

Early markers for protective mechanisms during rush venom immunotherapy

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Abstract

Background: Allergen-specific venom immunotherapy (VIT) represents the only rational-based option to treat allergic sensitizations against bee and wasp venom. So far, there is not much knowledge about early induction of protective and tolerogenic pathways during VIT.

Objectives: To identify the earliest markers for protective mechanisms against allergic reactions in the peripheral blood during the build-up phase of VIT.

Methods: PBMC and monocytes were isolated, and serum samples were taken before and during a five day build-up phase from 65 hymenoptera venom allergic patients. Expression level of tolerogenic markers was analyzed on mRNA and protein level. Serum levels of different soluble tolerogenic factors were measured.

Results: We observed significantly enhanced tryptophan degradation, elevated ILT4 expression of monocytes as well as IL-10 production of CD3⁺ T cells only a few hours after the first injection on day 1, followed by increased IL-10 serum levels, monocyte apoptosis and elevated intracellular cAMP levels of monocytes on day 3 combined with a higher ILT3 protein expression and IL-10 secretion of monocytes on day 5.

Conclusion: From these data, we conclude that tryptophan depletion, ILT3/4-mediated inhibition, higher IL-10 production as well as intracellular cAMP might contribute to early induction of protective mechanisms against allergic reactions during the build-up phase of VIT.

Allergen-specific immunotherapy with hymenoptera venoms (VIT) represents a well-established long time treatment of hymenoptera venom allergy to avoid life-threatening anaphylaxis (1). Mechanisms related to VIT classically include tolerance induction in the periphery, which comprises of immunological ignorance of an antigen, deletion by induction of apoptotic pathways, inhibition – achieved by upregulation of inhibitory co-receptors as well as induction of anergy and regulatory T cells (2). Furthermore, a fine tuning of co-stimulatory and co-inhibitory signals mediated in part by CD80 or CD86, the inducible costimulator ligand (ICOS-L) and mole-

cules of the immunoglobulin-like transcript (ILT) family expressed by antigen presenting cells (APCs) play an important role in deriving the nature of outcoming immune responses (3, 4). Beyond, the composition of the microenvironment, i.e. excess of soluble factors promoting tolerogenic properties of cells such as IL-10 or TGF- β as well as reduction of factors in the microenvironment, which support T-cell activation and proliferation or promote upregulation of tolerogenic surface markers by APCs might direct a pro-tolerogenic state and attenuate overactivation of T cells. The classical soluble mediators of tolerogenic mechanisms are interleukin IL-10 and TGF- β , both secreted by regulatory T-cell subtypes (5, 6). Further on, development of CD4⁺ CD25⁺ T cells, one of the best characterized regulatory T cell subtypes, is controlled by the forkheadbox protein 3 (Foxp3) transcription factor (7). The enzyme indoleamine 2,3-dioxygenase (IDO) degrades the amino acid tryptophan.

Abbreviations

APC, Antigen presenting cell; DC, Dendritic cell; Fc ϵ RI, High affinity receptor for IgE; IDO, Indoleamine 2,3-dioxygenase; IgE, Immunoglobulin E; IL, Interleukin; Kyn, Kynurenin; SIT, Specific immunotherapy; Try, Tryptophan; VIT, Venom immunotherapy.

Tryptophan starvation induced by high IDO activity has been demonstrated to suppress T-cell responses leading to anergy (8) as another mechanism associated with the induction of tolerance. It is very likely that a complex sequence of events leading to the induction of protective mechanisms early during VIT belongs to a sophisticated network, which consolidates the establishment of allergen-specific tolerance (9).

However, markers for early induction of protective mechanisms and tolerance during VIT have not been elucidated in detail so far. Mainly, tolerance during VIT develops in two steps: Protective mechanisms leading to nonresponsiveness to the allergen the individual is sensitized to occur at very early time points, i.e. during the build-up phase. Later on, allergen-specific tolerance develops during the maintenance phase and persists for years, even after the treatment is stopped. Most of our knowledge on induction of T cell anergy and allergen-specific tolerance derive from later phases of VIT, such as the maintenance phase and after termination of VIT (10–12). However, insights into the very early immunologic changes already during the build-up phase, which might operate against severe allergic reactions at this initial state are rare. In this context, it has been shown very recently, that inducible type 1 regulatory T cells, which produce IL-10 were detectable at the end of the build-up phase (13).

