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Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects

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The current study was carried out to elucidate the immunoregulatory changes induced by venom immunotherapy (VIT) in bee or wasp allergic subjects. All subjects included in this study had a history of severe systemic allergic reactions to stings of the respective insect as well as positive skin tests with the respective venom or venom-specific IgE in the sera. Parameters assessed in peripheral blood mononuclear cells (PBMC) before and after initiation of VIT (rush therapy reaching a maintenance dose of 100 µg venom injected subcutaneously within 1 week) were expression of CD3, CD4, CD8, CD45RA, CD45RO, interleukin (IL)-2 receptor (R)α, IL-4R, IL-12R, FcεRII, CD40, and CD40 ligand (CD40L), cells producing interferon (IFN)-γ and IL-10 after stimulation with phorbol 12-myristate 13-acetate + ionomycin in the presence of monensin measured by flow cytometry; secretion of IFN-γ, IL-4, and IL-10 measured by ELISA (IFN-γ and IL-10 were additionally measured by PCR), and proliferation after stimulation with the respective venom. Significant decreases were observed after VIT for proliferative response to venom and venom + IL-4, IL-4 secretion, FcεRII, CD40, and CD40L expression. Significant increases were observed after VIT for IFN-γ concerning the amount secreted and the number of producing cells, and IL-10. IL-10 was mainly produced by CD4⁺ cells that were negative for IFN-γ, but some double-positive (IL-10 and IFN-γ) cells were always detected. Addition of blocking anti-IL-10 antibodies, but not isotype control antibodies, prevented down-regulation of proliferation (but not IL-4 secretion) and further enhanced IFN-γ secretion after VIT. These data indicate that in insect venom allergic subjects, VIT not only induces a rapid shift in cytokine expression from Th2 to Th1 cytokines, but also leads to induction of the immunosuppressive cytokine IL-10, which may be important for the limitation of potentially harmful allergen-specific Th1 responses. The described changes in cytokine expression may be responsible for subsequent increases in allergen-specific IgG and decreases in IgE production, as well as suppressive activity observed in earlier studies.

1 Introduction

Venom immunotherapy (VIT), *i.e.* subcutaneous injection of graded doses of venom, is very effective to protect bee- or wasp-allergic subjects with serious IgE-mediated systemic allergic reactions of the immediate type after stings of the respective insect [1–4]. In older studies, induction of blocking IgG antibodies, especially IgG4 was thought to be responsible for this protective effect [4–6]. The generation of CD8⁺ suppressor cells which modulate allergen-specific IgE production was also described [7]. Overall, a decline of allergen-specific IgE and an increase of

allergen-specific IgG production can be observed after immunotherapy [5, 8, 9]. However, the immunoregulatory mechanisms by which VIT induces these changes are not fully understood.

IgE production is induced and enhanced by the Th2 cytokine IL-4 [10–13] and inhibited by the Th1 product IFN-γ [14–17]. In humans, IL-13 can also induce IgE and IgG4 production [18–20]. Besides IL-4 or IL-13, the interaction between CD40 and its ligand CD40L has been identified to be necessary for isotype class switching [21–25]. PBMC from atopic and normal donors have been shown to produce both IgE and IgG4 upon stimulation with IL-4 [26–28], but the levels of IgE and IgG4 are conversely regulated by IL-4: high IL-4:IFN-γ ratios favor the production of IgE, while IgG4 production declines [29].

Recent studies provide evidence that immunotherapy (including bee VIT) may alter the pattern of cytokine synthesis by allergen-specific T cells by inducing a shift in cytokine expression from Th2 (IL-4, IL-5) to Th1 (IFN-γ) cytokines [30–32].

The purpose of our study was to extend these observations to wasp allergy, to investigate additional parameters besides Th1 and Th2 cytokines, to analyze cytokine produc-

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Abbreviations: VIT: Venom immunotherapy CD40L: CD40 ligand

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tion at a single-cell level for correlation with other parameters, to assess changes induced early on after initiation of VIT, and to avoid culturing of T cells, as the latter may strongly influence the cytokine spectrum produced [33].

2 Materials and methods

2.1 Subjects

Wasp- and bee-allergic patients ($n = 102$ and 12 , respectively) were included in the study. All suffered from severe systemic reactions to stings of the respective insect (*i.e.* generalized urticaria, angioedema, hypotension, or shock). Specific sensitization was documented by positive skin tests to the respective venom (intracutaneous injection of $0.1 \mu\text{g/ml}$ venom) or detection of venom-specific IgE in the sera (radioimmunosorbent assay or RAST class ≥ 2). Of the included patients, 46% were atopic, suffering from allergic rhinoconjunctivitis, asthma, atopic eczema, or had a total serum IgE greater than 100 U/ml ; and 54% were not atopic. Patients with other known immunologic disorders or systemic immunomodulating therapy were excluded.

2.2 Rush VIT

VIT was carried out according to the manufacturer's descriptions (ALK-lyophilized SQ, Scherax, Hamburg, Germany), reaching a maintenance dose of $100 \mu\text{g}$ venom injected subcutaneously within 1 week.

2.3 Timing of blood sampling

Heparinized blood (30 ml) was obtained on the day of hospital admission (before VIT) and 1 week later at the day of discharge (after the initiation of VIT) when the maintenance dose of $100 \mu\text{g}$ was reached.

2.4 Preparation of cells

PBMC were isolated from heparinized blood by Ficoll-Paque (1.077 g/cc) (Biochrom, Berlin, Germany) density centrifugation and washed three times with PBS containing 1 mM EDTA. For some experiments, CD4^+ or CD8^+ T cells were removed from PBMC with the anti-CD4 mAb OKT4 (mouse IgG2b, American Type Culture Collection; ATCC, Rockville, MD), or the anti-CD8 mAb OKT8 (mouse IgG2b, ATCC) and sheep anti-mouse IgG magnetic beads M-450 (Dynal, Hamburg, Germany). Depletion was controlled by flow cytometry.

