

Wasp venom immunotherapy induces a shift from IL-4-producing towards interferon-gamma-producing CD4⁺ and CD8⁺ T lymphocytes

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Summary

Background Venom immunotherapy (VIT) has proven to be very effective in hymenoptera venom anaphylaxis. However, the underlying immunoregulatory mechanisms of venom immunotherapy remain poorly understood. Recent studies measured the total amount of cytokine in culture supernatants, suggesting a shift in cytokine production from Th2 to a Th1 cytokine profile during VIT. We wanted to examine the contribution of specific T lymphocyte subpopulations, which is impossible using an extracellular method to determine cytokines in supernatants.

Objective The present study was designed to evaluate the effect of VIT on the percentages of type 1 (IL-2, interferon-gamma (IFN- γ)) and type 2 (IL-4) CD4⁺ and CD8⁺ T lymphocytes of patients with wasp venom anaphylaxis during immunotherapy.

Methods Peripheral blood mononuclear cells of 20 individuals with a history of wasp stinging anaphylaxis and a positive serum wasp venom specific IgE were isolated and *in vitro* stimulated with phorbol-12-myristate-13-acetate and ionomycin before VIT, at the end of a 5-day semirush VIT and at 6 months during VIT. Three-colour flow cytometric analysis was used for intracellular cytokine (IL-2, IFN- γ , IL-4) detection in CD4⁺ (CD3⁺CD8⁻) T lymphocytes and CD8⁺ (CD3⁺CD8⁺) T lymphocytes.

Results At the end of a 5-day semirush VIT, there was a significant decrease in percentage of IL-4-producing CD4⁺ and CD8⁺ T cells, compared with cytokine-producing cells before VIT ($P = 0.0002$ and 0.004). After 6 months of VIT, patients showed an increased number of IL-2-producing stimulated CD4⁺ and CD8⁺ lymphocytes compared with values before VIT ($P = 0.002$ and $P = 0.0003$). A higher amount of IFN- γ -producing stimulated CD4⁺ and CD8⁺ cells was found after 6 months of VIT ($P = 0.001$ and $P = 0.0006$). There was no correlation between cytokine-producing cells and specific IgE for wasp.

Conclusion Venom immunotherapy induced a shift from IL-4-producing towards IFN- γ -producing CD4⁺ as well as CD8⁺ T lymphocytes.

Keywords: hymenoptera allergy, immunotherapy, cytokines, Th1/ Th2, Tc1/ Tc2, wasp venom, CD4⁺, CD8⁺

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Introduction

Venom immunotherapy (VIT) has proven to be very effective in hymenoptera venom anaphylaxis, providing protection against future sting-induced anaphylaxis [1–3]. However, the immunoregulatory mechanisms involved in VIT remain unclear. In older studies, induction of blocking IgG antibodies, especially IgG4, was thought to be responsible for the protective effect of immunotherapy [4]. The generation of CD8⁺ T suppressor cells, which modulate allergen-specific IgE production, was also described [5,6]. It has been postulated that a decline of allergen-specific IgE and an increase of allergen IgG production can be observed after VIT [4,7,8]. Also, IL-10-induced anergy in T lymphocytes against bee venom was demonstrated in bee allergic patients during VIT [9,10]. Recent evidence suggests that a shift in cytokine production from Th2 to a Th1 cytokine profile may effect immediate and long-term hyporesponsiveness [9–14]. These studies measured the total amount of cytokine in culture supernatants, suggesting a shift of the predominance of type 2 cytokine-producing cells towards a type 1 cytokine-producing cell. However, it is still unclear whether the cytokine shift is the result of changed capacity of cells to produce cytokines or of changed percentages of specific cytokine-producing T cell subpopulations. Furthermore, we wanted to examine the contribution of specific T lymphocyte subpopulations, which is impossible using an extracellular method to determine cytokines in supernatants. The purpose of our study was to investigate percentages of type 1 (IL-2 and interferon-gamma (IFN- γ)) and type 2 (IL-4) cytokine-producing cells, not only among CD4⁺ T cells but also among CD8⁺ T cells of patients with hymenoptera allergy at a single-cell level by using flow cytometry.

