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IMMUNE SYSTEM CORRELATION DYNAMICS IN ULTRA-RUSH WASP VENOM IMMUNOTHERAPY

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ABSTRACT

INTRODUCTION: Venom Immunotherapy (VIT) stands as an effective approach for addressing Hymenoptera venom allergies, yet the intricate journey of reprogramming the immune system's hypersensitive response to wasp venom allergens remains a frontier ready for exploration. Generally, VIT orchestrates a shift in the polarization of Th2 and Th1 lymphocytes, fostering a greater prevalence of tolerogenic reactions mediated by Treg lymphocytes and dendritic cells. This orchestrated shift precipitates a decline in the activity and abundance of eosinophils, basophils, and mast cells — key effector cells in allergic reactions. These transformative alterations stem from intricate cell-to-cell communication modulated by cytokines.

MATERIAL AND METHODS: In this investigation, correlated was data derived from the immune system analysis of individuals with wasp venom allergy (Muller's scale III and IV) undergoing VIT, drawing samples at intervals of 0, 2, 6, and 24 weeks post-initial dose. These findings were compared with outcomes from a control group experiencing mild inflammatory reactions post-wasp sting. The authors' scrutiny encompassed 50 diverse immune system components, spanning white blood cell subpopulations, complement components (C3, C4, and C5), concentrations of histamine and tryptase, and selected cytokine concentrations encompassing interleukins, tumour necrosis factor-alpha, interferon-gamma, transforming growth factors beta, chemokines, growth factors, and the IL-1 receptor antagonist. Through meticulous analysis, the present extensive dataset was categorized into distinct groups based on the effects elicited by VIT: immediate, delayed, late effect, and temporary.

RESULTS: Our findings unveiled compelling correlations in various VIT response types, particularly linked to IL4, IL7, IL12, IL15, IL17, and CCL3.

CONCLUSIONS: These observed shifts underscore the pivotal roles individual cytokines and their interactions play in the desensitization process induced by VIT treatment. However, a comprehensive understanding of this intricate process warrants further in-depth investigation.

KEYWORDS: desensitization; wasp venom; VIT; immune system; immunotherapy; allergy

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INTRODUCTION

Hymenoptera stings, inflicted by a diverse order of insects comprising bees, wasps, and ants, are not only a common occurrence in daily life but also a matter of significant medical concern. In addition to food and drug allergies, Hymenoptera venom allergy stands as one of the foremost triggers of anaphylaxis on a global scale [1]. Often perceived as a mere inconvenience, these stings can pose substantial threats to human health due to their potential to induce a wide spectrum of medical conditions. Although the majority of Hymenoptera sting reactions are limited to mild local symptoms, a noteworthy fraction of individuals experience severe and, in some cases, life-threatening systemic reactions [2]. Among the most prevalent manifestations (occurring in up to 26% of cases) are localized oedema, erythema, pain, and pruritus. However, systemic reactions are observed in 0.3% to 7.5% of the European adult population and escalate to an alarming 32% among beekeepers [3, 4]. In the United States, between 2000 and 2017, there were a cumulative 1,109 fatalities resulting from Hymenoptera stings, equating to an annual mean of 62 fatalities. Notably, males accounted for approximately 80% of these recorded deaths [5]. These systemic reactions encompass dermatological, gastrointestinal, respiratory, and cardiovascular symptoms, either singularly or concurrently. Notably, anaphylactic reactions following insect stings can trigger rapid fatality, with initial cardiorespiratory arrest occurring within 5 to 10 minutes of venom exposure. Moreover, Hymenoptera stings impose a substantial healthcare burden on society, contributing to elevated healthcare expenses, including emergency department visits and hospitalizations.

Hence, venom-specific immunotherapy, commonly referred to as desensitization, involves a subcutaneous allergen administration regimen, initially characterized by escalating doses (induction phase) leading to the establishment of tolerance at a maintenance dose, succeeded by regular maintenance doses. The induction phase's duration varies depending on the protocol: it can span from three to five hours (ultra-rush, ultra-fast immunotherapy), several days (rush, rapid immunotherapy), to several weeks (conventional immunotherapy). Previously, the study investigations assessed the short-term (at 2 and 6 weeks) and long-term (at 24 weeks) effects of ultra-rash wasp venom desensitization on parameters such as white blood cell (WBC) counts, lymphocyte phenotypes, the population of natural regulatory T cells (nTreg), levels of specific complement components (C3, C4, and C5), concentrations of histamine and tryptase in peripheral blood [6]. Moreover, the impact of venom immunotherapy (VIT) on serum cytokine concentrations was examined, encompassing interleukins (IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), transforming growth factors beta 1 to 3 (TGF-β1-3), chemokines (MIP-1a (CCL3), MIP-1b (CCL4), MCP-1, RANTES (CCL5), Eotaxin (CCL11), IP-10), growth factors (G-CSF, GM-CSF, PDGF, bFGF, and VEGF), and the receptor antagonist of IL-1 [7].