2.5 Cell cultures

For cytokine production and proliferation assays *in vitro*, cells were suspended in RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley, Scotland) with 2 mM L-glutamine supplemented with $100 \mu\text{g/ml}$ streptomycin, 100 U/ml penicillin, $5 \mu\text{M}$ 2-mercaptoethanol, 20 mM HEPES, 1 mM sodium pyruvate, 24 mM sodium hydrogen-carbonate, and 5% AB serum (transfusion center, Mainz, Germany).

For determination of IL-4, IL-10, and IFN- γ secretion, 5×10^5 cells ($200 \mu\text{l/well}$) were cultured for 48 h in 96-well flat-bottom tissue-culture plates (Costar, Bodenheim, Germany). Wasp or bee venom (ALK lyophilized SQ, Scherax) was added at a concentration of 250 ng/ml . The antigen tetanus toxoid was used at a concentration of $10 \mu\text{g/ml}$. PHA ($2.4 \mu\text{g/ml}$) and PMA + ionomycin (10 ng/ml and $0.5 \mu\text{M}$, respectively) were used as positive controls, and incubation with medium alone as negative control. Cells were cultured at 37°C in humidified 5% $\text{CO}_2/95\%$ air. After 2 days of culture, supernatants were harvested and stored at -70°C until analysis. As insect venoms contain cytotoxic components, the remaining cells were harvested and analyzed for their viability by the trypan blue method or by staining with propidium iodide detected by flow cytometry.

For proliferation assays, 2×10^5 PBMC were incubated in 96-well flat-bottom tissue-culture plates in $200 \mu\text{l/well}$ in triplicate for 4 days. The following reagents were added: 250 ng/ml bee or wasp venom, $10 \mu\text{g/ml}$ tetanus toxoid, 400 U/ml rIL-4, rIL-4 + bee or wasp venom, rIL-4 + tetanus toxoid, rat anti-human IL-10 mAb ($1 \mu\text{g/ml}$; JES3-19F1.1.1, ATCC), rat anti-human IL-10 mAb + bee or wasp venom, or PMA + ionomycin (10 ng/ml and $0.5 \mu\text{M}$, respectively).

2.6 Determination of cytokine content in tissue culture supernatants

Human IL-4 and IFN- γ was measured by ELISA kits (Bio-source, Camarillo, Ca) according to the manufacturer's descriptions. ELISA plates were analyzed in a microplate ELISA reader Model 450 (Bio-Rad, München, Germany) using the microplate manager software (Bio-Rad). The cytokine concentrations were calculated according to standard curves. The detection limits were 8 pg/ml for IL-4 and 15 pg/ml for IFN- γ .

Human IL-10 protein concentrations were also measured by ELISA according to instructions of the manufacturer of the employed pair of antibodies (PharMingen, San Diego, CA). Briefly, Maxisorp Nunc Immunoplates (Nunc GmbH, Wiesbaden, Germany) were coated overnight at 4°C with $1 \mu\text{g/ml}$ purified rat anti-human IL-10 mAb (JES3-9D7) diluted in 0.1 M NaHCO_3 , pH 8.2. After washing with PBS containing 0.05% Tween 20, the plates were blocked for 2 h at room temperature with PBS containing 10% FCS (Gibco BRL). Then, serial dilutions of tissue culture supernatants and the provided human IL-10 standard (PharMingen) were incubated overnight at 4°C . The next day, plates were washed and incubated at room temperature for 1 h with $1 \mu\text{g/ml}$ rat biotinylated anti-human IL-10 mAb (JES3-12G8) diluted in PBS containing 10% FCS. After additional washing, $1 \mu\text{g/ml}$ alkaline phosphatase-conjugated egg white avidin (Jackson ImmunoResearch Laboratories Inc., Baltimore, PA) was added for 1 h. After development with *p*-nitrophenylphosphate (Sigma, Deisenhofen, Germany), the plates were read in a microplate ELISA reader Model 450 (Bio-Rad) at 405 nm . The cytokine concentrations were calculated according to the standard curves. The limit of detection was 15 pg/ml for IL-10.

2.7 Determination of proliferative response

[³H]thymidine (1 μ Ci; ICN, Irvine, CA; 5 mCi/ml) was added to the cultures for the last 6–8 h. Cells were harvested with a Skatron AS cell harvester (Skatron, Norway) and [³H]thymidine incorporation was measured with a liquid scintillation counter 1205 Betaplate (LKB Wallac, Turku, Finland) after addition of scintillation liquid (Roth, Karlsruhe, Germany).

2.8 Determination of surface marker expression and cytokine production by flow cytometry

Surface phenotyping of PBMC was performed by staining 2×10^5 cells with marker-specific mAb for 30 min on ice. Antibodies used were phycoerythrin (PE)-conjugated anti-CD3 UCHT-1 (mouse IgG1, Immunotech, Marseille, France), PE-conjugated anti-CD4 13B8.2 (mouse IgG1, Immunotech), PE-conjugated anti-CD8 B9.11 (mouse IgG1, Immunotech), PE-conjugated anti-CD45RA HI100 (mouse IgG2b, PharMingen), PE-conjugated anti-CD45R0 UCHL1 (mouse IgG2a, Immunotech), PE-conjugated anti-HLA-DR B8.12.2 (mouse IgG2a, Immunotech), PE-conjugated anti-CD23 (Fc ϵ RII) EBVCS-5 (mouse IgG1, Becton Dickinson, San Jose, CA), PE-conjugated anti-CD25(IL-2R α) B1.49.9 (mouse IgG2a, Immunotech), PE-conjugated anti-CD40 B-B220 (mouse IgG1, Camon, Wiesbaden, Germany), PE-conjugated anti-CD40L 24-31 (mouse IgG1, Ancell Corporation, Bayport, MN), anti-IL-4R Sf21 (mouse IgG2a, Sigma) and anti-IL-12R 2-4E6 (mouse IgG1, kindly provided by Dr. Maurice Gately, Hoffmann-LaRoche Inc., Nutley, NJ). After washing in PBS supplemented with 2% FCS (Gibco BRL), cells were stained (only when unconjugated mAb had been used) with donkey PE-conjugated anti-mouse IgG (Jackson ImmunoResearch) for 15 min on ice, washed twice, and analyzed on a FACScan (Becton Dickinson) equipped with Lysis II software.