Materials and methods

Patients

Patients were recruited from the out-patient university Hospital service. Twenty patients aged from 17 to 67 years, who had exhibited systemic anaphylaxis to yellow jacket stings (*Vespula vulgaris*) with a positive serum wasp venom-specific IgE, were included. All were treated according to a standardized protocol with wasp venom (Pharmalgen-ALK Wasp venom; Pharmacia, Brussels, Belgium). Each patient was initially administered subcutaneously a dose of 1 pg of venom. The dose was progressively increased over 5 days. On the day 5 of build-up therapy 80 and 100 μ g were injected. A maintenance dose (100 μ g per injection) was administered at days 12, 26, 37 and once every 4 weeks. Blood samples were obtained just before VIT, after 5 days of semirush VIT and

after 6 months of maintenance VIT. All patients gave informed consent for the study.

Total and wasp venom-specific IgE

Quantification of serum total IgE and serum-specific IgE for wasp venom was performed with the Immuno-CAP assay (Upjohn-Pharmacia CAP system, Brussels, Belgium). Assays were performed according to the manufacturer's instructions. Results were expressed quantitatively as kilounits IgE per litre (kU/L). A serum concentration for total IgE < 120 kU/L was considered as a non-atopic value. A serum concentration for specific IgE < 0.35 kU/L was considered as negative.

Cell isolation

Peripheral blood (30 mL) was collected in EDTA tubes (Vacutainer®; Becton Dickinson, Meylan, France). Blood was diluted 1:2 with PBS pH = 7.4 (Life Technologies, Gibco, Gent, Belgium) and subsequently layered on 13 mL Nycoprep (d = 1.077 mg/mL; Nycomed, Oslo, Norway), centrifuged at room temperature for 20 min at 1000 g. Peripheral blood mononuclear cells (PBMC) were collected and washed twice with PBS for 10 min at 200 g. Cells were counted and concentrated at 5×10^6 cells/mL in AIM-V culture medium (Life Technologies, Gibco).

Analysis of monocytes and lymphocyte subsets

As previously described by Hagendorens *et al.*, 100 μ L isolated mononuclear cells were incubated for 15 min in the dark at 4°C with 10 μ L of multicolour monoclonal antibody panels (CD3–PerCP + CD4–FITC + CD8–PE; CD45–PerCP + CD3–FITC + CD19–PE; CD14–PE + CD45–FITC; Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium) [15]. Subsequently, the remaining erythrocytes were lysed with 2 mL of a lysing solution (155 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA) for 20 min at room temperature. Cells were pelleted at 400 g for 10 min and fixed with 1% paraformaldehyde (Merck, Darmstadt, Germany) in PBS. Afterwards, the cells were resuspended in 1% paraformaldehyde (Merck) in PBS and analysed by FACSscan flow cytometry (Becton Dickinson) within 24 h.

Cytokine assay

Mononuclear cells were cultured and stained as previously described [16]. Briefly, the isolated mononuclear cells were cultured for 6 h at 37°C and 5% CO₂. One part of the cells was stimulated in the presence of 50 ng/mL phorbol-12-mirystate-13-acetate (PMA; Sigma, St Louis, MO, USA),

Table 1. Peripheral blood counts and lymphocyte subsets

	Day 0	Day 5	Month 6
Leucocytes ($10^9/L$)	6.0 (3.4–11.4)	5.2 (4.0–7.0)*	6.4 (3.9–11.6)
Neutrophils (%)	54.3 (37.8–77.6)	65.1 (39.5–78.4)*	60.0 (34.0–86.1)
Lymphocytes (%)	32.3 (16.3–44.5)	23.1 (12.5–40.5)*	28.0 (16.7–51.0)
Monocytes (%)	6.5 (2.6–10.0)	6.4 (4.4–9.2)	5.8 (0.2–9.5)
Eosinophils (%)	2.7 (0.7–15.0)	2.5 (0.7–11.5)	2.5 (0.7–11.7)
Basophils (%)	0.5 (0.2–2.0)	0.4 (0.1–2.0)	0.5 (0.0–1.5)
CD3 ⁺ (%)	77.8 (13.0–88.6)	77.6 (9.6–87.9)	77.1 (60.9–85.3)
CD4 ⁺ (%)	51.7 (28.4–69.5)	50.9 (31.5–65.9)	50.8 (32.2–65.8)
CD8 ⁺ (%)	19.5 (5.1–37.5)	20.8 (7.0–34.1)	21.7 (7.2–39.4)
CD19 ⁺ (%)	5.9 (1.3–7.4)	6.6 (1.9–9.2)	6.4 (1.3–15.2)

Results are expressed as median (range).

