

Venom immunotherapy induces monocyte activation

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Summary

Background Venom immunotherapy (VIT) is an efficient treatment of hymenoptera venom allergy. The mechanism of VIT is based on the induction of tolerance of allergen-specific Th2 cells. The mechanisms of this T cell modulation are unknown, and could depend on cytokines produced by other cell types such as interleukin (IL)-12, tumour necrosis factor (TNF)- α and IL-10 by monocytes.

Objective To assess if VIT modifies the monocyte production of IL-12, TNF- α and IL-10 during the 45 first days of treatment.

Methods Fourteen patients and seven controls were included. Blood samples were taken once in controls and at day (D)1, D30 and D45 of VIT in patients. Monocytes were isolated, cultured with and without lipopolysaccharide (LPS), and the culture supernatant was harvested. IL-10, IL-12 and TNF- α were assayed in supernatants by ELISA.

Results Baseline cytokine levels were not statistically different between patients and controls. During treatment, an increase of spontaneous monocyte production of IL-12 and TNF- α was observed at D15 and D45. The production of IL-10 increased at D15 and D45 but not significantly. After LPS-stimulation, IL-12, TNF- α and IL-10 monocyte production was not modified by VIT.

Conclusion VIT induces a monocyte activation characterized by a delayed overproduction of IL-12 and TNF- α . These cytokines could be relevant to the inhibition of Th2 cells during VIT. Therefore, VIT-induced tolerance could depend not only on the specific action of venom antigens on T cells, but also on a secondary non-specific action of monocytes.

Keywords: immunotherapy, IL-12, IL-10, TNF- α , Th1, Th2, allergy, monocyte, hymenoptera venom.

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Introduction

Venom immunotherapy (VIT) is widely recognized as an efficient treatment of hymenoptera venom allergy [1]. As in all IgE-mediated diseases, venom allergy is governed by activated allergen-specific Th2 cells, producing interleukin (IL)-4 and IL-5 but no or little interferon (IFN)- γ . Recent studies have showed that the success of VIT was based on

the induction of specific anergy in T cells, reflected by a lack of proliferation of T cells from treated patients in response to specific and non-specific stimuli [2,3]. IL-10 plays a pivotal role in this process [4]. Initially considered as a Th2 cytokine inhibiting specifically the IFN- γ producing (Th1) T cells, IL-10 is now considered as an anti-inflammatory cytokine, suppressing both kinds of T cell activation [5]. Secreted by venom-specific T cells, IL-10 induces anergy in an autocrine fashion, resulting in a decrease of IL-4 production, and thus suppression of IgE and enhancement of IgG4 synthesis [4]. Upon stimulation

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by IL-2 or IL-15, tolerized (Th0) specific T cells from VIT patients can be committed to a Th1 pattern of cytokine production [6].

Some studies have shown a decrease in IFN- γ production during VIT, parallel to the decrease in IL-4 production [4]. Secrist and coworkers [7] generated CD4⁺ T cell clones from patients treated for allergic rhinitis. They found a decrease in IL-4 production by these clones and no change in IFN- γ secretion. However in other experimental conditions, several studies reported an increase in the production of IFN- γ by T cells during VIT. Jutel and coworkers [8] have shown in rush desensitization to bee venom an increase in IFN- γ production upon stimulation with the major bee allergen, phospholipase A (PLA), as early as 6 h after treatment. This elevation progressively increased until day (D)50. By contrast, McHugh and coworkers [9] have found an early transient decrease of IL-4 secretion upon specific stimulation, followed by an increase of specific IFN- γ production only 2 months after treatment. This delayed increase was concomitant of a secondary decrease of IL-4. Akoum and coworkers [10] showed a decrease in T cells expressing the IL4 mRNA and an increase in T cells expressing the IFN- γ mRNA at D15, enhanced at D90. Durham and coworkers [11,12] showed that after grass pollen immunotherapy, the number of cells expressing the IFN- γ gene was increased *in situ*, in response to a specific dermal or nasal stimulation. At the maintenance dose of rush immunotherapy for house dust mite-sensitive asthma, an increase of IFN- γ production by CD4⁺ T cells was shown by flow cytometry [13]. In addition, Pierkes *et al.* showed that IFN- γ participates with IL-10 to the decreased release of histamine and sulfido-leukotrienes by leucocytes after VIT [14].

Therefore, it is still unclear to what extent the modulation of T cell function during VIT can be qualified as a Th2-to-Th0 shift (IL-10-driven induction of anergy), as a Th2-to-Th1 commitment (IFN- γ -based immune deviation) or both [2]. Nevertheless, mechanisms underlying this modulation are unknown and can involve non-T cells such as monocytes.