However, detailed knowledge about mechanisms protecting the individual from allergic reactions at this early stage of treatment would be important to optimize build-up schemes, reduce unwanted side reactions and putatively identify or discriminate individuals with suboptimal clinical responses. Therefore, it was the aim of this study to identify pathways impacting on markers of early protection during the build-up phase of VIT.

Material and methods

Characterization of patients

Venom immunotherapy was applied to 65 patients (mean age 49 ± 13 years, age range: 18–74 years, 29 women, 36 men; mean total serum IgE 180 ± 195 kU/l) sensitized to venom from honey bee ($n = 10$, mean specific serum IgE 5.2 ± 14.5 kU/l) and wasp ($n = 55$, mean specific serum IgE 20.3 ± 26.6 kU/l) according to the rush protocol (ALK-lyophilisiert SQ, ALK Scherax, Hamburg, Germany) (Table 1) (14). All patients had a history of a systemic anaphylactic reaction to a hymenoptera sting. Clinical symptoms and severity of anaphylactic reactions to hymenoptera sting were evaluated with a standard questionnaire. All patients

received an H1R-antagonist (1 tablet of 10 mg cetirizine) every morning from day 2 to day 5, up to thrice daily, if required. After informed consent, peripheral venous blood was obtained immediately before VIT, a few hours after onset of VIT on the first day as well as after the last injection every day during the 5 days lasting build-up phase. The protocol was approved by the local ethics committee. None of the patients developed any moderate or severe side reactions despite transient local erythema or swelling at the injection site.

Reagents

F(ab')² goat-anti mouse IgG (GaM-IgG) was from Jackson Immuno-Research Laboratories (West Grove, PA). Monoclonal antibody (mAb) against CD80 (mIgG1)(L307.4), CD86 (mIgG1)(2331), CD83 (mIgG1)(HB15e), MHC I (mIgG)(G46-2.6), MHC II (mIgG2a)(G46-6) and Phycoerythrin (PE)-labeled mAb against CD14 (mIg2a)(M5E2), PE-labeled mAb against CD3 (mIgG)(UCHT1) and PECy5-labeled mAb against CD16 (mIgG)(3G8) were from BD Biosciences Pharmingen (Heidelberg, Germany), unconjugated mAb against B7H1 (mIgG)(MIH1) was from eBiosciences (San Diego, USA), unconjugated mAb against B7H2 (mIgG2b)(136716), unconjugated mAb against ILT3 (mIgG2a)(293623) and unconjugated mAb against ILT4 (mIgG2a)(287219) were from R&D systems (Wiesbaden, Germany). Normal mouse and rabbit serum for blocking purposes was obtained from Dianova (Hamburg, Germany), and 7-amino-actinomycin-D (7-AAD) was from Sigma Aldrich (Taufkirchen, Germany). All other reagents were obtained from Sigma Aldrich unless otherwise indicated.

Preparations and cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinised blood by density gradient centrifugation as described in detail elsewhere (15). Monocytes were isolated from peripheral blood as described before (16). Purity of monocytes was assessed by flow cytometric staining.

Cell surface and intracellular phenotyping of monocytes by flow cytometry

Extracellular and intracellular staining of freshly isolated monocytes before VIT, a few hours after the onset of VIT on day 1 and after the 3rd day and 5th day of the build-up phase of rush immunotherapy was performed as reported in detail elsewhere (15).

Table 1 Dosing schedule of the build-up phase of VIT

Day	d1	d1	d1	d1	d1	d2	d2	d2	d2	d3	d3	d4	d4	d5	d5
Concentration in (µg/ml)	0.1	0.1	0.1	1	1	1	10	10	10	10	100	100	100	100	100
Applied dose in ml	0.2	0.4	0.8	0.2	0.4	0.8	0.2	0.4	0.6	0.8	0.1	0.4	0.6	0.8	1

Annexin-V staining

Evaluation of apoptotic cells was performed using the Annexin-V staining kit according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA). Flow cytometry was carried out using a FACS-Canto (BD Biosciences, Heidelberg, Germany) and analyzed by FACSDiva (BD Biosciences) and FLOWJO (TreeStar Inc., Ashland, OR, USA) software.