* $P \leq 0.001$ vs. day 0 and vs. month 6.

1 $\mu\text{g/mL}$ ionomycin (Sigma) and 2 μM monensin (Sigma). The other part of the cells remained unstimulated in the presence of 2 μM monensin. After stimulation, 1 mL PBS was added, cells were centrifuged at room temperature for 5 min at 1000 g and resuspended at 1×10^7 cells/mL in PBS. Cells were labelled with conjugated antibodies (CD3–PerCP, CD8–FITC; Becton Dickinson Immunocytometry Systems) during 15 min in the dark at room temperature. Afterwards, cells were fixed with 4% paraformaldehyde (Merck) in PBS. Cell membranes were made permeable with 0.3% saponin (Sigma) in PBS and incubated with anti-cytokine antibodies (mouse anti-human IL-2–PE, mouse anti-human IL-4–PE, mouse anti-human IFN- γ –PE (Fast Immune; Becton Dickinson)) during 15 min in the dark at room temperature. Cells were spun for 5 min at 1000 g and resuspended in PBS.

Flow cytometric analysis

Ten thousand cells were analysed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) and processed with WinMDI 2.8 software (provided by J. Trotter through <http://facs.scripps.edu/software.html>). Analysis gates were set on lymphocytes according to forward and sideways scatter properties. CD4⁺ T cells were defined as CD3⁺CD8[–] and CD8⁺ cells as CD3⁺CD8⁺. According to PerCP and FITC emission, CD3⁺CD8[–] and CD3⁺CD8⁺ lymphocyte populations were gated out in a compensated fluorescence plot. Within this gate, intracellular cytokine production (PE emission) was measured in CD3⁺CD8[–] and CD3⁺CD8⁺ lymphocytes. Markers were set on the 99th percentile of isotype-matched irrelevant control antibodies (PE–IgG1 mouse antibodies; Serotec, Oxford, UK) as a reference. Results were

expressed as the percentage of cytokine-producing cells and as absolute numbers.

Statistical analysis

Differences of cytokine-producing cells between day 0, day 5 and month 6 were assessed using Friedman test and Wilcoxon rank test. Correlations were calculated using the Spearman rank correlation. A P -value of < 0.05 was considered significant.

Results

Total and specific IgE

Total IgE after semirush VIT (128 kU/L (0.60–648 kU/L)) (median (range)) and after 6 months of maintenance VIT (81 kU/L (0.50–633 kU/L)) did not alter significantly compared with values before VIT (135 kU/L (0.59–621 kU/L)).

There was no significant difference in specific IgE for wasp venom between the patients before (4.8 kU/L (0.49–27.5 kU/L)), at the end of semirush VIT (4.6 kU/L (0.48–37 kU/L)) and after 6 months of maintenance VIT (4.8 kU/L (0.46–38.4 kU/L)).

Distribution of leucocytes

The number of leucocytes and the percentage of lymphocytes decreased after 5 days' semirush VIT, compared with baseline values (respectively $P = 0.0004$ and $P = 0.0006$) (Table 1). The percentage of neutrophils increased significantly, whereas the percentage of lymphocytes decreased significantly after 5 days of VIT ($P = 0.002$ and

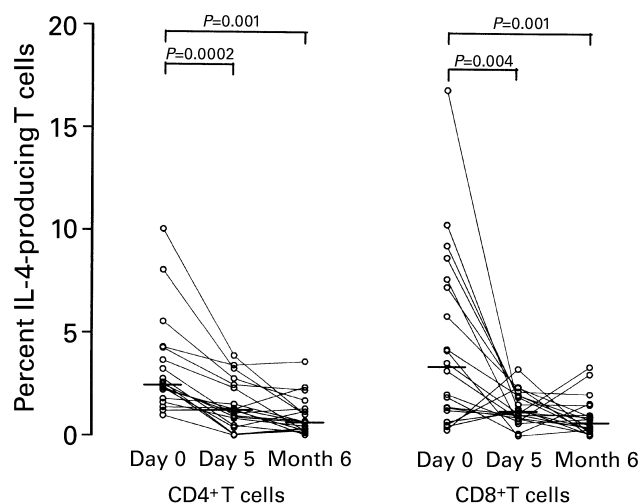


Fig. 1. Percentage (%) of IL-4-producing CD4⁺ and CD8⁺ T cells in patients with hymenoptera allergy before (day 0), after 5 days of semirush immunotherapy (day 5) and after 6 months of maintenance immunotherapy (month 6). Bars represent median values.