Indeed, as antigen-presenting cells (APC), monocytes modulate T cell activation. Their action on T cells varies according to the dose of antigen. The IL-4 production by allergen-specific T cells is decreased when monocytes are used as antigen-presenting cells in the presence of high doses of allergen [15]. Monocytes modulate T cell activation notably by producing various cytokines including IL-10, IL-12 and tumour necrosis factor (TNF)- α . As IL-10 produced by T cells, monocyte-derived IL-10 could play a role in VIT-induced T cell anergy. IL-12 strongly stimulates T cells to synthesize IFN- γ [16] and is pivotal in the Th1 commitment. TNF- α is a pro-inflammatory cytokine which also stimulates the production of IFN- γ

by T cells [17]. These properties of monocytes and their products prompted us to assess the course of monocyte activation during VIT, by measuring the production of these three cytokines *ex vivo*.

Material and methods

Skin tests

The presence of venom-specific IgE was shown by intradermal skin tests with serial tenfold dilutions of yellow jacket, wasp and honeybee venom (Dome Hollister Stier Laboratories, Paris, France), starting at the lowest concentration of 10^{-5} $\mu\text{g/mL}$. Skin tests were considered positive if reactions occurred at a concentration of 1 $\mu\text{g/mL}$ or less. To select non-atopic subjects, a battery of skin prick tests including the frequent aeroallergens was performed. Skin tests were considered positive if the weal diameter 20 min after allergen injection reached at least half the weal diameter induced by codeine phosphate and more than the weal induced by saline 9%. In any case, a positive (codeine phosphate 9%) and a negative test were performed.

Study population

Fourteen patients (8 male, 6 female) 32–70-year-old (median 50.5 years) were included in the study. Inclusion criteria were the requirement of desensitization to yellow jacket venom, i.e. a general reaction due to sting anaphylaxis, a positive skin test to yellow jacket venom, and the presence in serum of IgE specific of yellow jacket venom.

Furthermore, to select a homogeneous population with regard to allergic background, the negativity of skin tests for honeybee venom and the absence of atopy, i.e. negative prick tests to common aeroallergens were required.

Sting anaphylaxis induced a shock in 3 cases, a laryngo-oedema in 8 cases, and a giant urticaria in 9 cases. Cutaneous prick tests were performed in all but one case. In this case, the positive control was negative, and skin tests were not performed. In the 13 other cases, skin tests were positive for yellow jacket. They were positive for wasp in 11 cases. Skin tests were positive at the concentration of 10^{-5} $\mu\text{g/mL}$ in 7 cases, at 10^{-4} $\mu\text{g/mL}$ in 3 cases, at 10^{-3} $\mu\text{g/mL}$ in one case and at 10^{-2} $\mu\text{g/mL}$ in 2 cases. Specific IgE were found in all cases. RAST were of class III in 7 cases and of class II in 7 cases. All the included patients completed the study without significant adverse effect of treatment.

Seven healthy controls (3 male and 4 female), 23–55-year-old (median 29.8 years) were included in order to compare baseline cytokine production between allergic and

non-allergic subjects. Controls displayed negative skin tests for insect venoms, and were non-atopic.

Immunotherapy regimen and study design

Only yellow jacket desensitization was considered in the present study. A 210 min protocol was used as previously described [18]. An initial dose of 0.1 µg of pure venom was followed by 1, 10, and 20 µg at 30-min intervals and then 30 and 40 µg at 60-min intervals. The cumulative dose was 101.1 µg. Patients were re-hospitalized for two booster injections of 50 µg on day 15 and one of 100 µg on day 45. Subsequently, monthly 100 µg boosters were given on an outpatient basis.

A sample of blood was taken before treatment (D1.1), after the first day of desensitization (D1.2), and then before the D15 and the D45 booster injections. A single blood sample was taken in controls. The ethical committee of Marseilles-I approved the protocol, and each subject gave an informed consent.

Monocyte culture and cytokine assays

Cell culture Peripheral blood mononuclear cells (PBMC) were prepared from freshly drawn heparin blood by ficoll density gradient separation. 10^6 cells were incubated for one hour at 37 °C in 24 well plates in 1 mL of RPMI containing 10% heat inactivated fetal calf serum (FCS) (Gibco Life Technologies, Eragny, France), 100 U/mL penicillin G and 100 µg/mL streptomycin. Wells were washed to remove non-adherent cells. More than 90% of adherent cells isolated by this methods were able to phagocyte latex beads, which proved their monocytic origin [19]. Cells were then cultured for 24 h in RPMI with or without 200 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6 (Sigma Chemical Co., Coger, Paris, France). Supernatant were removed, centrifuged, decanted and stored at -20 °C until ELISA assays.