For determination of intracellular IFN- γ and IL-10 production, 2×10^6 PBMC/ml were incubated in 24-well tissue-culture plates for at least 4 h with 10 ng/ml PMA, 0.5 μ M ionomycin, and 2.5 μ M monensin (Sigma) at a final volume of 2 ml/well. Then, the cells were harvested, washed twice with HBSS, and fixed with 4% paraformaldehyde for 5–10 min on ice. After surface phenotyping (see above) cells were treated with 0.1% saponin diluted in HBSS and stained with 10 μ g/ml anti-human IFN- γ B-B1 mAb (mouse IgG1; Camon) or 5 μ g/ml anti-human IL-10 mAb (rat IgG, JES3-19F1.1.1, ATCC) for 30 min on ice. After washing with HBSS/0.1% saponin, the cells were stained with rat FITC-conjugated anti-mouse IgG (Camon) or donkey PE-conjugated anti-mouse IgG (Jackson ImmunoResearch) and goat FITC-conjugated anti-rat IgG (Sigma) for 30 min on ice, washed twice with HBSS, and analyzed on a FACScan (Becton Dickinson).

2.9 Determination of IL-10 and IFN- γ mRNA expression by reverse transcription (RT)-PCR and Southern blot hybridization

PBMC were prepared as described above and RNA was extracted with TRIzol[®] (Gibco BRL) following the instruc-

tions of the manufacturer. The extracted RNA was reverse-transcribed and the cDNA was amplified by 25 cycles using the Perkin Elmer RT-PCR kit (Perkin Elmer, Überlingen, Germany) in a GeneAmp PCR System 9600 machine (Perkin Elmer). Briefly, 100 ng RNA were added to a 1 mM solution of each dATP, dCTP, dGTP, dTTP, with 50 U reverse transcriptase, 100 pmol of each primer, and 2.5 U AmpliTaq[®] DNA polymerase. The cycling conditions chosen were 1 min at 95°C (denaturation), 1.5 min at 57°C (annealing), and 2 min at 72°C (extension). The primer sequences for IL-10 are 3' TCT CAA GGG GCT GGG TCA GCT ATC, and IL-10 5' ATG CCC CAA GCT GAG AAC CAA GAC, and for IFN- γ 3' ACC GAA TAA TTA GTC AGC TT, and IFN- γ 5' AGT TAT ATC TTG GCT TTT CAG. In each experiment, amplifications were performed without addition of RNA and primers as a negative control. PCR products were separated for 1 h by agarose gel electrophoresis (2%) and stained with ethidium bromide. For analyzing the molecular size of the PCR amplicates, a 100-bp DNA ladder (Pharmacia, Freiburg, Germany) was used as a standard. Then, the PCR amplicates were blotted on a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) by a Southern transfer technique using 20 $\times$ SSC buffer. The DNA was immobilized by baking the membrane at 120°C for 30 min. Thereafter a nonisotopic Southern blot hybridization was performed with an IL-10 or IFN- γ specific internal digoxigenin (DIG)-labeled probe (IL-10 int GAG CAG GTG AAG AAT GCCTTTAAT AAG GTC, IFN- γ int GGC ATT TTG AAG AAT TGG AAA GAG GAG AG) to confirm the origin of the PCR products using the DIG oligonucleotide tailing kit (Boehringer Mannheim). The DIG-labeled nucleic acids were detected by enzyme-linked immunoassay using an anti-DIG alkaline phosphatase conjugate, a luminescence substrate (DIG luminescent detection kit, Boehringer Mannheim) and subsequent exposure to X-ray film (Amersham, Little Chalfont, GB).

2.10 Statistics

Student's *t*-test and paired *t*-test were employed to test the statistical significance of results; *p* < 0.05 is considered significant.

3 Results

3.1 VIT reduces bee or wasp venom- and IL-4 + bee or wasp venom-specific proliferation of PBMC from venom-allergic patients

First, venom-specific proliferation of PBMC before and after initiation of VIT were measured. Before VIT, 91 of 114 patients responded to the respective venom, while 23 patients did not respond (mean index of stimulation = 1.98 ± 1.32 , *n* = 114). The venom-specific proliferation was diminished almost to the level of unstimulated PBMC after the patients had undergone induction of VIT (mean index of stimulation = 1.13 ± 0.5 , Fig. 1). Since the presence of IL-4 inhibits the development of Th1 cells [16, 17], we also analyzed the proliferation to allergen + IL-4, which may reflect the allergen-specific proliferative response of Th2 (or Th0) cells. IL-4 and IL-4 + bee or wasp venom-induced proliferation was also significantly