$P = 0.008$). The percentage of monocytes, eosinophils and basophils was not significantly different between the three time points (Table 1).

The distribution of circulating lymphocyte subsets was similar before, after semirush VIT and after 6 months of VIT with respect to CD3⁺ (T lymphocytes), CD4⁺, CD8⁺ and CD19⁺ (B lymphocytes) (Table 1).

Intracellular cytokines

At the end of a 5-day semirush VIT, the percentage of IL-4 CD4⁺ T lymphocytes (1.23% (0.01–3.89%)) (median (min.–max.)) was significantly lower than before VIT (2.46% (0.97–10.06%)) ($P = 0.0002$) (Fig. 1). Also, the percentage of IL-4-producing CD8⁺ T cells was significantly decreased after 5 days' semirush VIT, ranging from 3.36% (0.27–16.87%) before VIT to 1.18% (0.01–3.26%) after 5 days of semirush VIT ($P = 0.004$) (Fig. 1). There was no significant difference in the number of IL-2- and IFN- γ -producing CD4⁺ T cells after 5 days' semirush VIT. A trend towards an increased percentage of IL-2- and IFN- γ -producing CD8⁺ T cells at the end of semirush VIT was seen (Figs 2 and 3). However, after 6 months of VIT, patients showed an increased percentage of IL-2-producing stimulated CD4⁺ and CD8⁺ T lymphocytes, compared with values before VIT (39.58% (13.4–51.23%) vs. 33.69% (7.02–48.76%) and 19.67% (6.88–39.05%) vs. 8.68% (1.43–28.66%)) ($P = 0.02$ and $P = 0.0003$) (Fig. 2). A higher amount of IFN- γ -producing stimulated CD4⁺ and CD8⁺ T cells was found after 6 months of VIT (20.75% (6.89–37.86%) vs. 28.59% (11.62–61.22%) and

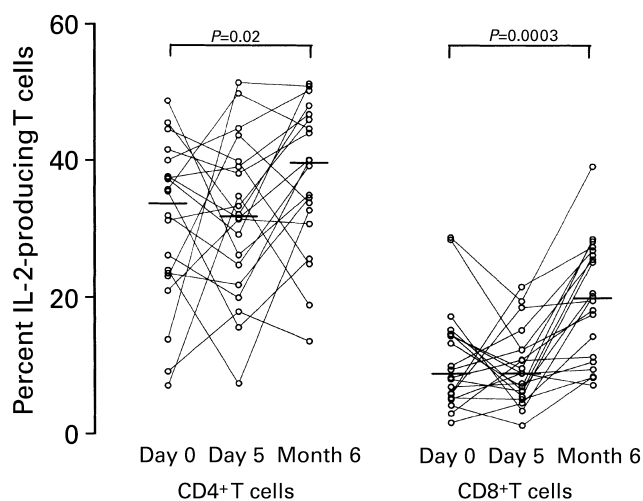


Fig. 2. Percentage (%) of IL-2-producing CD4⁺ and CD8⁺ T cells in patients with hymenoptera allergy before (day 0), after 5 days of semirush immunotherapy (day 5) and after 6 months of maintenance immunotherapy (month 6). Bars represent median values.

30.16% (14.34–47.93%) vs. 45.50% (19.53–73.78%)) ($P = 0.001$ and $P = 0.0006$) (Fig. 3). The percentage of IL-4-producing CD4⁺ and CD8⁺ T cells (0.63% (0.03–3.60%) and 0.62% (0.02–3.36%)) was still significantly decreased after 6 months of VIT, compared with values before VIT (2.46% (0.97–10.06%) and 3.36% (0.27–16.87%)) ($P = 0.001$ and $P = 0.0008$) (Fig. 1).

