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COMMD8 changes expression during initial phase of wasp venom immunotherapy

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Abstract

Background: Hymenoptera venom allergy (HVA) is of great concern because of the possibility of anaphylaxis, which may be fatal. Venom immunotherapy (VIT) is the only disease-modifying treatment in HVA and, although efficient, its mechanism remains partially unknown. Gene expression analysis may be helpful for establishing a proper model of tolerance induction during the build-up phase of VIT. The present study aimed to analyze how the start of VIT changes the expression of 15 selected genes.

Methods: Forty-five patients starting VIT with a wasp venom allergy were enrolled. The diagnosis was established based on anaphylaxis history (third or fourth grade on the Mueller scale) and positive soluble immunoglobulin E and/or skin tests. Two blood collections were performed in the patient group: before and after 3 months of VIT. One sample was taken in the control group. Gene expression analysis was performed using a reverse transcriptase-polymerase chain reaction with microfluidic cards and normalized to the 18S housekeeping gene.

Results: *Commd8* was the only gene that changed expression significantly after the start of VIT ($p = 0.012$). Its expression decreased towards the levels observed in the healthy controls. Twelve out of 15 genes (*commd8*, *cldn1*, *cngb3*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *prlr*, *slc16a4*, *snx33*, *socs3* and *twist2*) revealed a significantly different expression compared to the healthy controls.

Conclusions: The present study shows that *commd8* changes significantly its expression during initial phase of VIT. This gene might be a candidate for VIT biomarker in future studies.

KEYWORDS

allergy, gene expression, immune response, RNA

1 | INTRODUCTION

Hymenoptera venom allergy (HVA), although not seen as frequently as other allergies, is of great concern because of the possibility of anaphylaxis, which may be fatal. The epidemiological data vary in different climate zones. Population-wise, 0.3% up to 8.9% of citizens would experience post-stinging anaphylaxis.^{1,2} Although the

mortality rate of anaphylaxis is low (0.03–1.6 per million citizens), it may be underestimated because an American *post mortem* study revealed elevated tryptase levels and soluble immunoglobulin E (sIgE) towards insect venom in 23% of people dying suddenly outdoors.³ A random study of 3000 Poles reported systemic reactions to insect stings at 8.9%; however, an allergy was confirmed via a skin test in only half of the cases.⁴ The greatest risk

of anaphylaxis concerns bee keepers. In this population, 6.5% report a systemic allergic reaction each year.

Patients with documented venom-specific IgE by skin or serum test who also have a history of sting anaphylaxis should receive venom immunotherapy (VIT).⁵ It is currently the only disease modifying treatment in HVA, with efficacy estimated at 93% for wasp, 80% for honeybee and 97% for ant venom.⁶ Although efficient, its main mechanisms corresponding to clinical efficacy remain unclear.⁷ Current hypotheses suggest that the allergen tolerance built during treatment is the result of a change in the profile of the B and T lymphocytes, a switch in a specific antibody class from IgE to IgG/IgG4, and inhibition of the migration and degranulation of effector cells.⁸ The scientific literature also highlights the role of T cells and their change from the Th2 towards the Th1 profile.^{9,10} All of these changes are probably caused by activated T regulatory cells (Treg).¹¹ These cells suppress allergic responses with interleukin (IL)-10 production, although they also create cell-to-cell interactions, even directly blocking allergen presentation by dendritic cells.

The same lack of knowledge applies to anaphylaxis. It is unknown why some people react to allergen exposure with anaphylaxis and others do not, despite having a high level of venom-IgE.¹² Gene expression analysis may be helpful in establishing a proper model of VIT. Stone *et al.*¹³ have conducted a whole genome expression study on six patients who were admitted to an emergency department because of anaphylaxis. The analysis was performed at three time points: on admission, after 1 hour and after 3 hours. The results revealed changes in the expression of 2, 67 and 2801 genes, respectively, compared to healthy controls, and also showed how the anaphylactic cascade progressed, as well as how many possible mechanisms were involved. To better understand the mechanisms behind those changes, the Genocodis database was used to group together a set of genes acting in the same pathways.^{14–16} This showed that, during an anaphylactic reaction, the first changes are connected to the Th1 response. Subsequently, an increase in proinflammatory and proapoptotic signals such as *MAPK*, *NFKB*, *STAT3*, *IL-5*, *IL-6*, *CREB*, *ESP300* and *MMP9* is observed.¹³ A change in gene expression associated with those pathways may predict an anaphylaxis onset on allergen exposure in the future. So far, the observations on patients treated with VIT confirm the results reported by Stone *et al.*¹³ Niedoszytko *et al.*^{17,18} performed two whole genome expression analyses to predict the gene expression markers of successful VIT by measurements performed before and after treatment. It was revealed that 43 and 89 genes, respectively, changed expression significantly; of these, 15 genes presented the most significant changes.

