
Changes in gene expression caused by insect venom immunotherapy responsible for the long-term protection of insect venom-allergic patients

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Background: Insect venom immunotherapy (VIT) is the only causative treatment of insect venom allergy (IVA). The immunological mechanism(s) responsible for long-term protection achieved by VIT are largely unknown. A better understanding is relevant for improving the diagnosis, prediction of anaphylaxis, and monitoring and simplifying treatment of IVA.

Objective: To find genes that are differentially expressed during the maintenance phase of VIT and after stopping, to get clues about the pathways involved in the long-term protective effect of immunotherapy.

Methods: Whole genome gene expression analysis was performed on RNA samples from 50 patients treated with VIT and 43 healthy controls. Patients were divided into three groups: (1) before the start of VIT; (2) on maintenance phase of VIT for at least 3 years still receiving injections; and (3) after VIT.

Results: Of all 48,804 probes present in the array, 48,773 transcripts had sufficient data for further analysis. The list of genes that were differentially expressed (at least $\log_2$ FC > 2; P < .05 corrected for multiple testing) during the maintenance phase of VIT as well as after successful VIT contains 89 entities. The function of these genes affects cell signaling, cell differentiation, and ion transport.

Conclusion: This study shows that a group of genes is differentially expressed both during and after VIT in comparison with gene expression in patients before VIT. Although the results of this study should be confirmed prospectively, the relevance of these findings is supported by the fact that they are related to putative mechanisms of immunotherapy.

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INTRODUCTION

Allergic reactions to insect venom allergens may vary from mild systemic dermal reactions to life-threatening anaphylactic shock. Insect venom immunotherapy (VIT) is able to prevent systemic reactions in up to 97% of treated patients.^{1–4} Insect venom immunotherapy has 2 phases: a buildup phase, in which increasing amounts are administered until the maintenance dose of allergen has been reached, and a maintenance phase, in which the dose is administered every 4 to 6 weeks. Depending on the protocol, the buildup phase lasts from 1

day (so-called ultra rush) to 15 weeks. At reaching maintenance dose, in most patients the risk of a systemic reaction to a subsequent sting is reduced from 70% (in this patient group before the start of VIT) to 3%.³ The maintenance phase has to be continued at least 3 years (in patients with mild systemic reaction, grade I–III Mueller) to 5 years (in patients with anaphylaxis, grade IV Mueller), enabling a long-time (possibly lifelong) prevention of anaphylactic reactions after stopping VIT in most patients.^{2,4}

The changes that occur during allergen immunotherapy differ between the initial buildup phase and during maintenance dosing. Several theories describe the mechanism of the early phase of immunotherapy^{5–9}; however, few data are available on the mechanisms responsible for long-term allergen tolerance.^{2,3,5} Insect venom immunotherapy seems to be a good model for studying the long-term effect of allergen immunotherapy (SIT), because of the clear symptomatology caused by *Hymenoptera* stings and the high efficacy of VIT, and because in other allergic diseases such as asthma, allergic rhinitis, or atopic dermatitis, the pathological condition is more complex. Studies on the immunological background of long-term protection induced by VIT may indicate new pathways that could be a target for further improvement of immunotherapy, or a combined pharmacological intervention, or it may help to find parameters to monitor the process of induction of tolerance.

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The aim of this study was to evaluate genes that are differently expressed during the maintenance phase of VIT and in the follow-up of the treatment after stopping VIT when long-term protection is achieved, to get clues about the pathways involved in the long-term effect of immunotherapy.

METHODS

Patients

Fifty patients treated with VIT in the Department of Allergology of the University Medical Center of Groningen were studied. Inclusion criteria were the diagnosis of IVA based on medical history (grade III or IV according to Mueller¹⁰ before VIT), positive skin tests, or sIgE. Insect venom allergy was diagnosed according to European Academy of Allergy and Clinical Immunology.² All patients except one who was bee allergic suffered from yellowjacket venom allergy. Exclusion criteria were lack of consent, pregnancy, severe chronic or malignant disease, or mastocytosis. Screening for mastocytosis was made using urinary N- τ methyl-histamine level or serum tryptase measurement. None of the subjects suffered from urticaria pigmentosa. Among co-morbidities, mild asthma (12%) and benign hypertension (20%) were the most prevalent. Other diseases were found in single patients.

Patients started VIT in the clinic, receiving 6 injections with increasing amounts of venom starting with 0.0001 μ g and reaching 10 μ g Pharmedgen *Vespula* Spp., respectively, *Apis Mellifera* (ALK; Abello, DK), and continued with Alutard *Vespula* Spp., respectively; *Apis Mellifera* (ALK-Abello, DK) in the outpatient clinic with one injection per week, increasing the amount of venom over approximately 6 weeks. Subsequently, a maintenance dose of 100 μ g Alutard was given every 6 weeks for 3 to 5 years.

The patients were divided into 3 groups (Table 1). Group 1 consisted of 16 patients (6/37% men and 10/63% women; mean age 54 [range, 35–80]) before the start of venom immunotherapy; in this group the risk of a re-systemic reaction to the culprit insect is estimated at approximately 70%.^{1,3,11} Group 2 consisted of 17 patients (6/35% men and 11/65% women, mean age 55 [range, 21–75]) still receiving

injections during the maintenance phase of VIT for at least 3 years. The risk of a re-systemic reaction to the sting by the relevant insect is lowest in this group and is estimated as 2%.^{1,3,4} The treatment period is considered to be long enough to induce long-term immunological memory.^{1,3,11} Group 3 consisted of 17 patients (9/53% men and 8/47% women; mean age 52 [range, 28–70]) with achieved and maintained long-term protection after VIT.^{1,3,11} They all underwent venom immunotherapy (mean duration of VIT was 3.15 years), and all were stung in the field at least twice (few patients had also sting challenges) after completing VIT and never experienced a systemic reaction after re-stinging with the relevant insect. The field sting occurred months or even years before the gene expression analysis and was tolerated by the patients. Neither systemic side effects during VIT, nor any other symptoms that could be a sign of ineffectiveness of VIT, were seen.