Measurement of IL-10 and enzymatic activity of indoleamine 2,3-dioxygenase in the sera

Amount of IL-10 in the sera and cell culture supernatants of the patients was analyzed with the help of the HS IL-10 ELISA (cut off 0.5 pg/ml) and the IL-10 Duo Set (R&D Systems) according to the manufacturer's instructions. Enzymatic activity of IDO *in vivo*, tryptophan (TRP) and kynurenine (KYN) concentration in plasma samples was determined using a modified HPLC method as described before (17). Accordingly, TRP was determined at +850 mV and 50 nA and KYN at +850 mV and 10 nA.

Isolation of IL-10 secreting cells

IL-10-secreting cells were enriched from PBMC isolated before and after the first day, the 3rd day and the 5th day of the build-up phase of rush immunotherapy as described in the manufacturer's manual of the IL-10 Secretion Assay, Miltenyi Biotec, and the percentage of CD14- and CD3-expressing cell subfractions was evaluated by flow cytometric staining with direct conjugated antibodies and analyzed using the FACS-Canto.

Real Time PCR

mRNA was isolated with the help of the RNeasy Mini kit (Qiagen, Hilden, Germany) including digestion of genomic DNA and was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified using TaqMan assay Master Mix (Applied Biosystems) according to the recommendations of the manufacturer in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primers were as follows: IL-10 (Hs00174086_m1), TGF- β (Hs00998129_m1), IL-4 (Hs00174122_m1), IFN- γ (Hs00989291_m1), IDO (Hs00158027_m1), B7H1 (Hs01125299_m1), B7H2 (Hs00391287_m1), FAS (Hs00531110_m1), FAS-L (Hs00899442_m1), IL-T3 (Hs00429000_m1), ILT2/T4 (Hs00601427_g1), Foxp3 (Hs01085835_m1). All primers as well as endogenous controls (18s) were from Applied Biosystems. All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed using the comparative $\Delta\Delta CT$ methods (18). All analyses were conducted in duplicates, and experimental samples were quantitated using sample dilutions insuring that the Ct values were in the linear proportion of the standard curve. Expression was calculated as relative expression normalized to baseline mRNA expression before onset of VIT.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Quantitative values were compared by nonparametric testing (Wilcoxon). Results are given in mean $\pm$ standard error of the mean (SEM), respectively. Any *P*-values are two-sided and subject to a significance level of 5%.

Results

Upregulation of IL-10, IDO, ILT3, TLR4 and FAS-L mRNA expression of PBMC during the build-up phase of VIT

Screening for changes of mRNA expression of several important markers, which have been shown to be associated with induction of protective or tolerogenic mechanisms of PBMC isolated from the peripheral blood of patients during the build-up and early maintenance phase of VIT revealed significant upregulation of IL-10 mRNA expression on day 2 (Fig. 1A), followed by increased pro-apoptotic FAS-L mRNA expression on day 4 (Fig. 1I) and elevated mRNA expression of the inhibitory surface receptors ILT3 and ILT2/T4 (Fig. 1G and H) as well as the tryptophan degrading enzyme IDO (IDO) on day 5 of VIT (Fig. 1F) compared to mRNA expression at baseline before onset of VIT. In addition, slight upregulation of IL-4 and TGF- β mRNA expression of PBMC was observed on day 5 (Fig. 1B and C), while increased mRNA of the forkhead box 3 (Foxp3) transcription factor characteristic for regulatory T cells as well as the Th1 cytokine IFN- γ was observed at later time points after the build-up phase, i.e. from day 10 of VIT (Fig. 1D and E).

Tryptophan degradation increases from the first day of the build-up phase of VIT

To correlate the early changes during VIT observed on the mRNA level of PBMC with modifications on the protein level, we performed several additional functional analyses of serum and cell samples. Tryptophan degradation initiated by the enzyme IDO is linked to the suppression of T cells responses and induction of tolerance in numerous immunologic mechanisms *in vitro* and *in vivo*. We observed significantly upregulated tryptophan degradation, mirrored by an increase of its metabolite kynurenine and decrease of tryptophan in the sera from the first day of the build-up phase only a few hours after the first allergen injection, as one of the earliest pro-tolerogenic mechanisms detectable during VIT (Fig. 2).