The present study aimed to analyze the change in expression of the 15 genes involved in the studies described above (*cldn1*, *cngb3*, *commd8*, *daz2*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *klf10*, *pcdhb10*, *prrl*, *slc16a4*, *snx33*, *socs3* and *twist2*) during the build-up phase of VIT and then to compare the results with their expression in healthy volunteers. Together with clinical assessment, we may identify the genes that would help predict efficacy at the start of VIT.

2 | MATERIALS AND METHODS

In total, 45 patients (30 women and 15 men; aged 18–77 years; mean age 41.8 years; Caucasian) starting VIT with wasp venom (*Vespula* spp.) were enrolled. Informed consent was obtained on enrollment. The diagnosis was established based on anaphylaxis history (third or fourth grade on the Mueller scale) and positive wasp-specific IgE (sIgE; *Vespula* spp. mix of allergens at least 3.5 kU/l; class 3 ImmunoCAP) and/or skin tests (skin prick tests and intradermal skin tests; natural extracts). The tryptase blood level was measured in all subjects. The diagnostic process was carried out in accordance with EAACI recommendations.¹⁹ Wasp-specific serum IgE was measured before and after VIT. The build-up phase of VIT was performed according to the ultra rush protocol.²⁰ The control group consisted of 38 healthy volunteers (19 women and 19 men; aged 25–77 years; mean age 46 years; Caucasian) with no previous abnormal post-stinging reactions.

For the purpose of gene expression analysis, two blood collections were performed in the patient group: before and after 3 months of VIT. One sample was taken in the control group. Blood was collected in Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) and stored at -80°C . The mRNA isolation was performed using Tempus™ Spin RNA Isolation Kits (Ambion, Austin, TX, USA) followed by reverse transcription using High Capacity cDNA Reverse Transcription Kits with RNase Inhibitor (Invitrogen, Waltham, MA, USA). The material was transferred to TaqMan Array Micro Fluidic Cards (Applied Biosystems) for a polymerase chain reaction (PCR) in a 7900HT Real Time Fast PCR System (Applied Biosystems). The microfluidic cards had 16 probes set up by the manufacturer for the detection of the *cldn1*, *cngb3*, *commd8*, *daz2*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *klf10*, *pcdhb10*, *prrl*, *slc16a4*, *snx33*, *socs3*, *twist2* and *18S* genes. *18S* ribosomal rRNA was used as a housekeeping gene for normalization. The number of PCR cycles until crossing set threshold was identified. The result was normalized with the housekeeping gene and related to the calibrator comprising 450 ng of Universal Human Reference RNA (Agilent Technologies, Santa Clara, CA, USA). Primary endpoint was relative quantification (RQ) calculated in accordance with MIQE guidelines.²¹ All laboratory procedures were performed in accordance with the respective manufacturer's instructions.

Comparisons were performed with Wilcoxon's (dependent variables) and Mann-Whitney (independent variables) *U* tests implemented in Statistica, version 12 (StatSoft, Inc., Tulsa, OK, USA).

The study was approved by the appropriate Independent Bioethics Commission at the Medical University of Gdansk (NKBBN/468/2013).

3 | RESULTS

Out of a total of 45 patients, 22 experienced third-degree anaphylaxis according to the Mueller scale (concerning symptoms of the respiratory system) and 23 had fourth-degree anaphylaxis (with

cardiovascular symptoms).²² Mean sIgE measured decreased during treatment from 4.07 kU/l to 3.33 kU/l. Considering known risk factors for severe allergic reactions¹: (i) two patients out of 45 had an elevated tryptase level (22 ng/ml and 27 ng/ml) and diagnosed systemic mastocytosis; (ii) three patients were at least 65 years old; and (iii) 23 patients had reported cardiovascular diseases, with these being exactly the same patients who had fourth-degree anaphylaxis. *commd8* was the only gene that changed expression significantly during the build-up phase of VIT (Figure 1 and Table 1). Its expression decreased from RQ 3.60 (SD 1.42) to RQ 3.33 (SD 1.36) compared to its expression in the healthy controls of RQ 3.00 (SD 2.51). Twelve out of 15 genes (*commd8*, *cldn1*, *cngb3*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *prlr*, *slc16a4*, *snx33*, *socs3* and *twist2*) showed a significantly different

expression in patients before VIT compared to the healthy controls (Table 1). With the exception of *commd8*, all genes presented lower expression before VIT compared to the control group. The possible connection of these genes to particular mechanisms is presented in Table 2.