Patients from groups 2 and 3 were also included in the analysis of the long-term efficacy of VIT, which has been published previously.¹¹

The control group consisted of 43 healthy subjects (22 [51%] men, 21 [49%] women; mean age 47.7 [range, 19–73]). They were nonrelated partners from patients with inflammatory bowel disease visiting the outpatient department of the inflammatory bowel disease unit of the University Medical Center of Groningen. No data regarding intolerance of the insect sting were available in this group.

Collection of Blood Samples

An RNA sample was collected using the PAXgene Blood RNA Tubes (Qiagen, Venlo, The Netherlands). All tubes were immediately frozen and stored in -20°C until RNA isolation (maximal period, 2 months). The RNA was isolated by using PAXgene Blood RNA Kit CE (Qiagen). All RNA samples were stored in -80°C until labeling and hybridization.

The quality and concentration of RNA was determined by using a 2100 Bioanalyzer (Agilent, Amstelveen, The Netherlands) and the Agilent RNA 6000 Nano Kit. Samples with

Table 1. Demographic and Clinical Patient Data

	Before VIT (group 1)	Maintenance phase of VIT (group 2)	Long-term protection (group 3)
No. of subjects (n)	16	17	17
Age (years, [range])	54 [35–80]	55 [21–75]	53 [28–70]
Sex male/female (%)	37/63	35/65	56/44
Years of VIT (n [SD])	0	4 (0.8)	3.15 (0.6)
Yellowjacket/bee allergy (%)	100/0	100/0	94/6
sIgE yellowjacket (kU/L mean [SD])	19.5 (27)	4.2 (4.7)	5.7 (7)
sIgE honeybee (kU/L mean [SD])	1.17 (3.3)	0.3 (0.5)	0.2 (0.5)
Tryptase (ng/mL mean [SD])	6 (3.4)	2.2 (4.3)	—
Methylhistamine in urine ($\mu\text{m}/\text{mkrea}$; mean [SD])	104 (41)	107 (41)	94 (36)
Asthma (n [%])	2 (13%)	2 (12%)	2 (12%)
Hypertension (n [%])	5 (31%)	4 (24%)	1 (6%)
N of re-stings after VIT mean (range)	—	—	5 (2–30)

RNA integrity number greater than 7.5 were used for further analysis on expression arrays.

Gene Expression

For amplification and labeling of the RNA with the Illumina TotalPrep 96 RNA Amplification Kit (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands), 200 ng of RNA from each sample was used. The Human HT-12_V3_ expression arrays (Illumina, San Diego, California) were processed according to the manufacturer's protocol. Slides were scanned immediately using Illumina BeadStation iScan, (Illumina).

Image and Data Analysis

First-line check, background correction, and quantile normalization of the data were done with Genomestudio Gene Expression Analysis module v 1.0.6 Statistics (Illumina). Entities in which at least 75% of samples had a signal intensity value above 20th percentile in 100% of the samples of at least 2 groups were included in further analysis.

Data analysis was performed using GeneSpring package version 8.0.0 (Agilent Technologies Santa Clara, California) according to the manufacturer's instruction. Genes for which expression was significantly different between compared groups were chosen based on a log₂-fold change of greater than 2 in gene expression, *t* test *P* < .05, and corrected for multiple testing by a Benjamin-Hochberg method (*P* < .05). Functional annotation of genes was described using Genecodis functional annotation Web-based tool.¹² Clinical data for this study were analyzed with Statistica 8.0 (StatSoft, Tulsa, Oklahoma). All patients gave their written informed consent. The study was approved by the Medical Ethical Committee of the UMCG (METc 2008/340).

RESULTS

Whole genome gene expression analysis was performed on RNA samples isolated from all blood cells in whole blood. From all 48,804 probes present in the array, 48,773 transcripts had sufficient data for further analysis.

Comparison of Gene Expression Profiles between the Different Groups

Comparing group 1 versus 2, a log₂-fold change difference greater than 2 in gene expression was found in 1,047 transcripts. A significant difference corrected for multiple testing was found in 593 transcripts analyzed. Comparing group 1 versus 3, a log₂-fold change difference greater than 2 in gene expression was found in 1,168 transcripts; a significant difference corrected for multiple testing was found in 649 transcripts analyzed. Comparing group 2 versus 3, a log₂-fold change difference greater than 2 in gene expression was found in 998 transcripts; a significant difference corrected for multiple testing was found in 572 transcripts analyzed.

Functional Annotation of Differently Expressed Genes

Functional annotation was assigned by Genecodis¹² (Table 2). In all compared groups, the main functions of the differentially expressed genes were related to multicellular organism development, cell differentiation, transcription, signal transduction, ion transport, cell proliferation, cell cycle, cell-cell signaling, and lipid metabolic processes.

The set of genes as described by Konno et al.^{8,9} in patients treated with VIT related with osteopontin pathway, expressed in monocytes, was analyzed. However, we only observed a difference in gene expression for interleukin receptor-associated kinase-1 binding protein 1 *IRAK1BP1* (*P* = .03).