Here, evaluated were correlations between immune system parameters following VIT treatment in patients with wasp allergy III/IV Müeller grade undergoing VIT treatment and the control group consisting of patients with wasp allergy I/II Müeller grade.

MATERIAL AND METHODS

Study participants

As previously described, the study involved 61 patients presenting with hypersensitivity to wasp venom, recruited from the Department of Infectious Diseases and Allergology at the Military Institute of Medicine in Warsaw, Poland [6, 7]the immune mechanism of acquiring rapid tolerance during VIT has not yet been fully understood. Therefore, we have analyzed (in 4-time points: 0, 2, 6, and 24 weeks after the initiation phase of VIT. The cohort encompassed individuals aged 20 to 70 years, with a median age of 50 years, who did not have any chronic conditions that would contraindicate immunotherapy. A few cases exhibited untreated hypertension without the use of beta-blockers. Patients with active cancer, autoimmune diseases in their active phase, or acquired immunodeficiency syndrome (AIDS) were ineligible, as were pregnant women. Throughout the study period, participants did not experience stings from Hymenoptera. The diagnosis was confirmed through skin and intradermal tests and sIgE measurements in patients with a history of allergic reactions following stings. Patient group allocation was determined based on Müeller criteria, with a focus on individuals displaying allergic reactions after wasp stings. In addition to clinical symptoms, there were conducted assessments of

total IgE and specific IgE (sIgE) levels corresponding to wasp, bee, and hornet venoms. The control group consisted of individuals who exhibited markedly lower sIgE concentrations for wasp venom, generally correlating with a Müeller grade of two or less. Additionally, patients in the control group demonstrated reduced levels of total IgE in their serum when compared to those in the study group. The control group consisted of 18 patients (Müller's grades I and II), while the study group included 43 patients (Müller's grades III and IV). The study was performed according to the Bioethics Committee resolution in the Military Institute of Medicine (No. 130/WIM/2018). All patients signed an informed consent form, and those undergoing VIT received oral antihistamines as a pretreatment before the ultra-rush induction phase and during the maintenance treatment.

Desensitization

Desensitization was exclusively administered to the study group, as comprehensively described previously [6, 7]. In brief, the ultra-rush protocol involved the administration of wasp venom (Venomenhal, 120 μg of wasp venom per vial) at escalating doses (0.1 μg, 1 μg, 10 μg, 20 μg, 30 μg, and 40 μg at 30-minute intervals, totalling 101.1 μg during the induction phase at "point 0"). Subsequently, every four weeks, 100 μg of Venomenhal was administered, commencing two weeks after the initial phase.

