

SERUM IL-4 AS A MARKER OF IMMUNOLOGICAL RESPONSE TO SUBLINGUAL IMMUNOTHERAPY

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Allergic rhinitis (AR) is characterized by a Th2 polarized immune response. Specific Immunotherapy modifies this bias restoring a physiologic Th1 profile. Sublingual immunotherapy (SLIT) is widely prescribed, but there is no early, simple marker of response. This study was undertaken in order to determine whether serum IL-4 might be a possible marker of SLIT immunological response in order to quickly and easily detect responder patients. Thirty-nine AR patients with a pollen allergy assumed pre-seasonal SLIT for 3 months. VAS for symptoms and medication efficacy were evaluated. Serum IL-4 was assessed before and 3 and 6 months after SLIT initiation. Eighty-two percent of patients (32/39) showed a clinical response to SLIT. Serum IL-4 significantly decreased at 6 months post-therapy in responders, whereas it increased in non-responders. In conclusion, these results may be considered clinically relevant proof that SLIT treatment induces a quick reduction in Th2 polarization. Serum IL-4 appears to be an early marker of immunological response to SLIT.

Allergic rhinitis (AR) is characterized by mucosal inflammation, sustained by mast cell and eosinophil activation. This inflammatory reaction displays a predominant T-helper (Th) 2 cell profile with greater interleukin (IL)-4 production than interferon (IFN)- γ expression by Th1 cells: the so called Th2 polarization (1). AR management includes patient education, allergen avoidance, drug therapy and, when appropriate, allergen-specific immunotherapy (SIT) (2). SIT is the practice of administering gradually increasing doses of the causal allergen in order to reduce allergic symptoms and the need for medication (3). Treatment efficacy depends on

achieving clinical tolerance of the causal allergen (4). There is evidence that SIT is effective in patients with allergic rhinitis and mild forms of asthma as stated in a World Health Organization (WHO) position paper (5).

SIT is classically based on the subcutaneous administration of allergen extracts (SCIT), but as this route of administration has exhibited a risk for serious adverse reactions, alternative routes of administration, including oral, sublingual (SLIT), and nasal, have been investigated and developed (6). The rationale of SLIT is that oral administration of high-dose allergens may reduce and prevent

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IgE responses. Methodologically, the extract is kept under the tongue for a few minutes and then swallowed: the “sublingual-swallow”. SLIT efficacy has been evaluated in one WHO position paper (5), in the EAACI/ESPACI document (6), in the ARIA document (7), and investigated in two meta-analyses on patients with AR and asthma (8-9).

The exact SIT mechanism of action is not completely understood (10). SCIT has been in practice for many years, and several studies have shown that subcutaneous administration of the allergen induces hyposensitization by redirecting peripheral and mucosal Th2-responses to a prevalent Th1-polarization (11-13). However, regarding SLIT, only two studies have demonstrated that it was able to increase allergen-driven IFN- γ production (14-15); other studies have not reported cellular changes (16-17). Moreover, a number of recent studies indicate that regulatory T cells (Tregs) play an important role in controlling the Th2-biased response (18). Indeed, a defect in T regulatory cells has been proposed by one study in allergic subjects (19). Very recently, also regarding SLIT, it was shown that the appearance of allergen-specific tolerance induction is highlighted by restored allergen-specific IL-10 and IFN- γ production (15, 19-20). On the other hand, only two studies have reported decreased allergen-induced Th2-dependent cytokine (IL-4 and IL-13) production after SLIT (21-22), while other studies did not confirm this result (15-17, 20, 23).

Both forms of SIT: SCIT and SLIT are long-term and expensive treatments as their administration is projected for 3-5 years. Moreover, a discrete percentage of allergic patients (about 20-30%) do not respond to this therapy (24). Considering that SIT prescriptions are numerically relevant in Europe and SLIT is more prescribed than SCIT, the aim of this study is to investigate whether serum IL-4 may be an easily detectable early marker of immunological response to SLIT. In addition, this information would be useful in identifying candidate responders.

MATERIALS AND METHODS

Patients

Thirty-nine patients with AR due to pollen sensitisation were enrolled; each subject underwent a complete evaluation and was then included in the study. The study was conducted with the approval of the local Ethics

Committee and performed with the written informed consent of all participants. AR was diagnosed according to validated criteria (7). The patients' blood samples for assessing serum IL-4 were collected before initiating SLIT (baseline, T0), at three months (corresponding to the end of the SLIT schedule, T1) and six months (corresponding to the pollen season peak, T2).