Cytokine assays IL-12 (p70) and IL-10 secretion was assayed by specific sandwich ELISA using antibody pairs according to the manufacturer's instructions (Quantikine R & D Systems, Abingdon, UK). The lower limit of detection was 0.3 pg/mL for IL-12 and 2 pg/mL for IL-10. TNF-α secretion was measured using specific sandwich ELISA kit (Immunotech, Marseilles, France). The limit of detection was 5 pg/mL. The inter- and intra-assay coefficients of variations were, respectively, below 13% and 10% for TNF-α and below 17% and 8% for IL-12.

Allergen-specific IgE assay

Allergen specific IgE against yellow jacket, honeybee and

wasp were measured in sera with Pharmacia CAP System RAST® RIA test. Briefly, allergen of interest, covalently coupled to ImmunoCap, reacts with the specific IgE in the patient serum specimen. After washing away non-specific IgE, radioactive labelled antibodies against IgE are added to form a complex. After incubation unbound anti-IgE ^{125} I is washed away and radioactivity of bound complex is measured in gamma counter (Cobra™ II-Auto-Gamma® Packard®, Packard Instruments, Rungis, France). To classify test results, counts for patient samples are compared directly with counts for standards run in parallel.

Statistical analysis

Non-parametric Mann and Whitney *U*-test was used to compare cytokine levels between patients' pretreatment samples and controls. Cytokine levels in samples taken during treatment were compared using non-parametric Friedmann repeated measures analysis of variance on ranks. When this analysis was significant, results of each day were compared to the predesensitization results by using a Wilcoxon signed rank test. Results significantly different from baseline were compared to each other by using a Wilcoxon signed rank test. Correlations between cytokine levels were done using the Spearman rank order correlation test. A *P*-value of 0.05 or lower was considered as significant. All the analysis were done by using the Sigmatat® Software, SPSS Inc.

Results

Production of IL-12 by monocytes

At baseline, spontaneous IL-12 monocyte production tended to be lower in patients as compared to controls, but the difference did not reach statistical significance (*P* = 0.092, Table 1, Fig. 1). After stimulation by LPS, IL-12 levels in culture supernatants were not different between groups (*P* = 0.82, Table 1).

During immunotherapy, spontaneous IL-12 monocyte production varied significantly (*P* = 0.021). No significant difference was found between the pretreatment and the post-treatment D1 samples (*P* = 0.73). A significant increase of spontaneous IL-12 monocyte production was observed at D15 (*P* = 0.023) and D45 (*P* = 0.027) as compared to pretreatment levels (Fig. 1). No significant difference was found between D15 and D45 (*P* = 0.76). However, this course was not observed in LPS-stimulated samples (*P* = 0.41). Median and 25% – 75% confidence intervals of cytokine levels during treatment are given in Table 2.

Table 1. Monocyte cytokine production at baseline in patients and control subjects. Median and interquartile ranges (25%–75%) of the data are given in pg/mL

	IL12	TNF- α	IL-10
Patients Unstimulated cells	0 (0–2)	128 (105–222)	7.3 (3–69.5)
Controls Unstimulated cells	2.9 (0.3–4.7)	200 (105–582.25)	4 (2.5–14.5)
Patients LPS stimulation	3 (0.37–11.8)	3122 (1180–5540)	505.6 (12.4–1118)
Controls LPS stimulation	4.7 (1.4–5.7)	2000 (2000–5770)	66 (47.25–325.5)

Production of TNF- α by monocytes.

At baseline and after stimulation by LPS, the monocyte TNF- α production was not different in allergic subjects as compared to controls ($P = 0.63$ and $P = 0.73$, respectively, Table 1, Fig. 1). During immunotherapy, a significant variation of spontaneous TNF- α production by monocytes was observed ($P = 0.011$). The TNF- α variation paralleled this of IL-12: No significant difference was observed between pretreatment and post-treatment samples on D1 ($P = 0.37$). Again, TNF- α levels increased at D15 ($P = 0.003$) and D45 ($P = 0.05$) (Table 2, Fig. 1) as compared to pretreatment data. No difference was found between D15 and D45 ($P = 0.94$). No difference between treatment days was found in TNF- α production after LPS-stimulation ($P = 0.182$, Table 2). A significant correlation was found between spontaneous IL-12 and TNF- α monocyte production ($R = 0.36$, $P = 0.007$).

Production of IL-10 by monocytes.