Table 2. Gene Co-occurrence Annotation Found by Genecodis (GOSlim Process Function) for the Genes Differentially Expressed (log₂ FC > 2 and *P* < .05,) Comparing Patients before VIT with Those on Maintenance Phase (Group 2 v 1) and Patients Before VIT with Those Who Achieved Long-Term Protection (3 v 1)

Genes 2 V1	Hyp 2 V1	Hyp* 2 V1	Annotations	Genes 3 V1	Hyp 3 V1	Hyp* 3 V1
22	2.9e-06	4.3e-05	GO:0007275:multicellular organismal development (BP) NGR 874	17	0.002	0.03
14	1.3e-05	0.0001	GO:0030154:cell differentiation (BP) NGR 434	9	0.003	0.01
29	1.6e-05	8.3e-05	GO:0006350:transcription (BP) NGR 1516	22	0.01	0.03
26	0.001	0.004	GO:0007165:signal transduction (BP) NGR1700	27	0.002	0.02
3	0.001	0.004	GO:0007275:multicellular organismal development (BP) NGR 29	—	—	—
11	0.002	0.005	GO:0006811:ion transport (BP) NGR 503	9	0.03	0.04
7	0.005	0.009	GO:0008283:cell proliferation (BP) NGR 258	—	—	—
8	0.01	0.01	GO:0007049:cell cycle (BP) NGR 376	9	0.006	0.01
6	0.01	0.01	GO:0007267:cell-cell signaling (BP) NGR 237	6	0.01	0.03
5	0.04	0.04	GO:0006629:lipid metabolic process (BP) NGR 232	6	0.01	0.03

Abbreviations: NGR, number of annotated genes in the reference list; NG, number of annotated genes in the input list.

P values have been obtained through hypergeometric analysis (Hyp) corrected by FDR method (Hyp*).

In a recent publication by our group, the gene expression profile of patients who gained tolerance after VIT was compared with patients who were not protected from a sting.¹¹ Six of the 18 genes described were also differently expressed in a comparison of groups 1 and 3 (C16ORF13 $P = .04$; HS.583392 $P = .03$, HS.532515 $P = .01$, PRLR $P = .03$; TWIST 2 $P = .02$; CLDN1).

Genes with Similar Differences in Gene Expression between Group 1 Versus 2 and Group 1 Versus 3

To assess the relevance of genes related to the longitudinal effect of VIT, we evaluated whether genes that are differently expressed during VIT (group 1 vs. 2) are also differently expressed in patients who stopped VIT and achieved long-term protection (at least $\log_2$ FC > 2 significant difference in gene expression corrected for multiple testing). The list consists of 89 genes (Table 3). The finding that the expression of genes is changed during VIT and remains differently expressed in patients with long-term protection may indicate that these genes are responsible for the mechanism of long-term protection of VIT. A functional annotation of the genes is presented in Table 4. A hierarchical clustering dendrogram of differently expressed genes of the patient groups before VIT and on maintenance phase of VIT (group 1 vs. 2) is presented in Figure 1, between patients before VIT and with long-term protection of treatment (group 1 vs. 3) in Figure 2.

Comparison of Gene Expression between Patients Allergic to Insect Venom and Healthy Controls

In the first analysis, we compared patients before (group 1) and after treatment (groups 2 and 3). Among 89 entities with similar differences in expression, 10 were also differently expressed when comparing groups 1, 2, and 3 with controls (CCDC96, DIRAS3, DYRK1A, FGFR1, HS.146933, KLF10, LOC145820, NMB, OR5AR1, TSPYL2).

In the second analysis, we compared only the patients from groups 2 and 3 (those tolerating stings) with controls. That comparison showed that the difference in only 2 of the 10 genes (DYRK1A and TSPYL2) was significant for patients treated with VIT. Thus, we concluded that patients tolerating stings have more similar pattern of expression to controls.

DISCUSSION

For this study, we analyzed the RNA isolated from whole blood. Because the mechanism of venom immunotherapy is largely unknown and probably is related to more than 1 or 2 specific cells and processes, the advantage of this method is that it allows for evaluation of all possible processes. Another advantage of this standardized method is that it also reduces the effect of sample handling on the result and allows for analysis of gene expression profiles in clinical practice in the future. The comparison of patients at different stages of the treatment was chosen to avoid the influence of blood sample storage on gene expression level in a prospective study.

The results of the current study show that a number of genes are differentially expressed during (group 2) and after the maintenance phase of immunotherapy (group 3) as com-

pared with before treatment of VIT (group 1). This suggests that these genes may be related to the achieved (long-term) protection, as confirmed by the tolerance to accidental or deliberate re-stings in these patients.^{1,2,4} Of course, other genes from these 2 groups also may possess a relevant function, but the difference in their expression level did not reach the $\log_2 > 2$ -fold change cutoff used in the current analysis. This means that the analysis of the function of genes presented here probably only describes the “tip of the iceberg” of genes involved in the mechanism of the immunological long-term protection of VIT. The confirmation of the function of 6 of 18 genes previously described¹¹ differentiating patients with tolerance induced by VIT from patients with failure of VIT may indicate that gene profiling might be of help, indicating relevant genetic processes that are important for the long-term protective effect of VIT.

The group of genes with similar differences during VIT and in patients with long-term protection includes 89 transcripts. A comparison of the gene co-occurrence annotation found by Genecodis (Table 2) shows that similar processes play a role in group 1 versus 2 and 1 versus 3. These processes include (1) cell differentiation; (2) cell–cell interactions, signaling, and signal transduction; and (3) transcription, ion transport, and signal transduction.