IL-10 serum levels increases from the 2nd day of the build-up phase of VIT

One of the most important soluble mediators of anti-allergic and protective mechanisms as well as tolerance is the cytokine IL-10. Therefore, we evaluated IL-10 protein in the sera of patients during the build-up phase of VIT and subtypes of

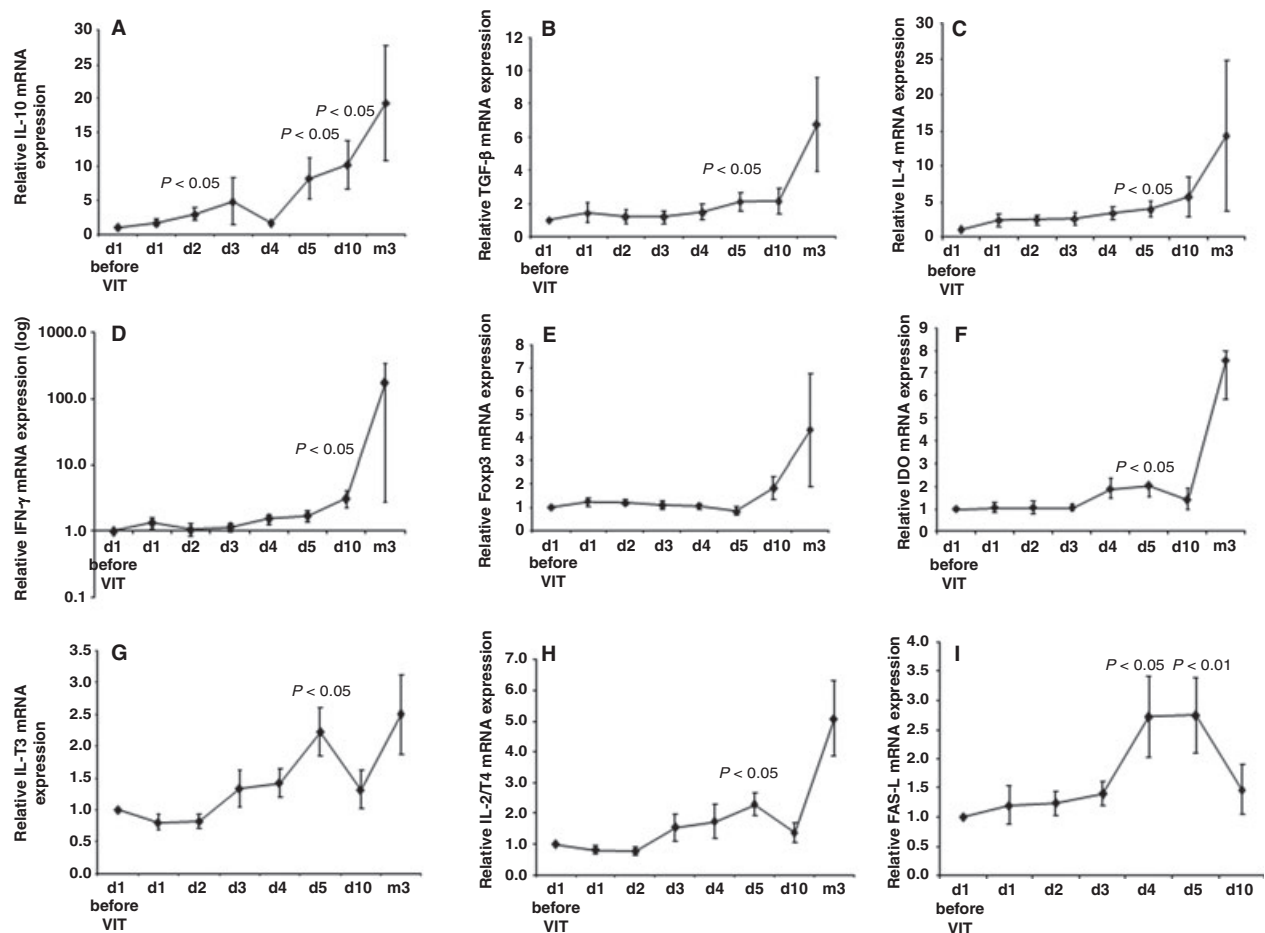


Figure 1 IL-10 mRNA expression of PBMCs increases on day 2, FAS-L mRNA on day 4 and ILT3, ILT2/T4 and IDO mRNA expression on day 5 of VIT. Mean values of relative mRNA expression

normalized to day 1 before SIT $\pm$ standard error of the mean (SEM) of $n = 11$ experiments is shown.