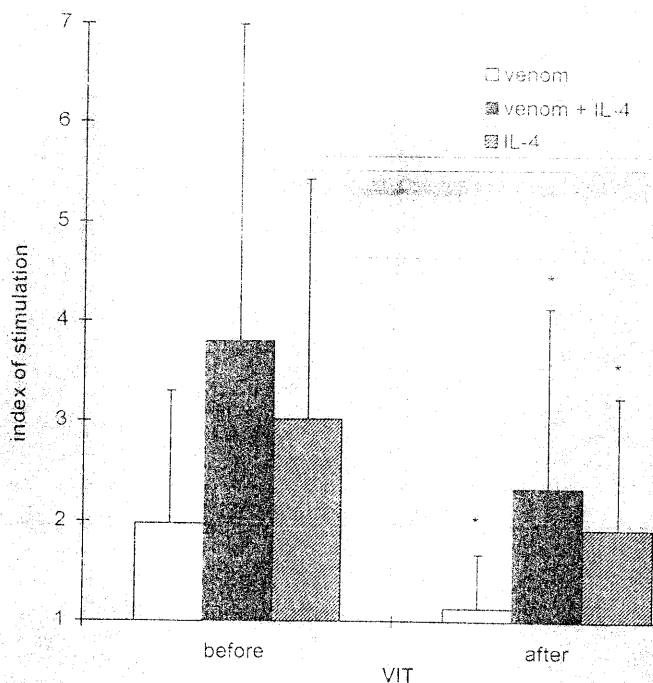


Figure 1. Allergen-specific proliferative responses of PBMC from insect venom-allergic subjects before and after initiation of VIT. PBMC (2×10^5) from insect venom-allergic subjects were prepared before and after induction of VIT and incubated for 4 days in the presence of 250 ng/ml specific bee or wasp venom, 400 U/ml IL-4, venom + IL-4, or medium alone as negative control. [^{3} H]thymidine incorporation was measured after addition of [^{3} H]thymidine for the last 6–8 h of culture. The data represent the means $\pm$ SD from 114 patients. * indicates results after initiation of VIT that differ significantly from results before VIT ($p < 0.01$). Stimulation index = venom specific proliferation / proliferation in medium alone of the cells of an individual donor.

reduced after VIT (Fig. 1). Proliferation to the antigen tetanus toxoid or an unspecific stimulus like PMA + ionomycin remained unaffected by VIT (data not shown). Nonspecific effects of venom components as a reason for decreased proliferation were excluded by viability tests by the trypan blue method and propidium iodide staining (flow cytometry). Additionally, toxicity of venom components should also affect allergen-nonspecific T cells and T cells before and after induction of VIT in the same way. Although high batch-to-batch consistency of the venom is guaranteed by the manufacturer, the same batch of venom was used to stimulate the cells of an individual donor before and after induction of VIT.

3.2 VIT decreases allergen-driven IL-4 production and increases allergen-driven IFN- γ production

To analyze whether cytokines that are representative for Th1 and Th2 cytokines are influenced by VIT, we measured the production of IFN- γ and IL-4 in response to the specific allergen before and after induction of VIT. Kinetic studies revealed that maximal cytokine production was observed after 48 h of culture. PBMC from bee or wasp allergic patients spontaneously produced only small amounts of IL-4, while allergen-driven IL-4 production was higher [19.4 ± 14.7 pg/ml (mean $\pm$ SD) versus 31.4 ± 19.3 pg/ml; Fig. 2a]. After initiation of VIT, the

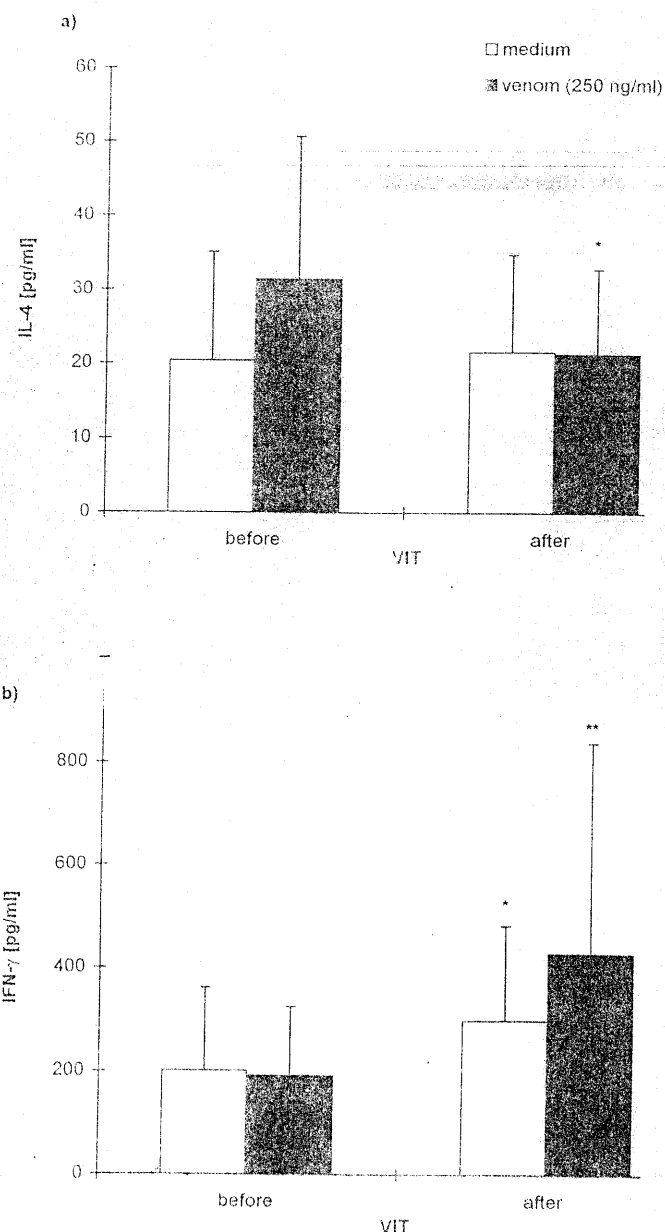


Figure 2. Allergen-driven IL-4 and IFN- γ secretion of PBMC from insect venom-allergic subjects before and after initiation of VIT. PBMC (5×10^5) were incubated with 250 ng/ml specific bee or wasp venom or medium alone as negative control. After 48 h of culture, supernatants were harvested and IL-4 production (a) and IFN- γ production (b) were measured by ELISA as described in Sect. 2.6. Shown are the means $\pm$ SD from 78 patients before and after induction of VIT. * $p < 0.05$ and ** $p < 0.01$ indicate results after initiation of VIT that differ significantly from results before VIT.

allergen-driven IL-4 production was significantly reduced and was only in the range of spontaneous IL-4 production (21.3 ± 11.5 pg/ml, Fig. 2a). The decrease in IL-4 production ranged between 3.4% and 97.3% in individual patients.