Furthermore, cell signaling, differentiation, and ion transport are main processes of the group of genes with similar expression patterns during VIT and after VIT in patients with long-term protection (1 vs. 2 and 1 vs. 3). Interestingly cell proliferation and anatomical structure morphogenesis were the only processes found when comparing the list of genes of group 2 versus 1. These processes may be responsible for the development of important cell subtypes such as T regs and B cells during immunotherapy. It also may indicate that these processes are only active during VIT.

Clinical and Pathological Relevance of Genes Differentially Expressed Both during the Maintenance Phase of VIT and in the Phase of Long-Term Protection

T. reg Differentiation and Induction of Allergen Tolerance.

We found a significant upregulation in KLF10 (Krüppel-like factor10). KLF10 belongs to the family of transcription factors regulating cell growth, development, and differentiation.^{1,13} KLF10 regulates CD4⁺CD25[−] T cell activation, targeting transforming growth factor- β 1 and Foxp3 CD4⁺CD25⁺ T reg differentiation and promotes CD4⁺CD25⁺ T reg suppressor function by regulating the level of transforming growth factor- β 1 and Smad2 phosphorylation.¹⁴ T reg cells have been shown to be important in inducing tolerance in VIT. Therefore, significant upregulation of KLF10 in patients who achieved tolerance during VIT confirms well-known mechanisms of SIT.^{6,15}

The upregulation of the suppressor of cytokine signaling 3 SOCS3 in groups 2 and 3 was a surprising result, because in vitro data from cell line studies indicate that SOCS3 decreases the proliferation of T reg cells¹⁶ and also because

Table 3. List of Genes That Were Differently Expressed after Reaching Maintenance Phase of VIT (in Comparison with Patients before VIT) Where the Difference Was Still Observed in Patients Who Were Treated in the Past and Achieved Long-Term Protection

Gene symbol	Gene name	r2 v1	r3 v1	r1 v3	r1 v2	Corr. p1 v3	p1 v3	Corr. p1 v2	p1 v2
AP4M1	Adaptor-related protein complex 4. mu 1 subunit	2.6	4.1	0.2	0.4	0.01	0.00001	0.04	0.00433
B3GAT1	Beta-1.3-glucuronyltransferase 1 (glucuronosyltransferase p)	1.8	2.3	0.4	0.6	0.04	0.01212	0.05	0.02391
C6ORF123	Chromosome 6 open reading frame 123	0.3	0.3	3.5	3.6	0.04	0.00528	0.04	0.00276
CAPN7	Calpain 7	3.0	3.2	0.3	0.3	0.04	0.00385	0.04	0.00274
CCDC96 ^a	Hypothetical protein flj90575	0.5	0.5	1.9	2.1	0.04	0.00613	0.04	0.00265
CNGB3	Cyclic nucleotide gated channel beta 3	0.4	0.3	3.9	2.7	0.04	0.00507	0.05	0.02487
CPSF6	Cleavage and polyadenylation specific factor 6. 68kda	1.9	1.9	0.5	0.5	0.04	0.01251	0.04	0.00716
CTF8	Decreased expression in renal and prostate	0.3	0.5	1.9	3.5	0.05	0.02109	0.04	0.00058
CYLC1	Cylicin, basic protein of sperm head cytoskeleton 1	0.6	0.6	1.7	1.7	0.05	0.02627	0.05	0.02561
DAZ2	Deleted in azoospermia 1	0.4	0.5	2.2	2.7	0.04	0.00296	0.04	0.00170
DIRAS3 ^a	Diras family gtp-binding ras-like 3	2.6	3.2	0.3	0.4	0.03	0.00023	0.04	0.00397
DKFZP434M131	dkfzp434m131 Protein	0.6	0.5	2.2	1.8	0.04	0.01393	0.05	0.02813
DSCR6	Down syndrome critical region gene 6	3.2	2.9	0.3	0.3	0.05	0.02566	0.04	0.01203
DUSP26	Dual specificity phosphatase 26 (putative)	1.7	1.9	0.5	0.6	0.04	0.00156	0.04	0.01086
DYRK1A ^a	Dual-specificity tyrosine-(y)-phosphorylation regulated kinase 1a	0.4	0.4	2.8	2.5	0.04	0.01165	0.04	0.01161
FADS1	Fatty acid desaturase 1	1.7	1.8	0.6	0.6	0.04	0.00331	0.04	0.01629
FGFRL1 ^a	Fibroblast growth factor receptor-like 1	3.0	2.5	0.4	0.3	0.04	0.00489	0.04	0.00603
GPR177	Mesoderm induction early response 1 homolog (xenopus laevis)	2.2	3.3	0.3	0.5	0.04	0.00273	0.05	0.02081
GRAMD3	Gram domain containing 3	2.2	1.8	0.6	0.5	0.04	0.00336	0.04	0.00131
GTPBP3	GTP binding protein 3 (mitochondrial)	0.3	0.4	2.8	3.3	0.04	0.01914	0.04	0.00717
HES6	Transcription cofactor HES-6	2.3	1.6	0.6	0.4	0.04	0.01123	0.04	0.00443
HLA-DRB5	Major histocompatibility complex class ii. dr beta 1	0.4	0.2	4.4	2.4	0.04	0.00757	0.05	0.02596
HS.128309		0.4	0.4	2.3	2.5	0.04	0.00352	0.04	0.00305
HS.146933 ^a		0.4	0.4	2.6	2.5	0.04	0.00139	0.04	0.00092
HS.153248		0.4	0.5	2.2	2.6	0.04	0.00434	0.04	0.00144
HS.188369		4.3	3.0	0.3	0.2	0.04	0.00138	0.04	0.00373
HS.283139		0.5	0.5	2.0	2.0	0.04	0.00795	0.04	0.00840
HS.31007		0.5	0.5	2.2	2.0	0.04	0.00916	0.05	0.02407
HS.327663		0.3	0.4	2.3	3.2	0.05	0.02616	0.04	0.00412
HS.334190		0.6	0.4	2.3	1.7	0.04	0.00437	0.04	0.01635
HS.46689		2.6	2.8	0.4	0.4	0.04	0.00753	0.05	0.02472
HS.538101		0.4	0.4	2.7	2.3	0.04	0.00213	0.04	0.01564
HS.539166		0.4	0.4	2.7	2.4	0.04	0.00459	0.04	0.01219
HS.540136		2.6	2.7	0.4	0.4	0.04	0.00293	0.04	0.01727
HS.541079		0.4	0.4	2.8	2.4	0.04	0.00352	0.04	0.00507
HS.542601		3.7	3.1	0.3	0.3	0.03	0.00020	0.05	0.02554
HS.543224		0.4	0.3	3.2	2.6	0.04	0.00250	0.04	0.00807
HS.550255		0.4	0.3	3.4	2.8	0.04	0.00485	0.04	0.01833
HS.565181		0.3	0.3	3.0	3.0	0.04	0.00636	0.04	0.00830
HS.565687		0.4	0.3	3.1	2.4	0.04	0.00100	0.04	0.00836
HS.567059		0.3	0.4	2.5	2.9	0.05	0.02592	0.04	0.00388
HS.569823		0.5	0.6	1.7	1.9	0.04	0.01780	0.04	0.01811
HS.571519		4.0	2.8	0.4	0.3	0.04	0.00132	0.04	0.00463
HS.99308		0.4	0.4	2.7	2.6	0.04	0.00963	0.05	0.02084
HTR3B	5-Hydroxytryptamine (serotonin) receptor 3b	0.3	0.5	2.2	3.0	0.05	0.02731	0.04	0.00523
IL17REL	flj41993 Protein	0.6	0.5	2.1	1.7	0.04	0.00204	0.04	0.01729
KCNJ16	Potassium inwardly rectifying channel; subfamily j. member 16	0.5	0.5	2.1	2.1	0.04	0.00712	0.04	0.01690
KIAA1754L	kiaa1754-like	2.1	2.4	0.4	0.5	0.04	0.00331	0.04	0.00866
KLF10 ^a	Krüppel-like factor 10	2.3	2.0	0.5	0.4	0.04	0.01299	0.04	0.00873