Specific Immunotherapy

Subjects undergoing SLIT received either a *Betula alba* pollen allergen extract (Anallergo, Florence, Italy; 17 cases, cumulative dose was 30 mcg), *Parietaria judaica* (15 cases, cumulative dose was 12 mcg), or a mix of three *Graminaceae* (7 cases, cumulative dose was 12 mcg) for at least three months before the pollen season, as pre-seasonal immunotherapy. The dose during the maintenance phase was 1,000 U.RAST/ml (internal reference): five drops 3 times per week.

Clinical evaluation

Allergy symptoms and the use of medication were scored at the end of the pollen season. The patient questionnaire included a visual analogue scale (VAS) of nasal symptoms (25). During the pollen season patients could assume lyophilised ebastine tablets (10 mg, 1 or 2) on demand as rescue medication. The medication scoring (MS) was: not at all (0), occasionally/seldom (1), occasionally/often (2), almost daily (3), continuously with the lowest dose (4), continuously with the maximal dose (5). Patients were arbitrarily considered SLIT responders when VAS was ≤ 5 and MS ≤ 2 .

Serum IL-4 assay

The human IL-4 Immunoassay (R&D Systems) employs the quantitative sandwich enzyme immunoassay technique and was performed according to the manufacturer's instructions and expressed as pg/mL. The minimum detectable dose, corresponding to the zero standard concentration, was arbitrarily assigned as 0.09 pg/ml. Reference values for IL-4 were 0-31.2 pg/ml.

Statistical analysis

Descriptive statistics were calculated on data at baseline (T0), at three months (T1) and six months (T2) following SLIT. Moreover, the differences in IL-4 serum levels were calculated at the different times: Δ_{T1-T0} , Δ_{T2-T0} , and Δ_{T2-T1} for each patient. The non-parametric Wilcoxon test was performed to evaluate the difference between responder and non-responder patients for both absolute values of IL-4 and Δ s. The same test was used to investigate the relationship between IL-4 and VAS. In addition, logistic regression was used to build a predictive model for clinical response to SLIT starting

from a temporal trend in serum IL-4. The logistic model result was considered correct if a patient's response/non-response was predicted with a probability higher than 0.5. All statistical analyses were performed using S-Plus (26).

RESULTS

The mean age of the analyzed AR patients (17 females and 22 males) was 37.5 years (range 14-75; median age 39).

Serum IL-4

Table I shows the descriptive statistics of IL-4 serum levels at T0, T1 and T2. Δ_{T1-T0} and Δ_{T2-T1} were significantly different between responder and non-responder ($p=0.042$ and 0.018 respectively), while Δ_{T2-T0} was not significantly different in the two groups. The model estimates the probability of clinical response by the following formula:

$$p(\text{response}) = \exp(2.285 + 0.028 * \Delta_{T1-T0} - 0.032 * \Delta_{T2-T1}) / [1 + \exp(2.29 + 0.028 * \Delta_{T1-T0} - 0.032 * \Delta_{T2-T1})]$$
 Logistic regression shows that an IL-4 temporal trend, in terms of Δ_{T1-T0} and Δ_{T2-T1} , significantly correlated with clinical response (Table II). The model prediction was considered correct when $p(\text{response})$ was less than 0.5 for a non-responder patient or ≥ 0.5 for a responder patient. Fig. 1 shows the model performance: its specificity (true negative rate) was 97% (31/32) and sensibility (true positive rate) 57% (4/7), with an overall efficiency of 90%.

IL-4 serum levels at base line, three months and six months following SLIT treatment, for responders and non-responders are reported in Fig. 2, together with linear regression lines. The two trends are clearly different as there is a significant decrease in serum IL-4 present in responder patients, whereas a significant increase is present in non-responders.

The relationship between VAS and IL-4 values at six months was also investigated, assuming an IL-4 cut-off value of 31.2 pg/ml, patients with normal IL-4 values showed a lower VAS (better clinical response), approaching statistical significance (Wilcoxon test $p=0.08$). Median and inter-quartile ranges for VAS in the groups were (5, 4-5) and (6, 5-7.75), respectively.