Immune system parameters

Serum cytokines concentration (interleukins-1b, –2, –4, –5, –6, –7, –8 (CXCL8), –9, –10, –12, –13, –15, –17A, TNF-α, IFN-γ, TGF-β1-3) chemokines (MIP-1a (CCL3), MIP-1b (CCL4), MCP-1, RANTES (CCL5), Eotaxin (CCL11), IP-10) growth factors (G-CSF, GM-CSF, PDGF, bFGF and VEGF) and receptor antagonist of IL-1), WBC and Lymphocytes phenotypes (T (CD3+), activated T (CD3+HLA-DR) cells, B (CD19+) cells, helper/inducer (CD4+) and suppressor/cytotoxic (CD8+) cells, and natural killer (NK) (CD16+CD56+) lymphocytes), nTreg cells analysis, complement system analysis as well as histamine and tryptase analysis were performed as described previously at 0, 2, 6, and 24 weeks following the induction phase [6, 7]. Briefly, cytokine levels were assessed using the Bio-Plex Pro TGF-β Panel 3-Plex (Bio-Rad) and Bio-Plex Pro Human Cytokine Grp I Panel 27-Plex (Bio-Rad, Poland). Analysis was conducted utilizing a Bio-Plex 200 system in conjunction with Bio-Plex Manager version 6.1.1, employing a 5-parameter (5-PL) nonlinear logistic regression curve fit model. White blood cell phenotypes were determined by morphological parameters (FSC/SSC) and CD45+/ /CD14+ identification using the Simultest™ — IMK Plus Kit (BD Biosciences, Poland). Lymphocyte immunophenotyping in peripheral blood involved incubation with specific antibodies: Leucogate™ (A CD45 FITC/CD14 PE), Isotype Control (IgG1 FITC/ /IgG2a PE), CD3 FITC/CD19 PE, CD3 FITC/CD4 PE, CD3 FITC/CD8 PE, and CD3 FITC/CD16 PE + CD56 PE. Subsequent erythrocyte lysis was carried out using BD FACS Lysing Solution (BD Biosciences, Poland) for 10 minutes at room temperature in the dark, followed by cell washing with PBS, fixation in 1% paraformaldehyde (PFA) in PBS, and flow cytometry analysis (FACS Calibur, BD Warsaw). Distribution percentages of white blood cell subtypes (lymphocytes, monocytes, granulocytes, neutrophils, eosinophils) were determined via CD45 FITC, CD14 PE antibody staining, and FSC/SSC assessment. Natural Treg cells were analyzed through cytometric methods involving staining with primary antibodies CD4-PerCP, CD25-APC, CD127-FITC (extracellular staining, BD Biosciences, Poland), or appropriate isotype controls in addition to CD4-PerCP antibody. The blood samples underwent staining, fixation, permeabilization, and subsequent flow cytometry analysis to identify nTregs defined as CD4+/CD25high/CD127low/ /FoxP3+. Serum complement C3 and C4 components were evaluated nephelometrically using MininephTM C3 or C4 Kit reagents (Binding Site Group Ltd., Birmingham, UK) and the MININEPHPLUS analyzer. Histamine and tryptase concentrations in serum were assessed via the ELISA method (FineTest®, Wuhan, China) using 50 μL of serum for histamine and 100 μL for tryptase, with all samples evaluated in duplicate.

Correlation analysis

The correlations between immune system parameters were evaluated using GraphPad Prism software (version 10.1.0; GraphPad Software, Inc., La Jolla, CA, USA). The initial assessment involved verifying data distribution through the Shapiro-Wilk test. For each defined time point (0, 2, 6, and 24 weeks post the induction phase), Spearman correlations were calculated for all immune system parameters under investigation. Subsequently, data exhibiting a correlation coefficient $|R| > 0.5$ and a p value

Table 1. Classification of correlation effects on immune system parameters

Control	0 weeks	2 weeks	6 weeks	24 weeks	Group
Correlation	No correlation	Correlation	Correlation	Correlation	Immediate Effect
No correlation	Correlation	No correlation	No correlation	No correlation	
Correlation	No correlation	No correlation	Correlation	Correlation	Delayed Effect
No correlation	Correlation	Correlation	No correlation	No correlation	
Correlation	No correlation	Correlation	Correlation	No correlation	Temporary Effect
No correlation	Correlation	No correlation	No correlation	Correlation	
Correlation	No correlation	No correlation	No correlation	Correlation	Late Effect
No correlation	Correlation	Correlation	Correlation	No correlation	

< 0.05 were extracted. The results were categorized based on the timing of correlation occurrence and classified into groups following the criteria outlined in Table 1. The defined categories include:

Immediate Effect: A pattern of correlation between immune system parameters in which the unification between control and study groups was observed shortly after the VIT induction phase.

Delayed Effect: A pattern of correlation between immune system parameters in which the unification between control and study groups was observed, but with a delay in their manifestation.

Temporary Effect: A pattern of correlation between immune system parameters in which the unification between control and study groups was observed but not sustained over the observation period.

Late Effect: A pattern of correlation between immune system parameters in which the unification between control and study groups was observed at the last time point during the study.

RESULTS

Spearman analysis revealed a total of 252 significant correlations in the control group, 131 in the 0 weeks group, 158 in the 2 weeks group, 195 in the 6 weeks group, and 81 in the 24 weeks group, with statistical criteria of $|R| > 0.5$ and a p value < 0.05. Figure 1 illustrates the heatmap corresponding to each time point/group.

Following the timing of correlation occurrence analysis, 22 correlation patterns were categorized in the Immediate Effect group, 6 in the Delayed Effect group, 18 in the Temporary Effect group, and 19 in the Late Effect group. The p value for each correlation was < 0.05. The results corresponding to each category are presented in Tables 2 to 5, respectively.