Table 3. Continued

Gene symbol	Gene name	r2 v1	r3 v1	r1 v3	r1 v2	Corr. p1 v3	p1 v3	Corr. p1 v2	p1 v2
LOC145820 ^a	Hypothetical protein loc145820	0.6	0.6	1.7	1.6	0.04	0.01422	0.04	0.01304
LOC389457	Hypothetical protein loc389457	2.8	3.3	0.3	0.4	0.04	0.00114	0.04	0.00710
LOC541469	Chromosome 19 open reading frame 33	0.2	0.1	8.0	4.8	0.02	0.00004	0.04	0.00098
LOC642539	Similar to glucosidase alpha acid	4.0	2.4	0.4	0.3	0.04	0.01577	0.04	0.00058
LOC643993	Hypothetical protein loc643993	0.3	0.3	3.4	3.1	0.04	0.00611	0.04	0.00761
LOC644743	Hypothetical protein loc644743	0.4	0.5	2.1	2.3	0.05	0.02030	0.05	0.02238
LOC645268	Hypothetical protein loc645268	0.2	0.3	3.4	4.4	0.04	0.01735	0.04	0.00220
LOC648069	Hypothetical protein loc648069	2.2	2.0	0.5	0.5	0.04	0.01938	0.05	0.02648
LOC648309	Hypothetical protein loc648309	3.1	2.7	0.4	0.3	0.04	0.00044	0.04	0.01222
LOC648934	Similar to citrate synthase precursor isoform a	0.4	0.5	2.1	2.6	0.04	0.00808	0.04	0.00366
LOC652565	Similar to t-cell surface glycoprotein cd8 beta chain precursor (antigen cd8b)	0.6	0.6	1.8	1.6	0.04	0.00439	0.04	0.01010
LOC653227	Similar to gamma-aminobutyric-acid receptor gamma-3 subunit precursor (gaba[a] receptor)	0.4	0.3	4.0	2.6	0.04	0.00115	0.05	0.02576
LOC653562	Similar to solute carrier family 6 (neurotransmitter transporter; creatine) member 8	0.3	0.3	3.2	3.0	0.04	0.01132	0.04	0.00568
LOC727811		0.3	0.3	3.2	3.9	0.04	0.00948	0.04	0.00191
LOC729558		0.6	0.5	1.9	1.6	0.04	0.00526	0.05	0.02619
MEIS2	meis1 Myeloid ecotropic viral integration site 1 homolog 2 (mouse)	0.5	0.5	2.1	2.2	0.04	0.01304	0.04	0.01226
MFAP3L	Microfibrillar-associated protein 3-like	3.7	3.5	0.3	0.3	0.04	0.00092	0.04	0.00040
MRM1	Mitochondrial rRNA methyltransferase 1 homolog (<i>S. cerevisiae</i>)	2.5	3.8	0.3	0.4	0.05	0.02314	0.04	0.01802
MS4A15	Hypothetical protein mgc35295	0.3	0.2	5.0	3.3	0.04	0.00094	0.04	0.01280
NARG2	NMDA receptor regulated 2	2.1	1.9	0.5	0.5	0.04	0.00882	0.05	0.02259
NMB ^a	Neuromedin b	1.3	1.5	0.6	0.7	0.05	0.02561	0.05	0.02804
NOG	Noggin	3.2	3.2	0.3	0.3	0.04	0.00635	0.04	0.00605
NTN4	Netrin 4	0.3	0.3	3.0	2.9	0.04	0.00573	0.04	0.00913
OMG	Oligodendrocyte myelin glycoprotein	3.6	3.0	0.3	0.3	0.04	0.00342	0.05	0.02386
OR5AR1 ^a	Olfactory receptor family 5 subfamily ar. member 1	0.5	0.3	3.9	2.2	0.04	0.00083	0.05	0.02539
PHF1	phd Finger protein 1	2.3	1.9	0.5	0.4	0.04	0.01513	0.04	0.00599
PIN4	Protein (peptidylprolyl cis/trans isomerase) nima-interacting. 4 (parvulin)	1.9	1.8	0.6	0.5	0.04	0.00554	0.04	0.00897
RAD50	rad50 Homolog (<i>S. cerevisiae</i>)	1.9	1.8	0.6	0.5	0.04	0.00638	0.04	0.01619
RNF43	Ring finger protein 43	0.3	0.3	2.9	2.9	0.04	0.00786	0.04	0.01426
SFRS12	Splicing factor arginine/serine-rich 12	3.8	4.4	0.2	0.3	0.03	0.00034	0.04	0.00244
SNRP70	Small nuclear ribonucleoprotein 70-kda polypeptide (rnp antigen)	2.1	2.0	0.5	0.5	0.04	0.00806	0.04	0.00452
SOCS3	Suppressor of cytokine signaling 3	3.0	1.9	0.5	0.3	0.05	0.02777	0.04	0.01376
SPTB	Spectrin beta erythrocytic (includes spherocytosis clinical type i)	0.6	0.4	2.4	1.8	0.04	0.01923	0.04	0.01729
SSFA2	Sperm specific antigen 2	1.5	1.6	0.6	0.7	0.04	0.01020	0.05	0.02614
ST6GALNAC3	st6 (alpha-n-acetyl-neuraminyl-2.3-beta-galactosyl-1.3)-n-acetylgalactosaminide alpha-2.6-sialyltransferase 3	2.4	2.0	0.5	0.4	0.04	0.00134	0.04	0.00689
TSPYL2 ^a	TSPY-like 2	1.9	1.7	0.6	0.5	0.04	0.00908	0.04	0.00211
USMG5	Upregulated during skeletal muscle growth 5 homolog (mouse)	3.4	3.0	0.3	0.3	0.04	0.00080	0.04	0.00117
VPS8	Kiaa0804	1.9	3.3	0.3	0.5	0.05	0.02618	0.04	0.01583
WDR33	WD repeat domain 33	0.6	0.5	2.0	1.6	0.04	0.01184	0.04	0.01547
ZNF367	Zinc finger protein 367	0.5	0.5	1.8	2.0	0.04	0.01640	0.05	0.02012