DISCUSSION

Allergic rhinitis is characterized by Th2-polarized inflammation. Th2-derived cytokines, such as IL-4 and IL-13, are the primary pathogenic factors which induce, maintain and amplify allergic inflammation. IL-4 and IL-13 orchestrate this inflammation by promoting IgE synthesis, up-regulating adhesion molecules selective for eosinophil recruitment, and causing increased mucus production and airway hyperreactivity (27). On the other hand, there is accumulating evidence that Th1-related cytokines, such as IFN- γ and IL-12, may suppress and counteract this Th2 response and vice-versa, as there is functional dichotomy between Th1 and Th2 cells (28).

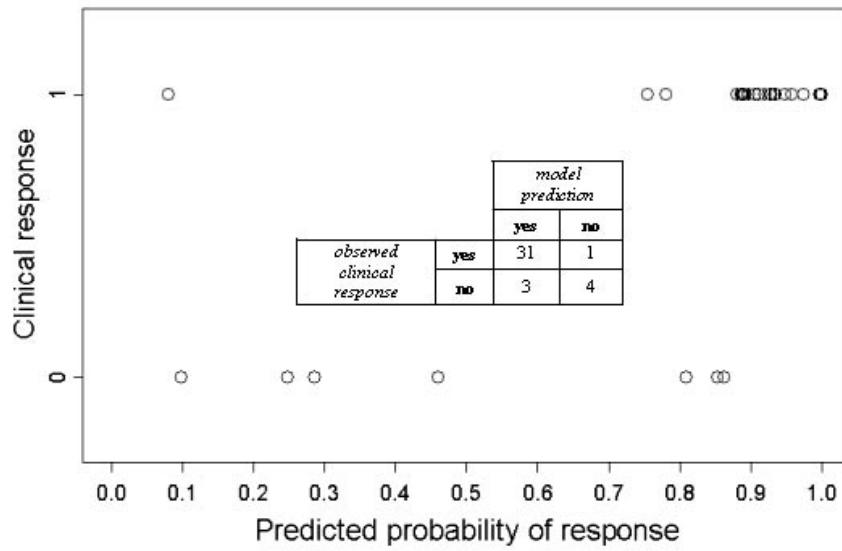
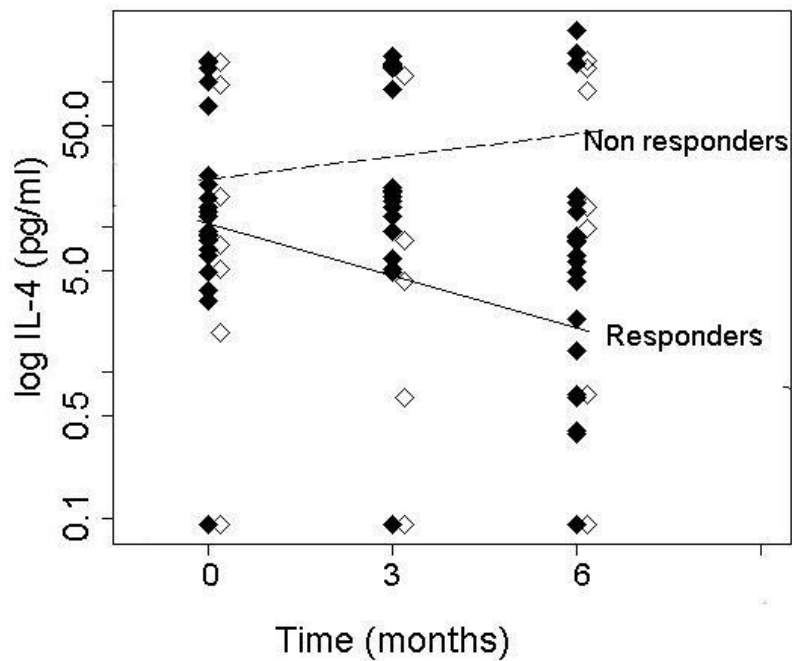
SIT is highly effective in the treatment of AR, and is the only treatment which promotes life-long tolerance against previously disease-causing allergens, through the restoration of normal immunity (29). SCIT was found to be associated with a decrease in IL-4 and IL-5 production by CD4+ Th2 cells, and a shift towards increased IFN- γ production by Th1 cells (11-13). SLIT is the preferred version of SIT, with a safer and more effective route of administration, however, its mechanism of action is still poorly understood, as there are few data

Table I. Temporal trend in IL-4 serum levels at T0 (base line), T1 (3 months) and T2 (6 months).