In parallel to IL-4 production, we also measured the IFN- γ production before and after induction of VIT. Before VIT, addition of allergen did not increase IFN- γ production [202.3 ± 160.5 pg/ml (mean $\pm$ SD) versus 192.7 ± 134.3 pg/ml; Fig. 2b]. After initiation of VIT, the IFN- γ produc-

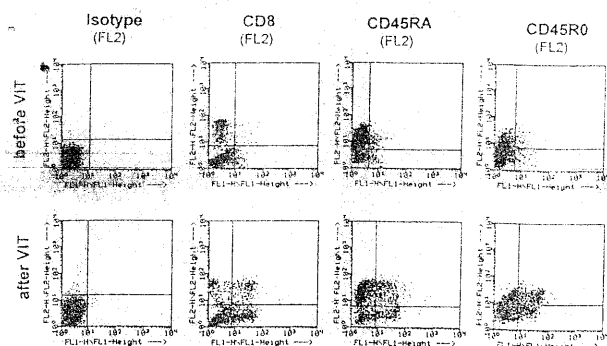


Figure 3. Percentage of IFN- γ -producing cells among different T cell subsets from insect venom-allergic subjects after nonspecific stimulation before and after initiation of VIT. PBMC (2×10^6 /ml) were incubated for at least 4 h with 10 ng/ml PMA, 0.5 μ M ionomycin, and 2.5 μ M monensin and intracellular IFN- γ production among different T cell subsets (CD3 $^+$, CD8 $^+$, CD45RA $^+$, and CD45R0 $^+$ T cells) was analyzed by flow cytometry as described in Sect. 2.8. The figure shows a representative example of the double stainings of one patient before and after initiation of VIT.

tion was significantly enhanced in stimulated (429.1 ± 411.6 pg/ml), but also in unstimulated cultures (299.2 ± 184.8 pg/ml; Fig. 2b). The increase of IFN- γ production ranged between 3.5 % and 2300 % in individual patients. In some patients, we also measured IFN- γ mRNA expression before and after induction of VIT. In accordance with the protein data, IFN- γ mRNA expression was also enhanced after induction of VIT in unstimulated and in venom-stimulated PBMC (data not shown).

3.3 VIT increases the number of IFN- γ -producing cells in different T cell subsets

To assess whether the increase in IFN- γ production was due to increased numbers of IFN- γ -producing cells and to determine which cells produced IFN- γ , we measured the IFN- γ production on a single-cell level by flow cytometry. To obtain detectable levels of intracellular IFN- γ production, PBMC were not stimulated with the allergen, but with PMA + ionomycin in the presence of the protein transport inhibitor monensin for at least 4 h. The number of IFN- γ -producing cells among PBMC was significantly increased after VIT [13.94 ± 5.69 % (mean $\pm$ SD) before VIT versus 18.84 ± 7.8 % after initiation of VIT; Fig. 3].

Double staining of PBMC to detect IFN- γ and CD45RA, CD45R0, or CD8 revealed that IFN- γ production was increased in all of these subsets to a similar extent (Fig. 3). The number of CD8 $^+$ IFN- γ $^+$ cells among PBMC increased from 3.96 ± 2.03 (mean $\pm$ SD, $n = 46$) before VIT to 5.81 ± 3.54 after initiation of VIT, the number of CD45RA $^+$ IFN- γ $^+$ cells increased from 6.09 ± 2.8 to 8.07 ± 4.94 and the number of CD45R0 $^+$ IFN- γ $^+$ cells increased from 6.39 ± 2.41 to 8.68 ± 3.97 . Application of the same technique for intracellular detection of IL-4 production did not yield quantitative results.

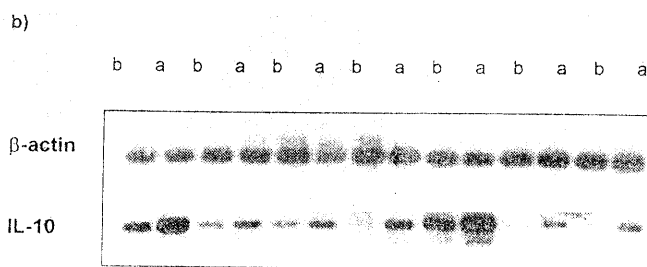
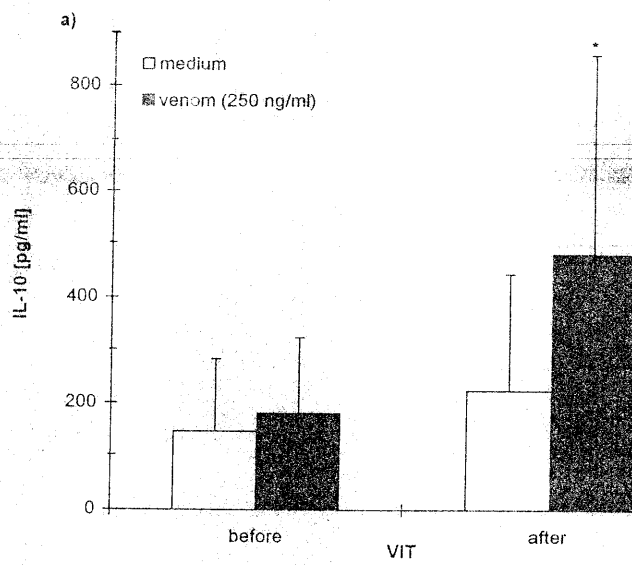
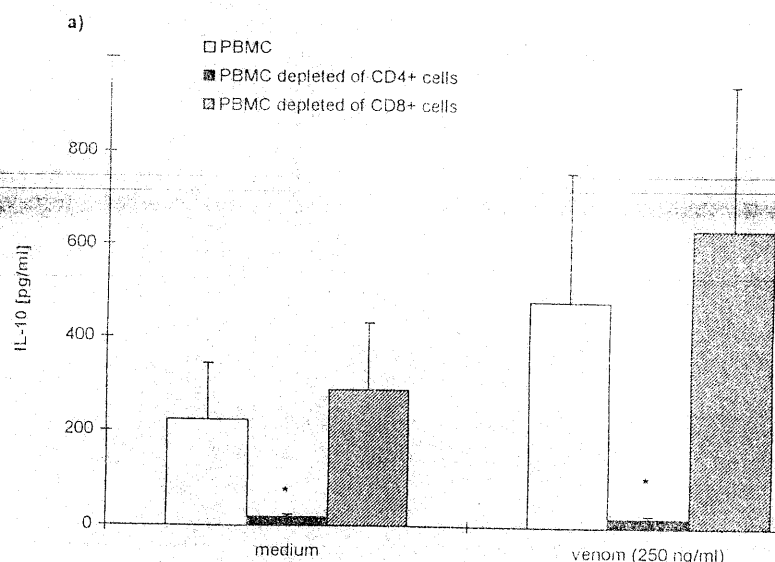