4 | DISCUSSION

A primary discovery in the present study is the decrease in *commd8* expression that was observed during the build-up phase of VIT. Copper metabolism Murr1 domain-containing (COMMD) proteins regulate numerous processes via the ubiquitination (inactivation by adding ubiquitin) of proteins. Well-described in the available literature, *commd1* is connected to copper metabolism, regulation of sodium concentration and hypoxia adaptation at the cellular level.^{23–27} It also regulates the well-known nuclear factor kappa B (NFKB) complex.²⁸ NFKB activation is dependent on the ubiquitination of the small IKB complex, which is a constitutive NFKB inhibitor.²⁹ Starokadomskyy *et al.*²⁸ described the CCDC22 complex that, in addition to *commd8*, is needed for IKB degradation, and thus the release of NFKB for nuclear translocation, as well as its influence on transcription. According to this model, an increase in *commd8* expression should lead to enhancement of the activity of NFKB. *Commd1*, working with the same CCDC22, would extract NFKB from the nucleus and present the opposite function to *commd8*.²⁸ NFKB is a transcription factor with multiple functions. It is a key activator of specific and non-specific immune reactions, and regulates apoptosis, and the cell-division cycle.³⁰ La Grutta *et al.*³¹ revealed a correlation between phosphorylated IKB and asthma symptoms in children.

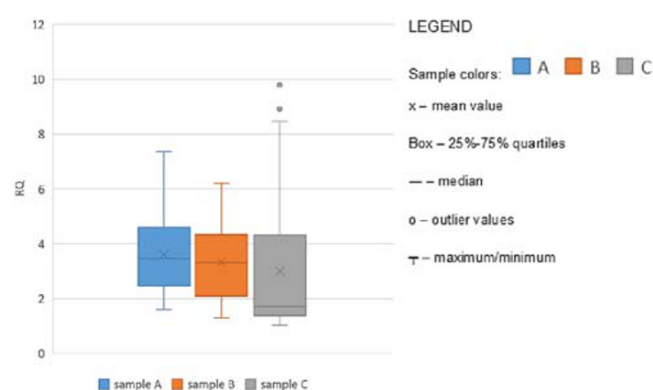


FIGURE 1 Expression of COMMD8 in patients starting wasp venom immunotherapy (A), after 3 months of treatment (B) and in healthy controls (C). RQ, relative quantification

TABLE 1 Comparison between samples for each gene expression using Wilcoxon's and Mann Whitney's tests

Gene	Comparison of gene expression before and after 3 months of VIT in patients		Comparison of gene expression before VIT and in healthy controls		Comparison of gene expression after 3 months of VIT and in healthy controls	
	Fold change	p value	Fold change	p value	Fold change	p value
<i>cldn1</i>	0.35	0.095931	5.41	0.000023	1.895271	0.009068
<i>cngb3</i>	0.85	0.345932	2.36	0.000035	2.01171	0.000055
<i>commd8</i>	1.08	0.012413	0.83	0.000515	0.901547	0.006366
<i>daz2</i>	2.15	0.845081	18.79	0.887951	40.38	0.830687
<i>fads1</i>	0.97	0.672089	2.92	0.033850	2.843901	0.032085
<i>hes6</i>	0.98	0.941512	1.39	0.007665	1.367892	0.018594
<i>Hla-drb5</i>	0.91	0.607544	3.43	0.010112	3.116107	0.012426
<i>htr3b</i>	0.85	0.357607	2.37	0.000040	2.012801	0.000066
<i>klf10</i>	1.03	0.058670	3.47	0.272316	3.578304	0.168949
<i>pcdhub10</i>	0.49	0.256628	4.62	0.126240	2.257041	0.339505
<i>Prlr</i>	0.98	0.286120	3.68	0.037961	3.587682	0.060341
<i>slc16a4</i>	0.93	0.100521	2.45	0.027395	2.278377	0.014486
<i>snx33</i>	0.91	0.852253	2.93	0.000763	2.658216	0.001692
<i>socs3</i>	1.07	0.055718	2.36	0.000848	2.526418	0.000083
<i>twist2</i>	0.74	0.208189	2.90	0.004828	2.141846	0.091728