DISCUSSION

The immune system's efficacy relies on establishing discernible patterns during the initial encounter between foreign entities and immune cell populations. This pivotal juncture dictates the immune cell's strategic response to impending threats through collaborative interactions with diverse somatic and immune cell types. The latter possesses an array of receptors, including but not limited to Toll-like receptors (TLR), NOD-like receptors (NRL), retinoic acid-inducible gene-I (RIG-I)-like receptors, tripartite motif-containing (TRIM) receptors, C-type lectin receptors (CLC), and T-cell immunoglobulin and mucin domain (TIM) receptors, that facilitate the recognition of foreign agents.

Cytokines serve as informational mediators regarding the identified threat, orchestrating the activation and augmented migration of specific immune cell subsets to the site of peril. Additionally, cytokines play a pivotal role in regulating the differentiation of naïve T-helper (Th) lymphocytes, transitioning them from a naïve state (Th0) to specialized phenotypes such as Th1, Th2, Th9, and Th17, among others. These differentiated Th lymphocytes subsequently assume command over the immune cell response. Following the eradication of the threat, a fraction of the specialized Th lymphocytes transform into memory cells, which take residence in secondary lymphatic organs, poised for subsequent encounters with the antigen. The sustained presence of lymphocytes in these locations is attributed to the expedited and reinforced immune system response upon subsequent antigen contact.

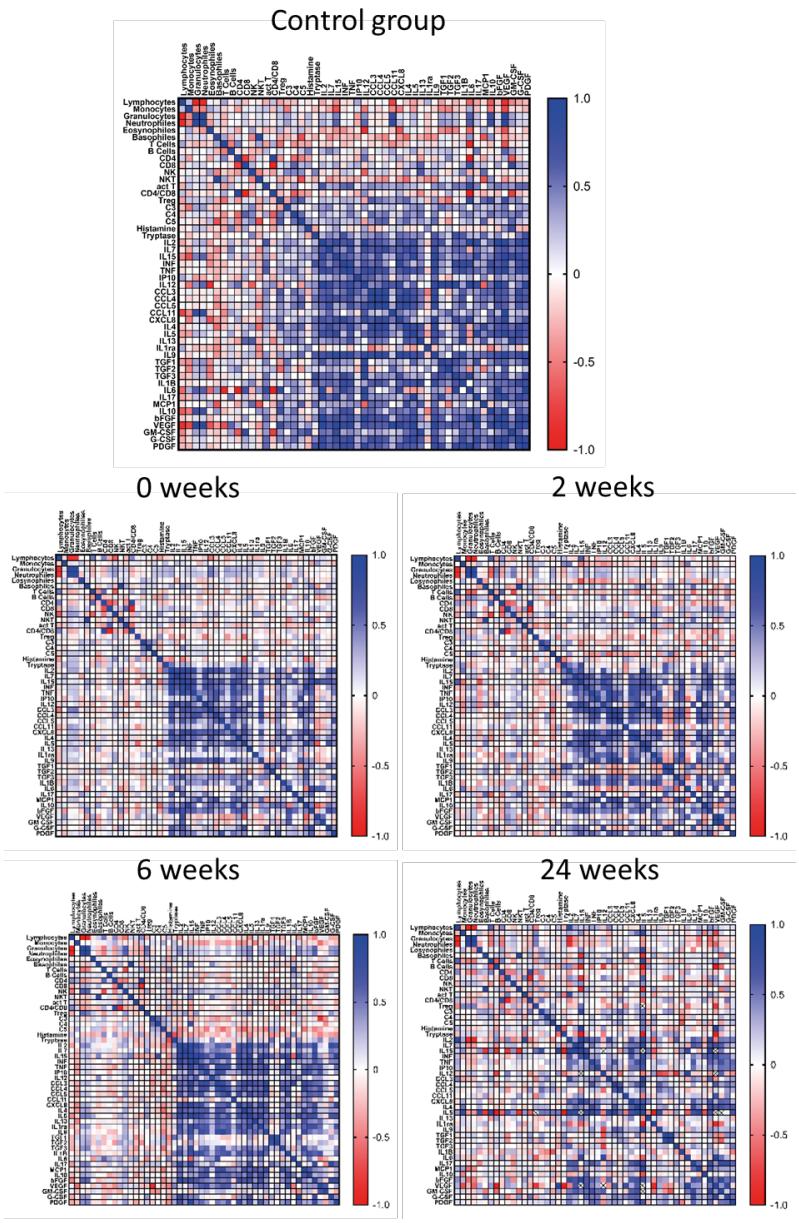

FIGURE 1. Heatmaps of correlations were identified in the control group, 0 weeks group, 2 weeks group, 6 weeks group and 24 weeks group using Spearman analysis. The bar on the right side of each map indicates the colour legend of the Spearman correlation coefficients