Genes that were differently expressed in patients with IVA and controls were marked with a.

^a Ratio of the expression levels for each individual gene when comparing patients before VIT and on maintenance phase (groups 1 vs 2) and before subjects who achieved success of VIT (groups 1 vs 3).

Table 4. Gene Co-occurrence Annotation Found by Genecodis (GOSlim Process Function) for Genes That Were Differentially Expressed during Maintenance Phase of VIT and Where This Difference Was Still Observed in Patients Who Stopped VIT and Achieved Long Term Protection

Genes	NGR	NG	Hyp	Hyp*	Annotations
FADS1 Fatty acid desaturase 1 KLF10 Krueppel-like factor 10 NMB Neuromedin-B precursor	237 (37,435)	3 (49)	0.003	0.01	GO:0007267:cell-cell signaling (BP)
HES6 Transcription cofactor HES-6 CYLC1 Cylicin-1 DAZ2 Deleted in azoospermia protein 2	343 (37,435)	3 (49)	0.01	0.02	GO:0007275:multicellular organis- mal development (BP) GO:0030154:cell differentiation (BP)
CNGB3 Cyclic nucleotide-gated cation channel beta-3 KCNJ16 Inward rectifier potassium channel 16 HTR3B 5-hydroxytryptamine receptor 3B Precursor	503 (37,435)	3 (49)	0.0281594	0.0469324	GO:0006811:ion transport (BP)

Abbreviations: NGR, number of annotated genes is shown in the reference list; NG, number of annotated genes in the input list. P values have been obtained through Hypergeometric analysis (Hyp) corrected by FDR method (Hyp*).

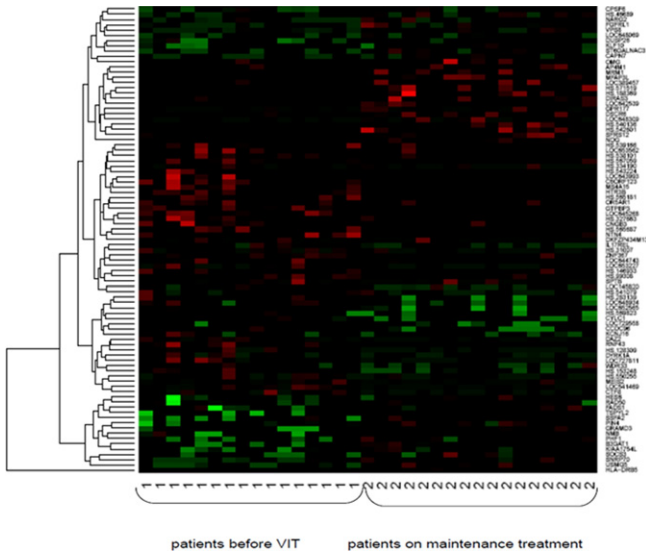


Figure 1. Hierarchical clustering dendrogram of differentially expressed genes between patients before VIT (group 1) and on maintenance phase of VIT (group 2). Each column represents the patient sample; each row, an individual gene. For each gene, the green color represents underexpression, the red color overexpression, and the black signal denotes missing data.