TABLE 2 Examined genes that showed significant differences in comparisons

Genes	General function	Potential involvement in VIT
<i>cldn1</i>	Major part of the tight junction complexes	Through NFKB, probably multiple mechanisms
<i>cngb3</i>	G-protein signal transduction	Take part in functioning of the nervous system that might connect with mast cells
<i>commd8</i>	NFKB complex nucleus translocation	Through NFKB, probably multiple mechanisms
<i>fads1</i>	Involved in biosynthesis of highly unsaturated fatty acids	Take part in functioning of the nervous system that might connect with mast cells
<i>hes6</i>	Suppresses HES1 – transcription factor in myogenesis	Take part in functioning of the nervous system that might connect with mast cells
<i>Hla-drb5</i>	Presents peptides from antigens on the antigen presenting cell surface for recognition by the CD4 T cells	Through IFN gamma, suppression of Th2 and IgE production
<i>htr3b</i>	Serotonin receptor	Reduction of mast cell activation, take part in functioning of the nervous system that might connect with mast cells
<i>Prlr</i>	Prolactin receptor, part in MAPK, JAK-STAT pathways	Through IFN gamma, TGF beta-dependent activation of T regulatory cells
<i>slc16a4</i>	Catalyzes the transport across the plasma membrane of monocarboxylates	Through NFKB, probably multiple mechanisms
<i>snx33</i>	Involved in organizing cytoskeleton, endocytosis and cellular vesicle transport. Acts in mitotic cell divisions	No data
<i>socs3</i>	Negative feedback system that regulates cytokine signal transduction through the JAK/STAT pathway	Through IFN gamma suppression of Th2 and IgE production
<i>twist2</i>	Suppresses proinflammatory cytokines such as TNF alfa and IL1B	Affects development of IL-10 producing B regulatory cells, though TNF alfa immunosuppressive effect

General functions according to GeneCards (Weizmann Institute of Science; <https://www.genecards.org>) database. For potential involvement, see the Discussion.

The impact of NFKB on tolerance induction is complex. It influences the maturation of immature dendritic cells (DCs) to tolerogenic DCs and, through them, a switch of naive T cells towards Treg and Th1.^{32,33} NFKB is also directly required for the maturation of Treg as a stimulus of *foxp3* expression and its presence on the cell surface.^{34,35} This would suggest that higher NFKB activity contributes to tolerance induction.

However, some studies focused on other pathways showed different relation. A recent study by Nakai *et al.*³⁶ on mice and *in vitro* cell lines showed a new interaction of *commd8* and *commd3*. The complex that interacts with G protein-coupled receptors (GPCRs) on lymphocytes that take part in cell migration. Deprivation of *commd3/8* impairs B cell migration with decreased signaling through chemo-attractant receptors.

The change in NFKB functions during the start of allergen specific immunotherapy has been described before. House dust mite immunotherapy on allergic mice resulted in significant inhibition of STAT6 and NFKB, together with a reduction of bronchial inflammation and specific IgE levels.³⁷ These observations were confirmed in human studies. Tsai *et al.*³⁸ showed that 1 year of immunotherapy application in children who were allergic to house dust mites resulted in a decrease of NFKB activation measured with its RelA subunit. Ventura *et al.*³⁹ investigated NFKB activation on lipopolysaccharide stimulation in asthmatics treated for an airborne allergy with immunotherapy. The phosphorylated IKB concentration decreased in correlation to the decrease in asthma symptoms. Several animal studies suggest the possibility of blocking NFKB activation to achieve clinical allergic effects. Taher *et al.*³² applied an NFKB selective inhibitor to mice allergic to ovalbumin, which resulted in the lowering of blood eosinophilia, IL-5 and IL-13, as well as an increase in IL-10. These changes suggest a tolerogenic effect of the NFKB blocker in food allergy. Therefore, there is a strong connection between NFKB and allergic diseases and immunotherapy, thus making it a new possible treatment target.