Allergic reactions manifest when the immune system aberrantly responds to typically innocuous substances. Initial exposure to wasp venom may not elicit an immediate allergic reaction, yet it contains a diverse array of proteins and peptides, including phospholipase A1 and A2, hyaluronidase, antigen 5, or mastoparan. Localized inflammation arises at the sting site, accompanied by the development of Th2 cells. Cytokines produced by Th2 cells exert influence on both innate and adaptive cells, instigating heightened production of eosinophils and basophils, as well as inducing a class switch in antibodies from IgG to IgE in B-cells. IgE antibodies bind to FcεRI receptors on mast cells and basophils, instigating the release of inflammatory mediators such as histamine, prostaglandins, and leukotrienes upon subsequent exposures. These mediators are culpable for the clinical symptoms associated with allergies. Efforts to recondition the immune system to cease its aberrant response to wasp venom prove challenging and protracted. Ultra-rash desensitization procedures involving the administration of wasp venom injections span a five-year duration, with a frequency of every four weeks in the first year, every

Table 2. Immediate Effect group. Correlation patterns — Spearman correlation coefficient R, p < 0.05

Correlation	Control	0 weeks	2 weeks	6 weeks	24 weeks
Lymphocyte x CCL3		–0.5074			
Neutrophile x CCL3		0.5011			
Treg x IL-5		–0.5961			
Histamine x IL-5		–0.6109			
Histamine x IL-10		–0.5539			
IL-7 x IL-12		0.6456			
IL-7 x CXCL8	0.5845		0.6448	0.6704	0.5098
IL-7 x IL-6		0.5301			
IL-15 x IL-10		0.5542			
TNF-α x IP10	0.5271		0.6544	0.6003	0.5981
TNF-α x PDGF	0.7535		0.5731	0.5543	0.5631
IL-12 x CXCL8		0.5957			
IL-12 x IL-13		0.5435			
IL-12 x IL-1B		0.5169			
CCL4 x IL-4	0.8638		0.5277	0.6250	0.5823
CCL11 x IL-5		0.5876			
IL-4 x IL-6		0.5613			
TGFb1 x TGFb2	0.7181		0.7631	0.7931	0.8607
TGFb3 x IL-10		0.5538			
IL-6 x VEGF		0.6129			
IL-6 x GM-CSF		0.6092			
IL-17A x G-CSF	0.7981		0.6683	0.8685	0.7874

Table 3. Delayed Effect group. Correlation patterns — Spearman correlation coefficient R, p < 0.05

Correlation	Control	0 weeks	2 weeks	6 weeks	24 weeks
IL-15 x IL-12		0.5741	0.8485		
IL-15 x IL-4		0.6506	0.5722		
CCL4 x IL-5	0.7277			0.5100	0.9747
CXCL8 x MCP1		0.5358	0.7377		
TGFb1 x G-CSF		0.5562	0.5825		
IL-17A x PDGF	0.6829			0.5652	0.5769

six weeks in the second year, and every eight weeks in the third to fifth years [8].

The meticulous examination of alterations occurring during desensitization is conducted with a dual purpose: to comprehend the dynamic changes unfolding within the immune system and to identify strategies for expediting this process. The initial stages of the immune response after VIT treatment are well-documented in the scientific literature [9, 10]. The early response to VIT is intricately linked to modifications in the activity of effector cells within the allergy-associated innate immune system, namely basophils and mast cells. These cells demonstrate a diminished capacity for activation, degranulation, and migration, attributed to a concomitant reduction in the binding of IgE particles to their specific FcεRI receptors [11, 12]. Furthermore, the observed decrease in IL-4 and IL-13 production and