overexpression of *SOCS3* was found in a mouse model of asthma.¹⁷ However, although an in vivo study on *SOCS3* expression in whole blood of allergic children also found upregulation of *SOCS3*, the level of *SOCS3* was positively correlated with proteins known to be upregulated during SIT (*Foxp3*, *Tbet*) and inversely with *IL4* (which is down-regulated during SIT), which is in accordance with our results.¹⁷ These differences are probably caused by the expression of *SOCS3* in other peripheral blood cells.^{17,18}

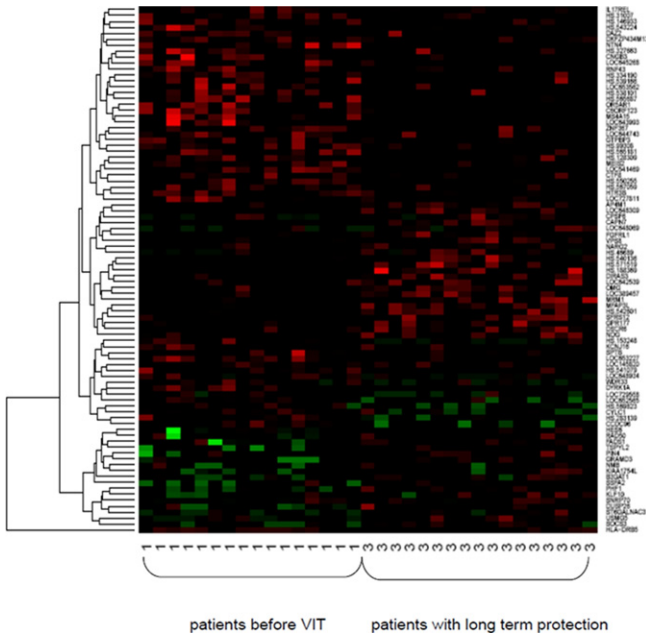


Figure 2. Hierarchical clustering dendrogram of differentially expressed genes between patient groups before VIT (group 1) and with long-term protection of treatment (group 3). Each column represents the patient sample; each row, an individual gene. For each gene, the green color represents underexpression, the red color overexpression, and the black signal denotes missing data.

Thus, overexpression of the gene in other cell subtypes may mask the differences in expression in T reg cells.

B Cell Stimulation

Downregulation of the dual-specificity tyrosine-(y)-phosphorylation regulated kinase 1a *DYRK1A* gene was found

in groups 2 and 3. The protein DYRK1A is required for phosphorylation and thus activation of STAT3,¹⁹ which subsequently is required for the production of IL-4 and IL-21, cytokines that induce the secretion of IgE from stimulated B cells.²⁰ Thus, downregulation of *DYRK1A* may result in suppressing the secretion of IgE from B cells.

Mast Cell Activation

5-Hydroxytryptamine (serotonin) receptor 3b *HTR3B* expression was downregulated in groups 2 and 3. Serotonin receptors are present, that is, on the surface of the mast cells.²¹ Low serotonin levels have been correlated with diarrhea, flushing, headaches, and bone pain in mastocytosis patients.²² The 5-HT_{3B} together with the 5-HT_{3A} receptor subunit forms a heteromeric receptor for serotonin.²³ The 5-HT_{3B} subunit is the major determinant of the receptor function.²³ This protein is expressed in the central nervous system but also in the spleen and intestine as well as in monocytes.²⁴ Kushnir-Sukhov et al.²² hypothesized that a low serotonin level might be related to mast cell activation and might be present in patients with allergic symptoms. Furthermore, they suggested that mast cell ablative therapy may lead to a rise in serotonin blood levels. The serotonin blood level is known to be inversely correlated with serotonin 5-HT_{3B} receptor expression.²⁵ As in our study, the expression of 5-HT_{3B} receptor was decreased after VIT; we hypothesize that this might be caused by an increased level of serum serotonin level. Therefore, our results might confirm the hypothesis of Kushnir-Sukhov on the role of serotonin in allergy.

Nervous System Involvement

Another explanation for the observed differences in gene expression found in groups 2 and 3 might point to a possible effect of VIT on the nervous system.²⁶ Siebenhaar et al.²⁷ showed that sensory nerves play a role in mast cell IgE-mediated activation. Among the genes differentially expressed during VIT, several transcripts might be involved in neuroimmune interactions, such as *HTR3B* (5-hydroxytryptamine—serotonin receptor 3b), *OMG* (oligodendrocyte myelin glycoprotein), *OR5AR1* (olfactory receptor, family 5, subfamily ar, member 1), *NMB* (neuromedin-B precursor), or *CNGB3* (cyclic nucleotide-gated channel beta3). However, the data available so far are too weak to draw definitive conclusions.

Differences in Expression between Patients with IVA and Controls

This analysis confirms the function of 8 genes with similar expression profiles in healthy controls and patients treated with VIT. Interestingly, *DYRK1A* and *TSPYL2* were differently expressed in patients during and after successful VIT as compared with healthy controls. Downregulation of *DYRK1A* might be related to suppression of IgE production in healthy controls.