In summary, a change in the expression of *commd8* may result from a proper immune system influence by VIT. Although, theoretically, NFKB is needed for Treg maturation, it appears that lowering its expression alongside with *commd8* would be beneficial to reduce allergic inflammation, IgE levels, eosinophilia and asthma symptoms. Judging by its pattern, VIT should decrease its expression to reach the levels in healthy persons (Figure 1). This change may also be linear and could help to predict when a particular patient crosses the tolerance threshold. This requires further investigation.

In *cldn1*, *cngb3*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *prlr*, *slc16a4*, *snx33*, *socs3* and *twist2*, the expression was lower than in the control group (Table 1). Some of these genes were grouped according to their function in the GeneCodis database.¹⁶ *fads1*, *htr3b* and *cngb3* are responsible for cell membrane transport and structure. HTR3B is a serotonin receptor, although it may also have a role in mast cell activation.^{40,41} *prlr*, *socs3* and *twist2* are involved in apoptosis processes; however, *prlr*, *socs3* and *hla-drb5* are connected to signal pathways of IFN gamma, which is considered one of a few key cytokines in tolerance development, and its expression significantly changes during

the build-up phase of grass pollen immunotherapy. It influences switch from Th2 to Th1 cells and suppresses IgE production.^{42,43} TWIST2 suppresses proinflammatory cytokines such as tumor necrosis factor (TNF) α and IL-1B, which play important role in allergic inflammation and might affect the immunosuppressive effect of T and B regulatory cells.⁴⁴

prlr expression also influences the mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signal pathways. Among many other functions, JAK kinases take part in transforming growth factor (TGF) β -dependent activation of T regulatory cells.^{45–47} STAT kinases stimulate Th2 differentiation and, through GATA3, inhibit FOXP3.⁴⁸ A mouse model also suggests that the absence of STAT3 generates hyper eosinophilic syndrome and activates NF- κ B.^{49,50} *Socs3* presents similar functions on STAT. In clinical studies on patients with asthma and atopic dermatitis, *socs3* expression correlates with disease severity.^{51,52}

Cldn1, *commd8* and *slc16a4* are also involved in the regulation of proinflammatory NF- κ B complex.^{28,53,54} *cngb3*, *htrb3*, *hes6* and *fads1* take part in the functioning of the nervous system, which could explain the hypothesis of Siebenhaar *et al.*⁵⁵ regarding the involvement of sense neurons in mast cell activation through a specific IgE.

From 2010 to 2013, Niedożytko *et al.*^{17,18} performed a genome-wide association study in patients receiving VIT. The expression of *cldn1*, *snx33*, *twist2*, *socs3*, *fads1*, *hes6* and *slc16a4* was significantly higher, and the expression of *commd8*, *prlr*, *hla-drb5*, *cngb3* and *htr3b* was significantly lower, in patients with VIT failure compared to those with treatment success. It was suggested that the changed expression is a result of the allergy being present, and treatment should return expression to the levels observed in the control group.^{17,18} As for the comparison between allergic patients and healthy control subjects, convergent results were obtained in the present study (with the exception of *prlr*).

As a comorbidity, cardiovascular disorders comprise the strongest risk factor for severe anaphylaxis,¹ which was evident in the present study. All patients with cardiovascular disease had fourth-degree reaction, whereas those without comorbidity had a third-degree reaction. There was no association with other risk factors such as gender, age or mast cell activation diseases. However, only two patients in the study group had systemic mastocytosis.

In summary, *cldn1*, *cngb3*, *commd8*, *fads1*, *hes6*, *hla-drb5*, *htr3b*, *prlr*, *slc16a4*, *snx33*, *socs3* and *twist2* show a significantly different expression in patients who are allergic to wasp venom. *Commd8* expression changes significantly during the initial phase of VIT. Further studies should concentrate on *commd8* expression changes and their relation to clinical efficacy, with a particular focus on its behavior in VIT failures.

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AUTHOR CONTRIBUTIONS

Karol Kempinski was responsible for study conception and design, analysis and interpretation of data, and drafting the manuscript. Jan Romantowski was responsible for data acquisition and drafting the manuscript. Agnieszka Maciejewska was responsible for data acquisition, analysis and interpretation of data, and drafting the manuscript. Ryszard Pawlowski was responsible for analysis and interpretation of data, and critical manuscript revision. Marta Chelminska was responsible for data acquisition and critical manuscript revision. Ewa Jassem was responsible for study conception and design and critical manuscript revision. Marek Niedożytko was responsible for data acquisition, drafting the manuscript, and critical manuscript revision. All authors approved the final version of the manuscript submitted for publication. All authors agree to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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