At the end of the 5-day semirush VIT and after 6 months of VIT, the ratio of percentage IL-4/percentage IFN- γ -producing CD4⁺ and CD8⁺ T lymphocytes differed significantly from the ratio found before VIT ($P = 0.002$; $P = 0.001$; $P = 0.004$; $P = 0.0003$) (Fig. 4).

Correlations between cytokine and total IgE, specific IgE and eosinophils

There was no correlation between total IgE and the percentage of IL-2, IL-4 or IFN- γ -producing CD4⁺ and CD8⁺ T cells. Neither was there a correlation between specific IgE for wasp venom and cytokine production. No correlation was found between percentage of eosinophils and total IgE or specific IgE for wasp. Nor was the percentage of eosinophils related to the percentage IL-2, IL-4 or IFN- γ -producing CD4⁺ and CD8⁺ T cells.

Discussion

In spite of the high efficiency of VIT, the molecular and cellular basis of the desensitization process is still not fully understood. *In vitro* studies on sequential specific wasp allergen-driven peripheral blood T cell lines have

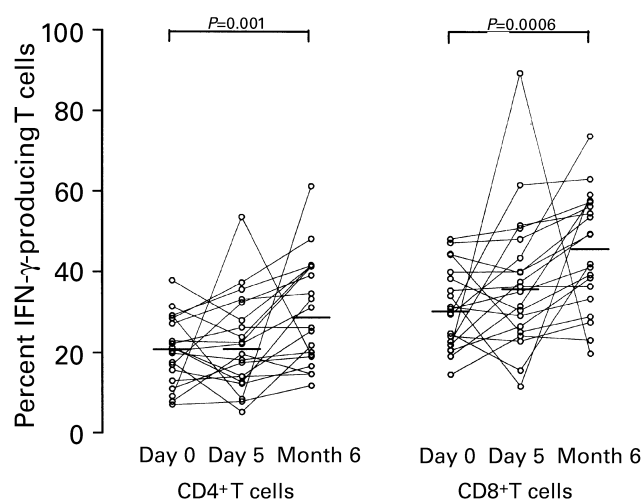


Fig. 3. Percentage (%) of IFN- γ -producing CD4⁺ and CD8⁺ T cells in patients with hymenoptera allergy before (day 0), after 5 days of semirush immunotherapy (day 5) and after 6 months of maintenance immunotherapy (month 6). Bars represent median values.

demonstrated a reduction of IL-4 secretion during both rush and conventional VIT [9–13]. A decreased expression of mRNA IL-4 was also reported in peripheral blood lymphocytes during VIT [17]. The production of other type-2 cytokines, such as IL-13 and IL-5, in allergen-driven peripheral blood T cell lines after rush and ultra rush VIT was also decreased [10,12]. The effect of VIT on type-1 cytokine secretion seems to be less consistent. Although increased IFN- γ production was reported after VIT [9,11,12], others observed decreased secretion of both type-2 and type-1 cytokines [10,14].

All these previous studies, using wasp allergen stimulation, applied extracellular methods that detect cytokines in culture supernatants. Applying flow cytometry, we wanted to identify the cytokine-producing cell type in order to investigate the contribution of T cell subsets in the type 1/type 2 imbalance in patients with hymenoptera allergy during VIT. Flow cytometry has the additional advantage of providing information about percentages of cytokine-producing T lymphocytes.

In this study, we demonstrated that the percentage of IL-4-producing T helper (CD4⁺) and T cytotoxic/suppressor (CD8⁺) cells was already lower after 5 days of semirush VIT, whereas the percentage of IFN- γ - and IL-2-producing T helper (CD4⁺) and T cytotoxic/suppressor (CD8⁺) cells was increased after 6 months of VIT.

The redistribution of Th2 cells from the peripheral blood compartment into the multiple late-phase injection sites after 5 days of semirush VIT could cause this reduction of IL-4-producing cells in the peripheral blood. Hamid *et al.* provided evidence of higher numbers of IL-4-producing