Table 4. Temporary Effect group. Correlation patterns — Spearman correlation coefficient R, p < 0.05					
Correlation	Control	0 weeks	2 weeks	6 weeks	24 weeks
IL-7 x TNF-α	0.7303		0.5852	0.5214	
IL-7 x CCL3	0.6135		0.6200	0.5101	
IL-7 x IL-5	0.7105		0.5543	0.5942	
IFN-γ x IL-1B	0.8070		0.6450	0.6891	
TNF-α x TGFb3	0.6266		0.6074	0.5166	
TNF-α x IL-1B	0.7774		0.6127	0.7011	
CCL3 x CCL5	0.8649		0.7124	0.5559	
CCL3 x IL-13	0.5101		0.5195	0.6322	
CCL3 x IL-1B	0.6125		0.6086	0.5593	
CCL3 x PDGF	0.5978		0.6340	0.5012	
CCL4 x IL-1B	0.6875		0.6384	0.6168	
IL-4 x IL-9	0.8658		0.6139	0.6051	
IL-5 x IL-13	0.5872		0.7146	0.6064	
IL-9 x TGFb3	0.6212		0.5850	0.6255	
IL-9 x IL-1B	0.7882		0.6177	0.5606	
TGFb1 x IL-17A	0.6829		0.5889	0.5296	
IL-1B x bFGF	0.5462		0.5190	0.5785	
IL-1B x PDGF	0.7649		0.6346	0.5113	

Table 5. Late Effect group. Correlation patterns — Spearman correlation coefficient R, p < 0.05					
Correlation	Control	0 weeks	2 weeks	6 weeks	24 weeks
act T x IL-2	0.6475				0.7653
IL-2 x IL-10		0.5490	0.5909	0.5591	
IL-7 x IL-15		0.7175	0.6690	0.8516	
IL-7 x IFN-γ		0.6146	0.6928	0.6081	
IL-7 x IL-17A	0.5226				0.6296
IL-15 x CCL3		0.6563	0.6045	0.8375	
IFN-γ x IL-12		0.6750	0.6575	0.6064	
IFN-γ x CCL3		0.6260	0.7039	0.5102	
IFN-γ x IL-5		0.5494	0.7076	0.8415	
IFN-γ x G-CSF	0.6916				0.5420
TNF-α x IL-17A	0.5551				0.6326
IP10 x CCL4		0.6545	0.6710	0.7205	
IP10 x MCP1		0.6793	0.7805	0.6869	
CXCL8 x IL-1B		0.5375	0.7392	0.6401	
IL-4 x G-CSF	0.7746				0.5868
IL-4 x PDGF	0.6347				0.6344
IL-5 x IL-10		0.7019	0.5507	0.7372	
VEGF x GM-CSF		0.5295	0.8525	0.6692	
G-CSF x PDGF	0.8221				0.5467

concomitant increase in the secretion of TGF-β, IL-10, and IL-22 contribute to alterations in response cells. These changes augment the antigen-tolerant potential of dendritic cells and T-helper cells, along with an elevation in the level of regulatory T cells [13–15]. Despite these advancements, gaps persist in understanding the subsequent stages of the desensitization process. Consequently, previous investigations focused on the analysis of selected immune system parameters at intervals of 2, 6, and 24 weeks post-ultra-rush VIT desensitization, as outlined in the prior publications [6, 7]. 6, and 24 weeks after the initiation phase of VIT.

Given the intricate interplay among immune system components, the present study adopts a comprehensive approach by scrutinizing correlations among obtained immune parameters within both control and experimental groups. These correlations are categorized into four groups based on temporal effects: immediate, delayed, late, and temporary effects. Notably, the initial analysis reveals pronounced correlations among the percentages of immune cells, specifically lymphocytes with granulocytes, lymphocytes with total neutrophils, and granulocytes with total neutrophils, within both control and experimental cohorts. These findings substantiate the robustness and validity of the statistical analyses employed in the investigation.

In the following investigation, a limited number of correlations among cells and cytokines was identified — three in the immediate response group and one in the late response group. Of particular interest was the relationship between CCL3 and neutrophils (positive) and lymphocytes (negative), observed solely within the experimental group. Notably, CCL3, a chemokine primarily associated with the progression of inflammatory and autoimmune conditions, exhibited a dysregulated pattern that normalized post-VIT treatment [16, 17].

