0.3)*
3	1.0 (0.4)	2.9 (0.8)	2.0 (0.5)	3.4 (1.4)
IL-18				
0	1.6 (0.8)	0.9 (0.2)	0.5 (0.1)	1.1 (0.3)
1	1.1 (0.2)	3.4 (1.0)**	3.7 (2.1)	3.2 (1.1)
3	1.7 (0.4)	2.8 (0.7)***	2.1 (0.6)**	3.2 (1.1)*
SLAM				
0	0.9 (0.1)	0.9 (0.1)	1.0 (0.1)	0.9 (0.1)
1	1.4 (0.3)	2.7 (0.6)***	3.4 (1.3)**	2.2 (0.4)**
3	1.3 (0.2)	1.7 (0.2)***	1.6 (0.2)*	1.8 (0.3)**
IL-10				
0	2.0 (0.7)	1.0 (0.3)	0.7 (0.1)	1.2 (0.4)
1	1.7 (0.5)	4.9 (1.1)***	6.7 (2.2)**	3.7 (1.0)
3	1.3 (0.3)	2.4 (0.5)*	2.8 (1.0)*	2.1 (0.6)

Results are shown as means (SEM).

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ compared to year 0 (Wilcoxon signed rank test).

¹ SIT efficacy: >40 percentage unit reduction or <40 percentage unit reduction in VAS symptom scores from year 0 to year 2.

and IL-10 ($p < 0.05$) expression remained up-regulated in the allergen-stimulated PBMC of SIT-treated patients after 3 years of SIT, albeit reduced compared to the first-year responses. In contrast, the immunomodulatory effects of SIT on the allergen-induced IFN- γ and Th2-type cytokine responses and the IL-4/IFN- γ ratio were no longer evident in the PBMC after 3 years of treatment. The decreases in the allergen-induced in vitro expression of IL-4 mRNA and the IL-4/IFN- γ ratio in the PBMC of SIT-treated patients after 1 year of treatment were associated with a good therapeutic outcome (>40 percentage

unit reduction in VAS), whereas in patients with less symptom improvement (<40 percentage unit reduction in VAS), there were significant increases in both IL-5 ($p < 0.02$) and IFN- γ ($p < 0.02$) mRNA responses and a tendency towards increased IL-4 mRNA expression during SIT. An increase in IL-10 mRNA expression was also associated more with lower efficacy of SIT than with a good therapeutic outcome after 1 and 3 years of treatment, while increases in the allergen-induced in vitro expression of IL-18 and SLAM mRNAs had no association with the clinical efficacy of SIT. No significant changes in Th2 (IL-4, IL-5)-, Th1 (IFN- γ , SLAM, IL-18)- or Treg (IL-10)-type immune responses to specific allergen were found in the PBMC of patients who had not been treated with SIT during the 3-year follow-up.

Discussion

In this report of a 3-year prospective study of SIT in pollen-allergic rhinitis patients, we demonstrate that SIT induces long-term changes in allergen-specific immune responses in PBMC. A transient decrease in allergen-induced Th2-type cytokine production (IL-5 and IL-4/IFN- γ ratio) was seen after 1 year of treatment, which was associated with significant increases in both Th1 (IFN- γ , IL-18, SLAM)- and Treg (IL-10)-type immune responses. The induced Th1- and Treg-type responses against specific allergen remained up-regulated in the PBMC after 3 years of SIT, albeit reduced compared to the first-year responses. These results thus support the induction of allergen-specific Treg cells that produce IL-10 and the immunodeviation in the allergen-specific Th2/Th1 cytokine balance as the 2 key mechanisms of SIT [4–8, 27, 38] and also concord with our previous results from high-dose sublingual immunotherapy in tree pollen-allergic children, where a peak in peripheral allergen-specific IL-10, IL-18 and SLAM mRNA responses was seen after 1 year of treatment but there was a decline back toward baseline after 2 years of treatment [16, 33]. Taken together, these findings suggest that there is a shift in allergen-specific immune responses from peripheral blood to target tissues after the first treatment year, during which they contribute to clinical improvement. This suggestion is also supported by the findings of Wachholz et al. [39], who found a shift from Th2 to Th1 responses in the nasal mucosa of patients treated for 2 years with SIT, but were not able to find a similar response in PBMC.

SIT has been proven clinically effective in numerous double-blind, placebo-controlled trials [for a review, see

1]. Although marked clinical improvement is often already achieved after one preseasonal treatment period, more pronounced and sustained clinical efficacy usually requires longer courses of treatment, typically for at least 3 years. In accordance with previous studies, we found that SIT was already highly effective in reducing seasonal symptoms and the need for rescue medication after the first treatment year, and a further improvement in both of these parameters was seen after the second treatment year, which was maintained at a similar level until the end of SIT. The decreased allergen-induced Th2-type cytokine responses after 1 year of treatment – in particular of IL-4 mRNA and the IL-4/IFN- γ expression ratio – were associated with a good therapeutic outcome with SIT. Conversely, increases in both Th2 (IL-5)- and Th1 (IFN- γ , SLAM)-type responses and IL-10 mRNA production during SIT were found in patients in whom SIT showed lower efficacy.

We have previously shown that allergen-specific IL-10 mRNA responses are increased early, by the time of reaching the maintenance dose, in PBMC of patients who benefited most from SIT [15]. A similar tendency was found for allergen-induced IL-18 and SLAM mRNA expression [15, 32], whereas the responses were delayed, not peaking until the end of the first treatment year, in PBMC of patients who had a less beneficial outcome. The early induction of allergen-specific IL-10 production in PBMC during SIT and sublingual immunotherapy has also been reported by other investigators, but often with no correlation with symptom improvement at later stages of SIT [31, 34, 40]. In this study, we extended the investigation of allergen-specific immune responses and their association with symptom improvement to cover the complete 3-year course of SIT. One and three years after starting SIT, we could not find any significant association between the increased allergen-induced IL-18 and SLAM mRNA responses in the PBMC and the clinical outcome, whereas higher IL-10 mRNA expression was found in patients with a less beneficial outcome. We therefore speculate that early immune deviation in peripheral allergen-specific immune responses is critical for a good therapeutic outcome with SIT.

In conclusion, our data demonstrate that SIT has long-term effects on allergen-specific immune responses in PBMC. The treatment induces tolerogenic Treg- and Th1 (IL-10, IL-18 and SLAM)-type responses that endure for at least 3 years of SIT, whereas decreased Th2-type cytokine responses are seen only during early therapy, after 1 year. The decline in these responses from peripheral blood by the end of SIT suggests a shift to local mucosal

tissues where they contributed to clinical improvement. Increased Treg- and Th1-type immune responses in allergen-stimulated PBMC after 1 and 3 years of SIT were not associated with the therapeutic outcome, suggesting

that the early induction of these responses, rather than their sustained increase, might be a more relevant factor in determining the ultimate clinical efficacy of this treatment.

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