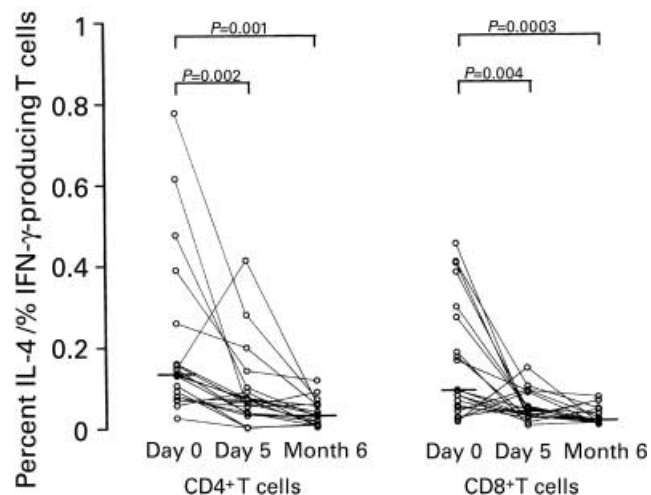


Fig. 4. Ratio of percentage (%) of IL-4/percentage (%) of IFN- γ -producing CD4⁺ and CD8⁺ T cells in patients with hymenoptera allergy before (day 0), after 5 days of semirush immunotherapy (day 5) and after 6 months of maintenance immunotherapy (month 6). Bars represent median values.

cells in skin biopsy specimens of skin lesions from patients with atopic dermatitis [18]. This could explain the decreased percentage of IL-4-producing T cells found in our study after 5 days of semirush VIT, which induces short-term hyporesponsiveness but does not explain the long-term tolerance for wasp venom after VIT. After 6 months, VIT can induce an immune deviation, by accelerating apoptosis of Th2 cells or even by converting Th2 towards Th1 cells and enhancing the generation and selective expansion of Th1 cells, resulting in the predominance of Th1 cells that produce IFN- γ and IL-2, as shown in our study. A possible mechanism of generation and expansion of Th1 cells can be that high cumulative doses of allergen, as administered during immunotherapy, are presented by monocytes/macrophages, which result in Th1 cytokine production instead of lower antigen doses that are presented by B cells [19,20].

The model for cytokine regulation becomes more complicated with the demonstration that also CD8⁺ T lymphocytes generate two subpopulations, called Tc1 and Tc2 cells, which might be important in immune modulation [21]. T cytotoxic/suppressor lymphocytes are no longer believed to act only as potent effector cells in the elimination of virally infected cells [22–24] but they also modulate the humoral response through suppressor activities in some allergic sensitization animal models [25,26]. In house dust mite-allergic patients, immunotherapy has also been shown to result in the expansion of allergen-specific CD8⁺ suppressor cells, which inhibit allergen-specific IgE production and allergen-specific proliferation *in vitro* [5]. In our study, we observed an increased

percentage of IFN- γ -producing CD8⁺ T cells. These results are in accordance with data from both *in vivo* and *in vitro* models that support the hypothesis that the induction of IFN- γ produced by CD8⁺ (Tc1) T cells during VIT inhibits the proliferation of Th2 allergen-specific cells [27–29].

In several studies, reciprocal roles for IL-4 and IFN- γ were described in the regulation of IgE synthesis *in vitro* [30–35]. Therefore *in vivo*, the decrease in IL-4 and increase of IFN- γ , due to VIT, may reduce IgE production and simultaneously enhance IgG4 [36,37]. However, there is considerable evidence that these parameters (IgE and IgG4) correlate poorly with clinical insensitivity [4]. Also in our study, specific wasp venom IgE did not alter significantly during the first 6 months after initiation of VIT, although the percentage of IL-4-producing T cells was clearly diminished after 5 days and 6 months of semirush VIT.

The question when to discontinue VIT also remains unresolved. Negative venom skin test responses and decrease of venom-specific IgE have been proposed as possible guidelines for stopping VIT but appeared to be not accurate [38–41]. Skin test can remain positive after maintenance immunotherapy during a period of 3–5 years in about 80% of subjects [41,42]. Reports claiming that increased concentrations of specific IgG would provide valuable information on clinical desensitization [42] have been questioned [4]. Recently, negatization of lymphocyte transformation test (LTT) during VIT is suggested as a possible indicator of clinical insensitivity [43]. Long-term follow up (2–5 years) of cytokine profiles in patients during VIT is required to consider determination of cytokine profiles as an additional *in vitro* method to decide when to stop VIT.

In conclusion, this study demonstrated that venom immunotherapy induces a shift from IL-4-producing towards IFN- γ -producing CD4⁺ T lymphocytes as well as CD8⁺ T lymphocytes.

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