Furthermore, the intriguing role of IL-5 in the immediate effect group merits attention. IL-5 exhibits a negative correlation with regulatory T cells and histamine while displaying a positive correlation with CCL11 (eotaxin). The dysregulation of Tregs, failing to inhibit the IL-5/CCL11 axis, promotes the development and migration of eosinophils to tissues. VIT induces alterations in these relationships, leading to an augmentation in the quantity, activity, and homing of Treg cells [18, 19]. Building on the findings of Caramalho et al. [20], who demonstrated an increase in the proliferation of regulatory T cells (Treg) during VIT with bee venom, defined as CD4+, CD127 (IL-7 receptor), CD25+, and Foxp3-positive, we delve into the critical IL-7/IL-7R axis. This axis plays a pivotal role in the homeostasis of all T cells, impacting allergen-induced memory CD4+ T cells [21]. Intriguingly, in patients with high Muller grade (III and IV), IL-7 concentration exhibits a positive correlation with IL-12 and IL-6, both cytokines known to promote Th1 development and mitigate Th2 influence [22]. Conversely, IL-7 shows no correlation with CXCL8, a well-known chemoattractant for various immune cells. Additionally, IL-12 concentration displays positive correlations with IL1B, CXCL8, and IL13, exclusively in the experimental group. Furthermore, a correlation between IL5 and CCL11 in the experimental group underscored the allergic nature of this cohort. Both IL-5 and eotaxin independently induced rapid and substantial blood eosinophilia in wild-type mice [23]. IL-5, moreover, amplified eotaxin-induced chemotaxis in the airway compartment [24]. The observed correlations within the immediate effect group did not correspond to significant alterations in cytokine concentrations between groups, as previously demonstrated in the authors' work. However, after VIT, these correlations normalized in the experimental groups compared to the control group [7].

The subsequent phase of the present analysis encompasses the delayed effects following VIT treatment. Notably, a significant number of correlations within this phase were identified involving IL-15. This cytokine plays a pivotal role in both inflammatory responses and protective immunity against microbial and parasitic challenges. Exhibiting remarkable pleiotropy, IL-15 exerts influence on cells involved in both innate and adaptive immune responses [25]. In a murine model of allergen-induced asthma, Venkateshaiah et al. demonstrated that IL-15 exerts an inhibitory effect on the airway obstruction triggered by environmental allergens [26]. In the present study, IL-15 demonstrated correlations exclusively within the experimental group, notably with two counteracting cytokines, IL-4 and IL-12. A correlation between IL-15 and IL-4 might suggest a potential interaction between the activation of T cells (IL-15) and the differentiation of Th2 cells (IL-4) in the context of the allergic response to Hymenoptera venom. On the other hand, a correlation between IL-15 and IL-12 might signify a potential crosstalk between the activation of T cells (IL-15) and the induction of Th1 responses (IL-12). Importantly, these correlations

remained unaltered immediately (2 weeks) following VIT treatment. The concurrent manifestation of these correlations may imply an augmented state of immunological activity. Therefore, monitoring these correlations during VIT could help understand how the treatment affects the balance between T cell activation and the allergic response mediated by Th2 cells. A disappearance of correlation might indicate a shift away from the allergic response. Further correlations were observed between CXCL8 and CCL2. Studies by Vantur et al. in human patients underscored the role of CCL2-mediated chemotaxis in the pathophysiology of basophil-induced anaphylaxis [27]. Noteworthy observations from the authors' prior work revealed a significant decrease in CCL2 concentration at 2 weeks post-treatment compared to the control group, followed by an increase during the extended period of VIT treatment [7]. These fluctuations suggest that VIT may initially attenuate the allergic response mediated by basophils. Furthermore, while correlations between IL-17A and PDGF were present in the control group but absent in the experimental cohort, VIT treatment reinstated this correlation after 6 weeks. IL-17A, recognized for its role in allergy promotion, has been linked to Th2 cell activation, eosinophil accumulation, and serum IgE production in studies employing IL-17A−/− or IL-17RA−/− mice [28]. VIT modulates the immune response, shifting it from a predominant Th2 response towards a more balanced state involving other T cell subsets, potentially including Th17 cells. As the treatment progresses, the balance between Th2 and Th17 responses might normalize, reinstating correlations seen in healthy individuals.

The subsequent group of dependency identified in the present analysis is characterized as a temporary effect, where correlations among immune system parameters manifest a discernible pattern of change between control and study groups that are not sustained over the observation period. Notably, intriguing correlations are observed in this group, particularly concerning the interactions involving CCL3, a chemokine implicated in activation and serving as a secondary signal for mast cell degranulation [29]. Elevated CCL3 levels have been documented following nasal allergen stimulation by pollen grass, especially in individuals with allergic conditions like seasonal allergic rhinitis [30]. In the control group, CCL3 exhibited positive correlations with IL-7, IL-13, CCL5, PDGF, and IL-1b.