Figure 4. IL-10 protein secretion and mRNA expression of PBMC from insect venom-allergic subjects before and after initiation of VIT. (a) PBMC (5×10^5) were incubated with 250 ng/ml specific bee or wasp venom or medium alone as negative control. After 48 h of culture, supernatants were harvested and IL-10 production was measured by ELISA as described in Sect. 2.6. The data represent the means $\pm$ SD from 46 patients before and after induction of VIT. * indicates results after initiation of VIT that differ significantly from results before VIT ($p < 0.01$). (b) Total cellular RNA was extracted from 1×10^7 freshly isolated PBMC before and after induction of VIT. RT-PCR and Southern blot hybridization were performed as described in Sect. 2.9. Shown are the signals for β -actin (221-bp template) and IL-10 (351-bp template) of the same RNA probes from seven representative patients before (b) and after (a) induction of VIT.

3.4 VIT increases IL-10 production on the protein and mRNA levels

Besides IFN- γ production, allergen-driven IL-10 secretion was also significantly enhanced after induction of VIT [from 181 ± 143 pg/ml (mean $\pm$ SD) to 481 ± 378.8 pg/ml, Fig. 4a]. To determine whether IL-10 expression is regulated at the transcriptional level, we analyzed IL-10 mRNA production before and after induction of VIT, and show that the amount of IL-10 mRNA produced was enhanced after the initiation of VIT. The enhancement of IL-10 mRNA expression was observed after 2 h of culture with the specific allergen (data not shown) as well as in freshly isolated PBMC after the patients had received their last injection (Fig. 4b).



b)

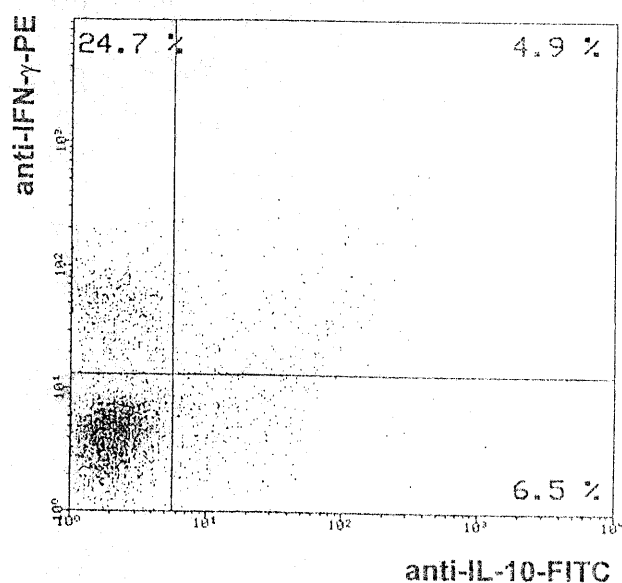


Figure 5. IL-10 is produced by CD4⁺ Th1 and Th2 cells. (a) CD4⁺ or CD8⁺ cells were depleted from PBMC prior to culture and 5×10^5 cells were incubated with 250 ng/ml specific bee or wasp venom or medium alone as negative control. After 48 h of culture, supernatants were harvested and IL-10 production was measured by ELISA as described in Sect. 2.6. The data represent the means $\pm$ SD from 23 patients after VIT. * indicates results that differ significantly from IL-10 production of undepleted PBMC ($p < 0.01$). (b) PBMC (2×10^6 /ml) were incubated for at least 4 h with 10 ng/ml PMA, 0.5 μ M ionomycin, and 2.5 μ M monensin and analyzed by flow cytometry for intracellular IFN- γ and IL-10 content as described in Sect. 2.8. The figure shows one representative result of ten independent experiments.

3.5 IL-10 is produced by CD4⁺ T helper cells, but not exclusively by Th2 cells

To determine which cell type produces IL-10, we depleted different cell subsets prior to cell culture. Depletion of CD4⁺ cells abolished IL-10 production, while depletion of CD8⁺ cells did not (Fig. 5a). Measurement of intracellular IL-10 production by flow cytometry revealed that a smaller part of the IL-10-producing cells also produced IFN- γ , while the majority did not (Fig. 5b). Again, after depletion of CD4⁺ cells, no IL-10 production was detected by this method (data not shown).

3.6 Addition of anti-IL-10 antibodies prevents the down-regulation of allergen-specific proliferation and further enhances IFN- γ production of PBMC after initiation of VIT

To address the biological significance of increased IL-10 production after induction of VIT, we investigated the effect of blocking anti-IL-10 antibodies added to proliferation assays and cytokine production assays. This resulted in a restoration of the proliferative response of PBMC from venom-allergic patients to venom after initiation of VIT, but did not restore the allergen-driven IL-4 production, whereas IFN- γ production was further enhanced [from 429.1 ± 411.6 pg/ml (mean $\pm$ SD) to 630.7 ± 400.2 pg/ml; Fig. 6]. Addition of isotype control antibodies had no effect on proliferation or cytokine production.