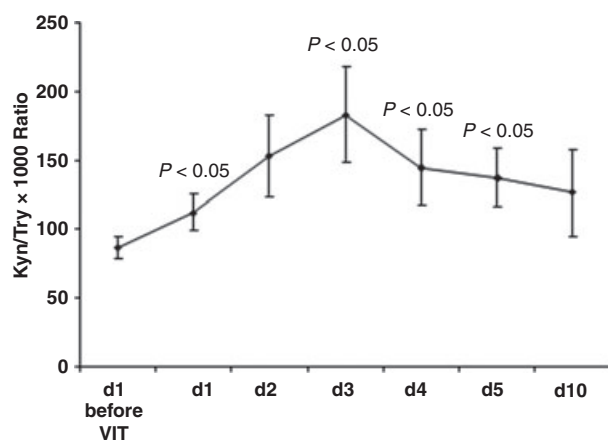


Figure 2 Enhanced tryptophan degradation in the sera of patients under VIT starts on day 1. Mean value of tryptophan (Trp)/Kynurenine (Kyn) ratio $\pm$ SEM of $n = 10$ experiments as an indicator for the activity of the tryptophan degrading enzyme IDO is depicted.

IL-10 secreting cells. We observed significant upregulation of IL-10 serum levels from day 3 of the build-up phase of VIT (Fig. 3A). Using IL-10 secretion assays, we could identify an early increase of IL-10 producing CD3⁺ T cells already on day 1 after the first injections, followed by an elevated IL-10 production by CD14⁺ monocytes in the peripheral blood of patients under VIT at day 5 (Fig. 3B and C).

Monocytes undergo higher apoptosis during the early build-up phase

Deletion and selective apoptosis of distinct cell subfractions or activated cells represents another way of tolerance induction by deletion. Therefore, we evaluated factors involved in the induction of programmed cells death of peripheral blood cells of patients under VIT. We found increased apoptosis of monocytes evaluated by Annexin-V staining on day 3 of VIT (Fig. 4A and B). However, CD95 and CD178 surface expression of monocytes did not change significantly (data not shown).

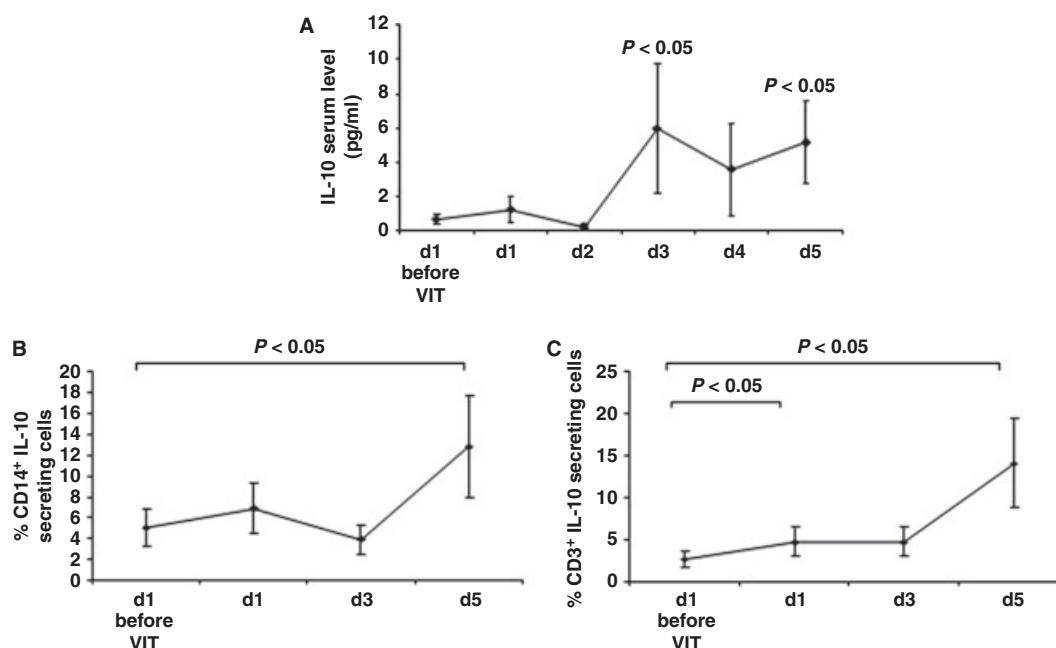


Figure 3 IL-10 serum level and percentage of IL-10 secreting CD3⁺ and CD14⁺ cells increases from day 3 of the build-up phase of VIT. (A) Mean value of IL-10 serum levels $\pm$ SEM of $n = 13$

donors as well as mean values $\pm$ SEM of the percentage of IL-10 secreting CD3⁺ cells (B) and CD14⁺ cells (C) for $n = 8$ experiments are shown.