In conclusion, the results of this study demonstrate several groups of genes that are differentially expressed

during the maintenance phase of VIT and that this difference remains after stopping successful VIT. This might relate to mechanisms that are responsible for the long-term protective effect of VIT. A better understanding of the protective mechanisms of VIT is relevant for a better understanding of anaphylaxis and immunotherapy. Although the results of the current study should be confirmed prospectively, the relevance of these findings is supported by the fact that they are related to putative mechanisms of immunotherapy.

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REFERENCES

1. Golden D. Stinging insect allergy. *Am Fam Physician*. 2003;67:2541–2546.
2. Bonifazi F, Jutel M, Biló B, Birnbaum J, Muller U. Prevention and treatment of hymenoptera venom allergy: Guidelines for clinical practice. *Allergy*. 2005;60:1459–1470.
3. Golden D, Kagey-Sobotka A, Lichtenstein L. Survey of patients after discontinuing venom immunotherapy. *J Allergy Clin Immunol*. 2000;105:385–390.
4. Golden D, Kwitrovich K, Kagey-Sobotka A, Lichtenstein L. Discontinuing venom immunotherapy: extended observations. *J Allergy Clin Immunol*. 1998;101:298–305.
5. Simons F, Frew A, Ansotegui I, et al. Risk assessment in anaphylaxis: current and future approaches. *J Allergy Clin Immunol*. 2007;120:S2–S24.
6. Jutel M, Akdis M, Blaser K, Akdis C. Mechanisms of allergen specific immunotherapy T-cell tolerance and more. *Allergy*. 2006;61:796–807.
7. Finegold I. Issues in stinging insect allergy immunotherapy: a review. *Curr Opin Allergy Clin Immunol*. 2008;8:343–347.
8. Konno S, Golden DB, Schroeder J, Hamilton RG, Lichtenstein LM, Huang SK. Increased expression of osteopontin is associated with long-term bee venom immunotherapy. *J Allergy Clin Immunol*. 2005;115:1063–1067.
9. Konno S, Hizawa N, Nishimura M, Huang SK. Osteopontin: a potential biomarker for successful bee venom immunotherapy and apotential molecule for inhibiting IgE-mediated allergic responses. *Allergol Int*. 2006;55:355–359.
10. Mueller HL. Diagnosis and treatment of insect sensitivity. *J Asthma Res*. 1966;3:331–333.
11. Niedoszytko M, Bruinenberg M, de Monchy J, et al. Gene expression analysis in predicting the effectiveness of insect venom immunotherapy. *J Allergy Clin Immunol*. 2010;125:1092–1097.
12. Nogales-Cadenas R, Carmona-Saez P, Vazquez M, et al. GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. *Nucl Acids Res*. 2009;37:317–322.
13. Bieker JJ. Isolation, genomic structure, and expression of human erythroid Krüppel-like factor (EKLF). *DNA Cell Biol*. 1996;15:347–352.
14. Cao Z, Wara AK, Icli B, et al. Kruppel-like factor KLF10 targets TGF- β 1 to regulate CD4+CD25– T cells and T regulatory cells. *J Biol Chem*. 2009;284:24914–24924.
15. Pyzik M, Piccirillo CA. TGF- β 1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets. *J Leukoc Biol*. 2007;82:335–346.

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16. Pillemer BB, Xu H, Oriss TB, Qi Z, Ray A. Deficient SOCS3 expression in CD4+CD25+FoxP3+ regulatory T cells and SOCS3-mediated suppression of Treg function. *Eur J Immunol*. 2007;37:2082–2089.
 17. Daegelmann C, Herberth G, Röder S, et al. Association between suppressors of cytokine signaling, T-helper type 1/T-helper type 2 balance and allergic sensitization in children. *Clin Exp Allergy*. 2008;38:438–448.
 18. Mieg D, Thierry-Mieg J. AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol*. 2006;7(Suppl 1):12–14.
 19. Matsuo R, Ochiai W, Nakashima K, Taga T. A new expression cloning strategy for isolation of substrate-specific kinases by using phosphorylation site-specific antibody. *J Immunol Methods*. 2001;247:141–151.
 20. Avery D, Ma CS, Bryant V, et al. STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. *Blood*. 2008;112:1784–1793.
 21. Kushnir-Sukhov N, Gilfillan A, Coleman J, et al. 5-Hydroxytryptamine induces mast cell adhesion and migration. *J Immunol*. 2006;177:6422–6432.
 22. Kushnir-Sukhov N, Brittain E, Scott L, Metcalfe D. Clinical correlates of blood serotonin levels in patients with mastocytosis. *Eur J Clin Invest*. 2008;38:953–958.
 23. Davies P, Pistis M, Hanna M, et al. The 5-HT3B subunit is a major determinant of serotonin-receptor function. *Nature*. 1999;397:359–363.
 24. Dubin A, Huvar R, D'Andrea M, et al. The pharmacological and functional characteristics of the serotonin 5-HT(3A) receptor are specifically modified by a 5-HT(3B) receptor subunit. *J Biol Chem*. 1999;274:30799–30810.
 25. Krzywkowski K, Davies P, Irving A, Bräuner-Osborne H, Jensen A. Characterization of the effects of four HTR3B polymorphisms on human 5-HT3AB receptor expression and signalling. *Pharmacogenet Genomics*. 2008;18:1027–1040.
 26. Golden DB. What is anaphylaxis? *Curr Opin Allergy Clin Immunol*. 2007;7:331–336.
 27. Siebenhaar F, Magerl M, Peters E, Hendrix S, Metz M, Maurer M. Mast cell-driven skin inflammation is impaired in the absence of sensory nerves. *J Allergy Clin Immunol*. 2008;121:955–961.
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