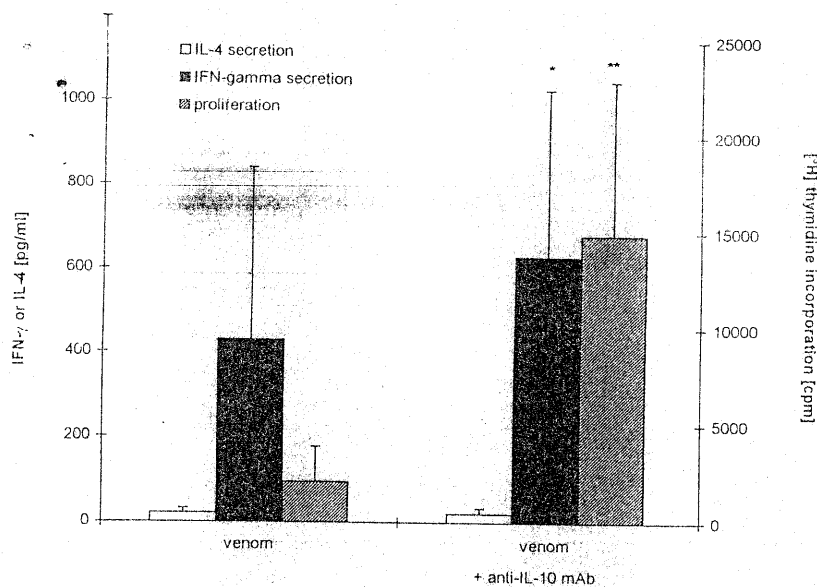


Figure 6. Cytokine secretion and proliferation of PBMC from insect venom-allergic subjects after initiation of VIT with or without blocking anti-IL-10 antibodies added. For cytokine production assays 5×10^5 PBMC were incubated with the specific bee or wasp venom (250 ng/ml) with or without addition of blocking anti-IL-10 antibodies (1 μ g/ml). Supernatants were harvested after 48 h and IL-4 and IFN- γ production were measured by ELISA as described in Sect. 2.6. Shown are the means $\pm$ SD (scale on left y-axis) from 26 patients after induction of VIT. For proliferation assays 2×10^5 PBMC were cultured with the 250 ng/ml specific bee or wasp venom with or without the addition of 1 μ g/ml blocking anti-IL-10 antibodies, and [^{3} H]thymidine incorporation was measured after 4 days of culture. The data represent the means $\pm$ SD (scale on right y-axis) from 62 patients after VIT. * $p < 0.05$ and ** $p < 0.01$ indicate results after induction of VIT that differ significantly from results before VIT.

3.7 VIT decreases expression of IL-4R, Fc ϵ RII, CD40, and CD40L

To determine whether VIT changes surface marker expression, we analyzed the expression of different T and B cell surface markers. As shown in Table 1, VIT not only significantly decreased IL-4 production, but also the expression of IL-4 receptor and Fc ϵ RII (CD23). The percentage of CD40 and CD40L-expressing cells was also significantly reduced, while other surface markers such as IL-2R (CD25), IL-12R, CD3, CD4, CD8, CD45RA, CD45RO, and HLA-DR were not significantly changed after initiation of VIT (Table 1).

4 Discussion

The presented data clearly indicate that in bee and wasp venom-allergic subjects, initiation of VIT not only induces a rapid shift in cytokine expression from Th2 (IL-4) to Th1 (IFN- γ) cytokines, but also leads to increased IL-10 production and down-regulation of the proliferative response to the venom. Inclusion of a control group of patients with insect venom allergy that were left untreated was impossible for ethical reasons, as these patients are vitally threatened by their sensitization and VIT represents the only approved successful therapy for them. However, in a few allergic patients that were not able to undergo VIT within

Table 1. Surface marker expression of PBMC from insect venomi-allergic subjects before and after VIT^{a)}

Surface marker	Before VIT (Percent positive cells)	After VIT	Statistical significance
IL-2R α (CD25)	60.4 $\pm$ 6.5	61.8 $\pm$ 10.6	n.s.
IL-4R <i>n</i> = 21	8.94 $\pm$ 3.21	5.38 $\pm$ 2.82	<i>p</i> < 0.05
IL-12R <i>n</i> = 35	12.5 $\pm$ 5.3	10.68 $\pm$ 3.97	n.s.
FcϵRII (CD23)	7.72 $\pm$ 3.07	6.3 $\pm$ 2.66	<i>p</i> < 0.05
CD40	16.22 $\pm$ 5.4	12.96 $\pm$ 4.3	<i>p</i> < 0.05
CD40L	6.49 $\pm$ 4.93	4.09 $\pm$ 3.01	<i>p</i> < 0.05
CD3	55.3 $\pm$ 9.2	58.75 $\pm$ 9.1	n.s.
CD4	36.9 $\pm$ 7.4	38.9 $\pm$ 7.6	n.s.
CD8	21.4 $\pm$ 6.3	20.3 $\pm$ 6.1	n.s.
CD45RA	51.4 $\pm$ 10.1	51.2 $\pm$ 12.8	n.s.
CD45RO	37.4 $\pm$ 12.9	39.7 $\pm$ 13.1	n.s.
HLA-DR	9.48 $\pm$ 3.7	9.69 $\pm$ 4.2	n.s.

a) PBMC from 53 insect venom-allergic subjects before and after induction of VIT were prepared by Ficoll density centrifugation and analyzed by FACScan. Expression of Fc ϵ RII (CD23), CD40, CD3, CD4, CD8, CD45RA, CD45RO, and HLA-DR was measured directly *ex vivo*, while expression of IL-2R α (CD25), IL-4R, IL-12R, and CD40L was measured after stimulation with the specific venom for 48 h. Results are shown as the mean $\pm$ SD (n.s. = not significant).

a short period of time, no significant changes of the described immunologic parameters were observed until they underwent VIT, excluding spontaneous changes of the described parameters (data not shown). Additionally, several patients were diagnosed and treated months after their last insect sting, also making spontaneous changes in their immune status unlikely. All observed changes in cytokine production were venom-specific, since responses to another antigen, tetanus-toxoid or nonspecific stimuli were not altered significantly after induction of VIT (data not shown).