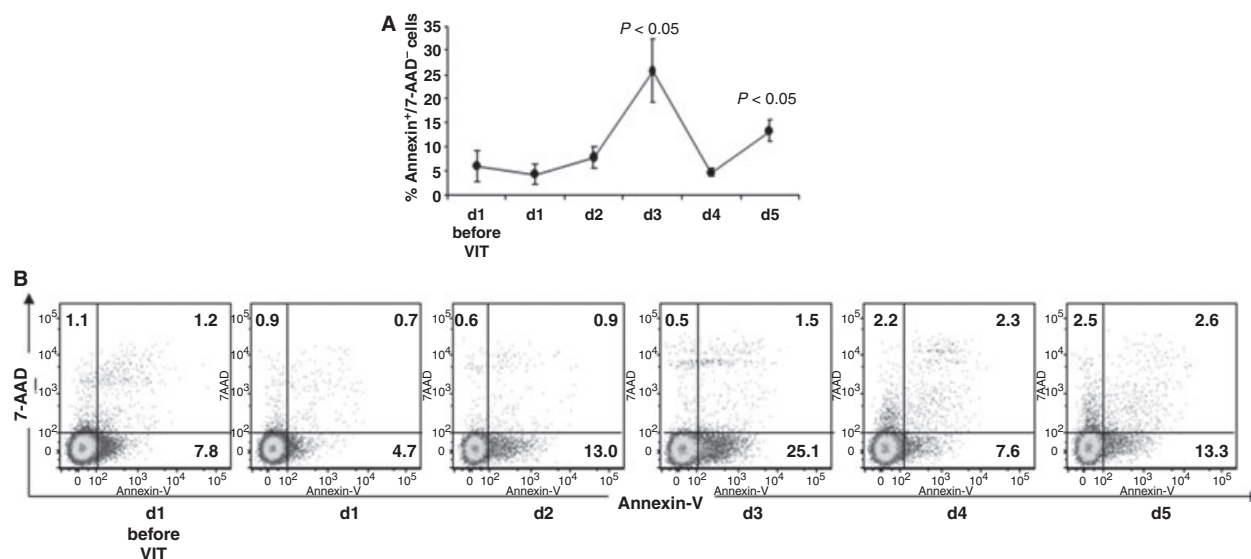


Figure 4 Annexin-V expression of monocytes is upregulated on day 3 and day 5 of VIT. (A) Mean value of percentage of Annexin-V⁺/7-AAD⁻ monocytes $\pm$ SEM of $n = 5$ experiments is shown. (B)

Representative dot plots of Annexin-V expression (log fluorescence index; x-axis) and 7-AAD expression (log fluorescence index; y-axis) are shown; percentage of positive cells is depicted in the corner.

ILT3/ILT4 protein expression of freshly isolated monocytes increases

ILT3 and ILT4 are inhibitory receptors, which negatively regulate activation of APCs and exert immunosuppressive activity on T cells. Therefore, we evaluated next ILT3 and ILT4 protein expression of monocytes of patients under VIT.

We found significant upregulation of monocytes' ILT4 expression on day 3 and ILT3 protein expression on day 5 of VIT (Fig. 5A and B). Concomitantly, expression of B7H1 and CD86 on monocytes decreased during the first 5 days of VIT (Fig. 5C and D), while no change of B7H2 and CD80 expression as well as MHC I and MHC II expression was observed (data not shown).