	IL-4 [pg/ml] RESPONDERS					IL-4 [pg/ml] non RESPONDERS				
	T0	T1	T2	Δ_{T1-T0}	Δ_{T2-T1}	T0	T1	T2	Δ_{T1-T0}	Δ_{T2-T1}
1st Quartile	0.09	0.09	0.09	-3.61	-11.37	3.55	0.09	5.27	-16.33	2.77
Median	6.73	5.66	1.06	0	0	7.60	0.67	13.93	-7.51	13.84
3rd Quartile	14.24	17.77	8.28	3.68	0.58	56.31	6.26	113.90	0	113.51

Table II. Logistic regression model of clinical response as a function of Δ_{T1-T0} and Δ_{T2-T1} .

	value	Std. Error	p-value
(intercep)	2.285	0.625	0.0001
Δ_{T1-T0}	0.028	0.013	0.02
Δ_{T2-T1}	-0.032	0.013	0.008

**Fig. 1.** The performance of the logistic model for clinical response prediction (0=no, 1=yes). Table inside the figure shows the number of cases correctly and incorrectly classified, using the response probability threshold of 0.5.**Fig. 2.** Regression lines showing the decreasing and increasing trend of IL-4 in responders (♦ black diamonds) and non-responders (◇ white diamonds), respectively. Logarithmic transformation of IL-4 has been used to improve distribution symmetry.

concerning the immunologic changes induced by SLIT (2). A decrease in serum levels of IL-13, as well as IL-4 has been demonstrated after SLIT therapy (21-22).

Regarding the balance between Th1-Th2 cells, some studies have shown a shift from Th2 towards a Th1 response (14-15, 20, 30), whereas others have failed to confirm this result (16-17). The discrepancies among these studies may depend on several methodological factors, including the type of allergen, duration of SLIT, age of patients and AR duration, dose of allergen extracts used in *in vitro* experiments, incubation kinetics, and the methods used to assess cytokines.

A very recent study indirectly supports the relevance of the Th2-Th1 shift through the increased expression of both IL-18 and the signalling lymphocytic activation molecule (SLAM) (30). IL-18 is a macrophage-derived cytokine which induces IFN- γ production from Th1 cells (31) and SLAM is a transmembrane, cell surface glycoprotein, whose activation promotes T-cell proliferation, and IFN- γ production, the latter is preferentially expressed in Th1 cells (32). Therefore, Th1 polarization represents an important change in the immune response after SLIT. However, all these studies were performed in the allergen-induced cytokine production model that is complicated and reserved only for *in vitro* studies as it can not be applied *in vivo*.

Importantly, there is still no prognostic parameter capable of identifying responders, which is an important draw back to this line of therapy, as a discrete percentage of subjects do not show any clinical benefit. Moreover, there is no agreement over the length of time necessary to detect responders. Thus, the definition of an easily detectable and early marker of immune response to SLIT would be clinically relevant. Therefore, this study was also designed to assess this parameter.

The first finding is that 82% of patients assuming SLIT showed a clinical response. This high percentage might depend on different factors, such as adequate screening of patients, their education and motivation. Overall, the most important outcome is that within six months of SLIT initiation, serum IL-4 levels significantly decreased in responder subjects. The trend of serum IL-4 values had a predictive correlation with response established as 90% efficient.

Moreover, it is noteworthy that serum IL-4 levels significantly increased during the peak pollen season in non-responder patients, whereas it significantly decreased in responders. Therefore, the present study highlighted that effective SLIT is capable of inducing an immunologic response directed at reducing Th2 polarization in allergic patients presenting with AR due to pollen sensitisation. Therefore, serum IL-4 reduction may be considered as an early marker of SLIT effectiveness.

In conclusion, these results may be considered clinically relevant proof that SLIT treatment induces a quick reduction in the Th2 response. This Th2 response may be useful to precociously identify responder patients. Therefore, the early evaluation of serum IL-4 during SLIT, i.e. at baseline and after 3/6 months, may help guide decisions in SLIT treatment. These preliminary findings should however be confirmed in further randomized studies.

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