In several studies, the antagonistic effects of IL-4 as an inducer and IFN- γ as a suppressor of IgE production have been described [14, 34–37]. Recently, Carballido et al. [29], who analyzed the regulation of IgE and IgG4 responses by allergen-specific T cell clones to bee venom phospholipase A₂ *in vitro*, demonstrated that low IL-4:IFN- γ ratios may decrease IgE production and simultaneously enhance IgG4 production and *vice versa*. Therefore, *in vivo*, the decrease in allergen-driven IL-4 and increase in allergen-driven IFN- γ production (the latter also at the transcriptional level) may be responsible for the decline in venom-specific IgE and the rise in venom-specific IgG4 antibodies in the sera observed in earlier studies [5, 38]. The decrease in IL-4R expression may also be due to decreased IL-4 production, as IL-4R expression is up-regulated in response to IL-4 [39, 40]. Decreased IL-4R expression may in turn contribute to decreased IgE production by B cells, as IgE production depends on IL-4 stimulation [41, 42]. Additionally, CD23 expression, which is also induced by IL-4 [43], was decreased, as has been described in earlier studies with other antigens [44].

Besides overall IFN- γ protein secretion, the number of IFN- γ -producing cells measured at the single-cell level was enhanced by VIT. The proportion of IFN- γ -producing cells was significantly increased also among CD8⁺ cells, indicating that VIT also induces changes in cytokine expression in CD8⁺ T cells and not only in CD4⁺ T helper cells. A general increase of CD8⁺ T cells was not observed and neither were changes of other T cell markers like CD3, CD4, CD45RA, and CD45R0. It has to be kept in mind that the changes in cytokine expression measured by flow cytometry do not reflect cytokine production in response to allergen, but rather to unspecific stimulation. Stimulation with allergen did not result in detectable induction of cytokine production assessed by this method (data not shown).

Our cytokine data concerning the Th2-to-Th1 shift are in accordance with other recent studies, which also indicated that immunotherapy altered the cytokine secretion by T cells from atopic individuals [30–32, 45]. Seerist et al. [30] demonstrated that allergen immunotherapy reduced IL-4 production by allergen-specific CD4⁺ T cells from grass pollen- and mite-allergic subjects, while IFN- γ secretion remained unaffected. However, Varney et al. [31] observed an enhanced IFN- γ mRNA expression after immunotherapy of patients with grass pollen allergy. Jutel et al. [32], who investigated the influence of VIT on cytokine secretion in phospholipase A₂-stimulated T cell cultures, observed a decrease in IL-4 and IL-5 and an increase in IFN- γ , but not in IL-10 production after VIT. The discrepancy to our data concerning the IL-10 production may be due to different experimental approaches taken. In the

study of Jutel et al. [32], PBMC were not only stimulated with the allergen phospholipase A₂, but also restimulated with plastic-bound OKT3 F(ab')₂ after 7 days of culture, and cytokine secretion was measured on day 8, while in our study, IL-10 production was measured *ex vivo* (IL-10 mRNA) or after 48 h of culture with the specific allergen (IL-10 protein). The increased IL-10 mRNA expression even in freshly isolated PBMC may be due to the fact that the blood was obtained directly after the last injection of 100 μ g venom. Overall, the observed induction in IL-10 production after VIT was especially interesting because it could be responsible for the prevention of a potentially harmful exaggerated allergen-specific Th1 response. This assumption is supported by the fact that the addition of blocking anti-IL-10 antibodies to the cultures further augmented the already enhanced allergen-driven IFN- γ synthesis and restored the proliferative response to the allergen, but not the allergen-driven IL-4 production, indicating that the presence of IL-10 may prevent the expansion of Th1 cells after VIT. Furthermore, this finding is consistent with results earlier reported by Del Prete et al. [46] showing that IL-10 inhibited antigen-specific proliferation and IFN- γ production.

The induced shift from Th2 to Th1 cytokines by VIT might be achieved by the high cumulative doses of insect venom administered during immunotherapy. This hypothesis is supported by the report that cytokine patterns change in response to the antigen concentration in phospholipase A₂-specific T cell clones [47]. Furthermore, the cytokine profile was also modulated by the involvement of different antigen-presenting cells [48]. Therefore, another important factor that could influence cytokine synthesis in allergen-specific T cells may be the route of allergen exposure. As VIT involves subcutaneous injections of bee or wasp venom instead of an intracutaneous insect sting, cutaneous mast cell-derived cytokines, especially IL-4 [49, 50], may be less involved and the allergen may be processed by different antigen-presenting cells, *e.g.* macrophages in the subcutaneous tissue versus dendritic cells in the epithelium.

Besides the described changes in cytokine expression, VIT also induced a reduced expression of CD40 and its ligand. This reduced expression may interfere with isotype switching in B cells, leading to a further decrease of IgE production and potentially preventing stronger increases in IgG production [24, 51]. On the side of the T cell, decreased CD40 and CD40L expression may contribute to reduced IL-4 production, as the CD40/CD40L pathway has been implicated in preferential IL-4 induction in T cells by B cells [52]. The observed changes in CD40 and CD40L expression were not due to changes in the composition of different cell types after VIT, as CD3, CD4, CD8, CD45RA, CD45R0, and MHC class II expression were not altered.

Overall, VIT induces a shift in cytokine production from Th2 to Th1 cytokines, and also induces the production of the immunosuppressive cytokine IL-10, inhibiting the potentially exaggerated Th1 responses.

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