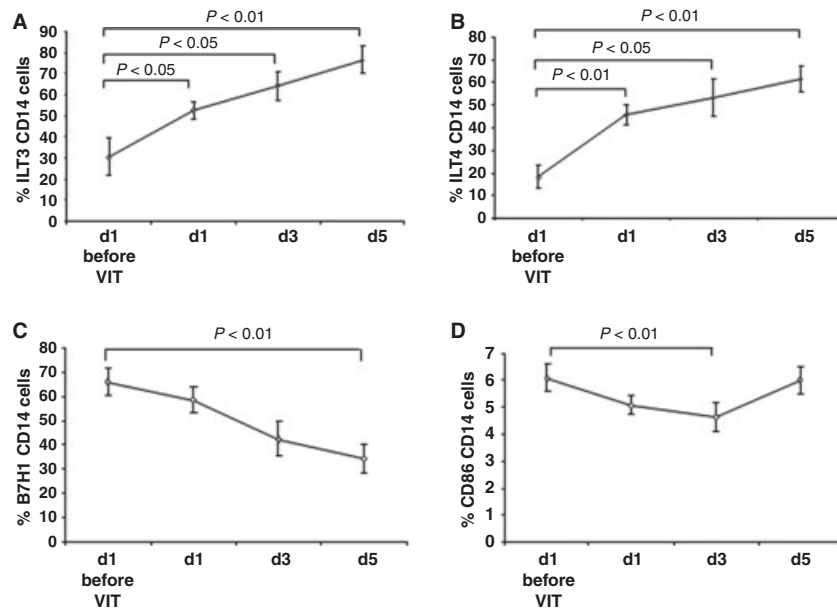


Figure 5 ILT3 and ILT4 surface expression of monocytes increases continuously during the build-up phase of VIT. Mean value $\pm$ SEM of percentage of ILT3 (A) and ILT4 (B) as well as

B7H1 (C) and CD86 (D) positive monocytes for $n = 16$ experiments is depicted on the y-axis, and time is shown on the x-axis.

cAMP levels in monocytes increases during the build-up phase of VIT

Increase of the second messenger cAMP has been described in the context of suppressive immune mechanisms. Therefore, we evaluated cAMP levels of monocytes enriched from the peripheral blood of patients under VIT. We observed significant upregulation of monocytes' cAMP levels on day 3 and 5 of VIT (Fig. 6).

Discussion

Mechanisms responsible for early protection against allergic reactions during VIT are of particular interest for the identification of target cells and putative markers for efficacy of VIT, mirrored by the establishment of stable allergen-specific tolerance. However, despite excellent clinical outcome of VIT in many, but not in all patients with insect venom allergy, the underlying molecular mechanisms are far from being completely understood (19). Induction of allergen-specific T cells is one of the major pathways supposed to go along with the development of allergen-specific tolerance and increase of regulatory T cells is detectable after 6 months of treatment in parallel with the increase of the venom-specific IgG4/IgE ratio (20). However, at present, there are no reliable markers available which can be used as parameters to appraise the efficacy of VIT in terms of a safety-risk assessment at early stages, i.e. the build-up phase, when most of the unwanted side reactions take part.

We could show that enhanced consumption of tryptophan by the enzyme IDO and resulting change of the micro-environment and availability of metabolites of this pathway such as kynurenine in the peripheral blood occurs as one of

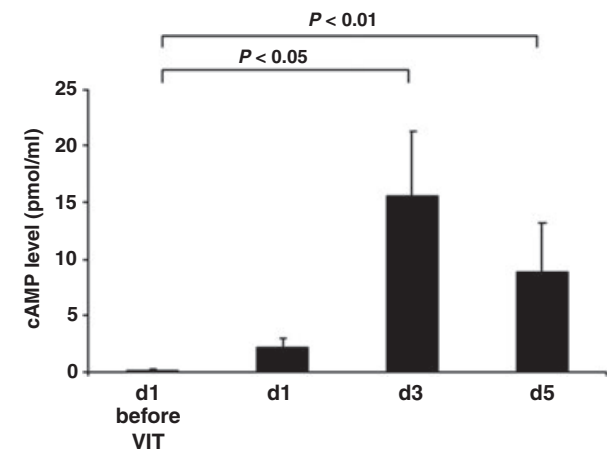


Figure 6 Intracellular cAMP levels of monocytes increases on day 3 and day 5 of the build-up phase of VIT. Mean values of intracellular cAMP levels $\pm$ SEM of monocytes purified from the peripheral blood during the build-up phase of VIT for $n = 9$ experiments are shown.