At baseline and after stimulation by LPS, the monocyte IL-10 production was not different between groups ($P = 0.57$, Table 1). During immunotherapy, an increase of the spontaneous IL-10 monocyte production was observed, but was not statistically significant ($P = 0.183$). No difference between treatment days was found in IL-10 production after LPS-stimulation ($P = 0.47$, Table 2).

Discussion

In this study, we demonstrate that VIT increases monocyte spontaneous IL-12 and TNF- α production, with a trend to the elevation of IL-10 levels. This effect is not observed at D1 but is delayed to D15. It is not enhanced by the D15 booster injection, but only sustained at D45. In some patients, this effect of VIT is not shown before D45,

suggesting that in these patients the D15 booster is necessary to induce it. The effect of the D45 booster injection was not studied. This variation of cytokine levels could be related to an increase of adherent cells recovered via a modification of cell adherence capacities, and/or to an increase of cytokine production per cell. As no correlation was found between cytokine levels and the number of adherent cells (data not shown), the latter mechanism is involved, eventually associated to the former.

The clinical relevance of the increase in IL-12 production is questionable. Indeed IL-12 levels are very low. However, this increase was observed in *ex vivo* culture without further *in vitro* stimulation. Furthermore, we assayed the heterodimeric (p70) form of IL-12, which is biologically active. Therefore the increase of IL-12 produced by monocytes could be relevant to the increase in IFN- γ production observed during VIT. IL-12 is a very powerful driver of the Th1 polarization of T cells [16]. In IL-12 knockout mice, IFN- γ production is impaired albeit IL-4 secretion is enhanced [20]. In various animal models, IL-12 acts as a suppressive agent of the allergic immune response [21]. Our results are in keeping with a previous study performed during grass pollen immunotherapy, in which Hamid *et al.* [22] have shown that the number of cells expressing the mRNA coding for IL-12 increased in skin 24 h after a local specific stimulation. They elegantly showed that the number of IL-12 mRNA-positive cells was directly correlated to the number of IFN- γ mRNA-positive cells and inversely correlated to this of IL-4 mRNA-positive cells. Parallel to the increase of IL-12, we observed an overproduction of TNF- α by monocytes during VIT. This result is also potentially relevant to the increase in IFN- γ production [17].

However, several arguments suggest that the effect of VIT on monocyte activation is driven by already re-committed Th1 cells and is not the trigger of an early Th2 to Th1 shift. Indeed, this activation is not seen at D1, but