the earliest events known to contribute to the induction of peripheral tolerance after the first allergen injection on day 1 of therapy. IDO is widely expressed in human tissue and by a high number of cell subsets including monocytes and regulatory DC subtypes (8). Upregulated activity of IDO has been identified as a key mechanism of acquired tolerance originally described for the initiation of materno-fetal tolerance in the placenta (21). Therefore, increased IDO activity and tryptophan degradation could represent one of the primary steps of suppression of T cell subtypes during the build-up phase of VIT. It is of notice that increased

tryptophan degradation occurs already a few hours after the first injections, while IDO mRNA expression increases with some delay on day 4. This might be because mRNA expression does not directly correlate with enzyme activity. Reduced level of tryptophan during *in vitro* culture of monocyte-derived DCs has been demonstrated to promote the generation of DCs with tolerogenic properties and high expression of the inhibitory receptors ILT3 and ILT4, which as a next step favor the induction of CD4⁺CD25⁺Foxp3⁺ regulatory T-cell subtypes (22). Upregulation of co-inhibitory surface molecules such as ILT has been observed on regulatory APC subtypes and is suspected to be involved in suppression of T cell responses (23). These APC are capable of transforming alloreactive T cells into regulatory T cells. An important soluble factor which upregulates ILT3 and ILT4 expression on DCs and endothelial cells is IL-10 (23, 24). In regard to the increase of IL-10 in the blood during the initial phase of VIT together with the reduced availability of tryptophan because of accelerated tryptophan degradation, both mechanisms together might contribute to the upregulation of ILT3 and ILT4 on monocytes and augment their protective properties during the build-up phase of VIT observed in this study.

In line with previous reports, we observed a sequential upregulation of IL-10 production starting with T cells and followed by monocytes later on (25). IL-10 belongs to the class 2 alpha-helical cytokines and mediates several protolerogenic pathways, such as induction or prevention of apoptosis, inhibition of cell growth and modulation of inflammatory immune responses (26). Its pivotal role in the induction of anergy of peripheral T cells in the context of allergen-specific immunotherapy or under physiologic circumstances of tolerance induction as well as tolerogenic APCs (3) has been clearly proven (25, 27). In addition, our data are in accordance with recent data demonstrating induction of IL-10 producing regulatory T cells during the build-up phase of SIT (13), pointing to a crucial role of IL-10 during this early phase.

Another mechanism which assigned to the induction of protective mechanisms and peripheral tolerance is directed deletion of specific cell subtypes via induction of programmed cell death. A temporarily enhanced rate of apoptosis of monocytes observed on day 3 of the built-up phase might account for this effect. However, despite upregulation of FAS-L mRNA expression in PBMC, no significant change of CD95 and CD178 (FAS-L) expression of purified monocytes was detected on the protein level. Additionally, no changes of soluble FAS-L levels in the sera of the patients were observed by ELISA (data not shown).

One of the thoroughly studied suppressive pathways of different cell types is the signal transduction of cyclic adenosine

3', 5'- monophosphate (cAMP). Binding of ligands to the receptor connects the ligand-receptor complex with cellular guanine nucleotide binding protein (G protein), activating adenylate cyclase, which induces the production of the second messenger cAMP (28). Both, adenosine and cAMP have been demonstrated to exert suppressive properties, in part by the inhibition of NF- κ B-mediated downstream signaling of immune receptors (29). Elevation of cAMP is linked to inhibition of cellular proliferation, differentiation and cytokine secretion (6, 30). Therefore, upregulation of intracellular cAMP levels in monocytes during VIT might contribute to a plethora of immunosilencing mechanisms and impact on the differentiation of APCs from these precursors, because cAMP was demonstrated to modulate functional plasticity of immature DCs (30). Because treatment with H1 antihistamines has been demonstrated to improve the efficacy and safety of VIT (31), it cannot be excluded that antihistamine medication applied from the first day of VIT to the patients in this study, impacts on the changes of protective markers observed here, too.

However, because so far no reliable serum parameter exists, which serves as a clear indicator for a protective effect of VIT, it cannot be excluded that part of the changes observed here might represent normal responses to the injections and do therefore not exclusively represent markers for a positive (i.e. protective) response. To finally answer the question whether the changes observed really represent early markers for protection, the changes of serum factors have to be directly correlated with the outcome if VIT and a positive protective effect should be proven by exposition to sting challenges or a field sting.

In view of the data summarized here, we demonstrate that reduction of tryptophan blood levels already a few hours after the first injection combined with an increase of IL-10 blood levels during the build-up phase represent earliest markers for tolerance induction, which might promote T3 and ILT4 from day 2 of VIT on monocytes as well as higher cAMP levels of these cells from day 3 of VIT, enforcing their tolerogenic and suppressive properties on immune responses and contributing to the development of protective mechanisms against allergic reactions during early stages of VIT.

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