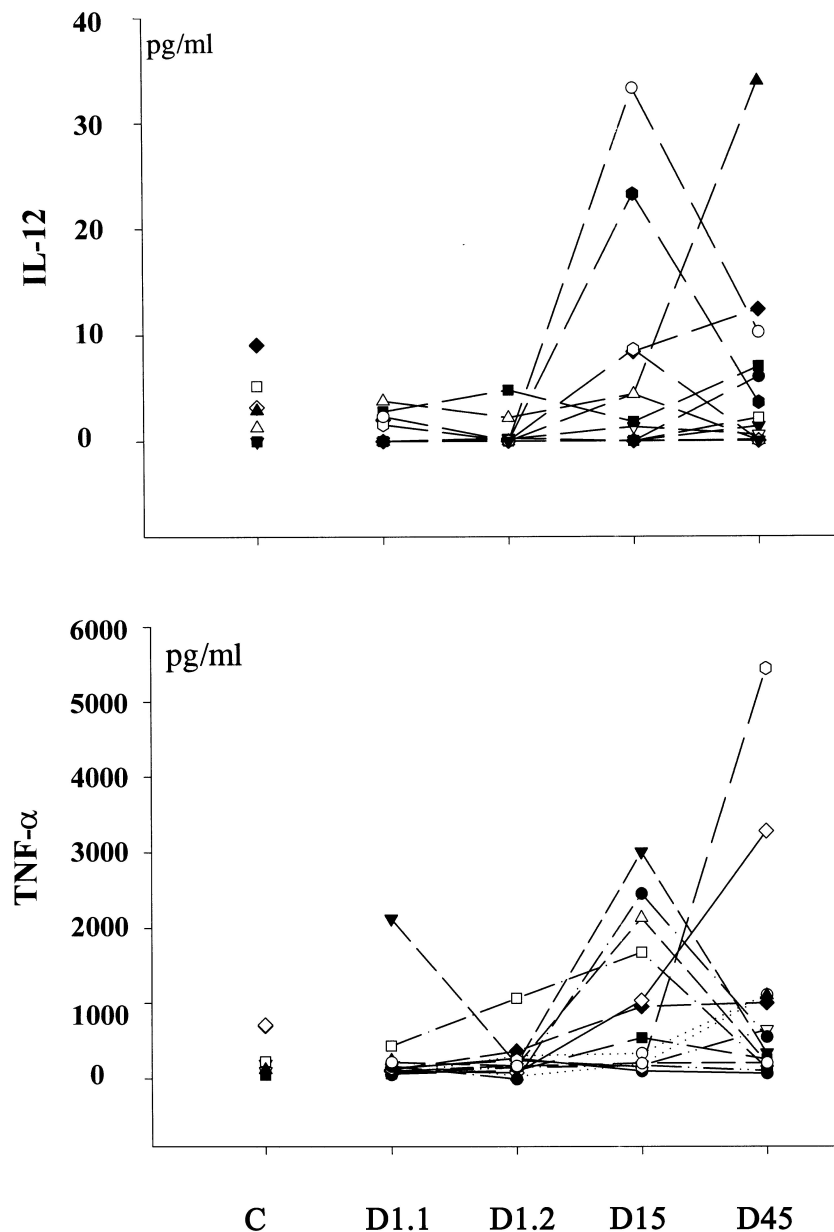


Fig. 1. Spontaneous monocyte cytokine production during venom immunotherapy. Blood samples were taken before (D1.1) and after (D1.2) the induction of venom immunotherapy at D1, and before the D15 and the D45 booster injections in 14 patients, and once (C) in 7 control subjects. Monocytes were isolated by adherence, cultured for 24 h and IL-12- and TNF- α -assayed in the culture supernatants. Data are expressed for each patient in pg/mL.

delayed to D15, although VIT is efficient as early as D1 and although some studies have found an increase of IFN- γ [8] or a decrease of IL-4 production [9] in the first hours of treatment. In addition, IFN- γ stimulates IL-12 production [23]. Furthermore the elevation of TNF- α was correlated to this of IL-12, which can result from a stimulation from Th1 cells. Indeed TNF- α production is enhanced by IFN- γ [23] and inhibited by IL-4 [24]. In addition, it was shown recently that a simultaneous monocyte IL-12 and TNF- α production is induced by the engagement of LAG-3, a MHC class II ligand expressed selectively on Th1 cells [25]. Lastly, TNF- α is poorly relevant to the Th1/Th2

balance: although it stimulates the IFN- γ production by T cells, TNF- α was found to have increased in a number of allergic diseases [26,27]. Therefore, the overproduction of IL-12 by monocytes would be more relevant to a late and sustained increase of IFN- γ than to an early specific Th2 to Th1 recommitment. Accordingly, Akoum and coworkers [10] showed that the late elevation of IFN- γ during VIT was not antigen-specific. In this view, the monocyte activation would boost the T cell modulation in an amplification loop.

IL-10 is a cytokine responsible for the T cell tolerance induced during VIT [3]. It is an immunosuppressive

Table 2. Monocyte cytokine production during venom immunotherapy. Median and interquartile ranges (25%–75%) of the data are given in pg/mL

	IL12	TNF- α	IL-10
	Unstimulated cells		
Pretreatment D1	0 (0–2)	128 (105–222)	7.3 (3–69.5)
Post-treatment D1	0 (0–2)	176.5 (112–274)	11.5 (4–18)
D15	1.5 (0–8.4)*	444.5 (181–1684)*	19.2 (8–90)
D45	1.735 (0–7)*	453 (215–1105)*	38 (10–80)
	LPS-stimulation		
Pretreatment D1	3 (0.37–11.8)	3122 (1180–5540)	505.6 (12.4–1118)
Post-treatment D1	5.3 (0.24–7)	5650 (3000–10050)	464 (255–687)
D15	3.8 (0.3–17.2)	5897 (3466–9300)	183 (86.25–639.75)
D45	8.18 (2.05–22.8)	5320 (3660–9540)	258.85 (151–522.3)

*Indicates statistically significant difference compared to pretreatment D1 data

cytokine, inhibiting the Th1- as well as the Th2-type activation [5]. This cytokine inhibits the allergic inflammation and was therefore proposed as a potential therapeutic agent in allergies [28]. During VIT, IL-10 production by peripheral mononuclear cells was shown to increase, and to result in T cell anergy [4,29]. However, the source of IL10 in these works was T cells themselves and not monocytes. This is in keeping with our results, in which the production of IL-10 by monocytes did not increase significantly during the first 45 days of VIT.

In conclusion, this study shows a delayed activation of monocytes within the first 45 days of treatment. This effect could participate in the modulation of T cell activation during VIT and be relevant to the sustained effect of the treatment.

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