CCL3 and CCL5 are chemokines that play roles in recruiting immune cells, especially monocytes and T cells, to sites of inflammation. IL-7 supports the survival and proliferation of lymphocytes. PDGF on the other hand, plays a role in tissue repair, fibrosis, and immune modulation [31]. In healthy individuals, correlations between CCL3 and IL-7, CCL3 and IL-1b, as well as CCL3 and IL-13, indicate a balanced interplay between chemokines (like CCL3) and cytokines involved in inflammation, immune cell regulation, and activation. Moreover, in healthy individuals, the correlation might reflect a coordinated response where immune cell recruitment (CCL3) is associated with subsequent tissue repair (PDGF). This balance is disrupted in allergic patients due to the skewed immune response, affecting the expected correlations. Another noteworthy observation in this group is the correlation involving the proinflammatory cytokine IL-1b and bFGF, PDGF, TNF-α, CCL4, and IL-9. IL-1b exerts its influence on various immune cells through a non-classical secretion pathway, encompassing processes such as exocytosis of secretory lysosomes, exocytosis of exosomes, shedding of plasma membrane microvesicles, or export through the plasma membrane via specialized transporters [32]. In conjunction with IL-6, IL-1b plays a role in the development of naïve Th0 to Th17 cells, stimulating the secretion of IL-17 by these cells [33]. The correlations observed within the temporary effect group, where parameters between control and study groups normalized briefly but were not sustained over time, likely mirror the mechanisms underlying the clinical long-term ineffectiveness of VIT in certain patients. Yet, discerning the precise factors responsible remains elusive based on current data.

In the present investigation, the final group scrutinized is the late effect group, characterized by a pattern of correlation among immune system parameters where normalization to the control group was observed at the study's last time point. The associations involving IFN-γ and IL-7, IL-12, CCL3, IL-5, and G-CSF are of particular interest. IFN-γ, a pivotal cytokine associated with Th1-type immune responses, plays a crucial role in activating macrophages, enhancing antigen presentation, and promoting the differentiation of Th1 cells. It holds significance in mediating cellular immunity against pathogens. IL-12, a cytokine that propels the differentiation of naive T cells into Th1 cells, stimulates IFN-γ production and contributes to the promotion of cellular immunity. These

correlations may signify the nature and balance of Th1 responses, which are instrumental in counteracting allergic Th2-dominated reactions [34]. Such interactions have the potential to influence the outcome and severity of allergic responses to venom allergens. Notably, IFN-γ might engage with G-CSF to regulate the intensity or duration of the inflammatory response elicited by Hymenoptera venom. An intriguing finding relates to the positive correlation observed between IP-10 and both CCL4 and MCP-1. Within allergy patients, the correlation between IP-10 and CCL4, alongside IP-10 and MCP-1, hints at a synchronized chemotactic reaction. IP-10, CCL4, and MCP-1 are chemokines known for their roles in the recruitment and activation of immune cells, notably T cells and monocytes/macrophages [35]. These correlations suggest a coordinated chemotactic response, likely orchestrating the recruitment of immune cells in response to hymenoptera venom, specifically within the altered immune milieu characteristic of allergic conditions. In the present investigation, positive associations were identified between IL-10 and IL-5 (Th2-related cytokine) as well as IL-2 (Th1-related cytokine). IL-10 stands as a pivotal component among immune-suppressive factors, crucial for inducing tolerance against allergens and mitigating allergic inflammatory responses mediated by mast cells, basophils, and eosinophils [36]. Additionally, the induction of dendritic cells that produce IL-10 is regarded as a mechanism to dampen allergen-specific Th2 responses [37]. Although the positive correlation between cytokines representing opposing T cell developmental pathways might appear puzzling, it predominantly underscores the suppressive function of IL-10 in modulating diverse inflammatory conditions, inclusive of those elicited by VIT. A schematic representation of the putative functions of selected immune system parameters is presented in Figure 2.

Unravelling these correlations and the altered patterns in venom allergy patients compared to healthy individuals can provide crucial insights into the regulatory networks underpinning allergic responses. These findings may unveil potential targets for therapeutic intervention or the identification of specific biomarkers associated with the allergic phenotype. Further comprehensive investigation is imperative to fully decipher these intricate immunological shifts.

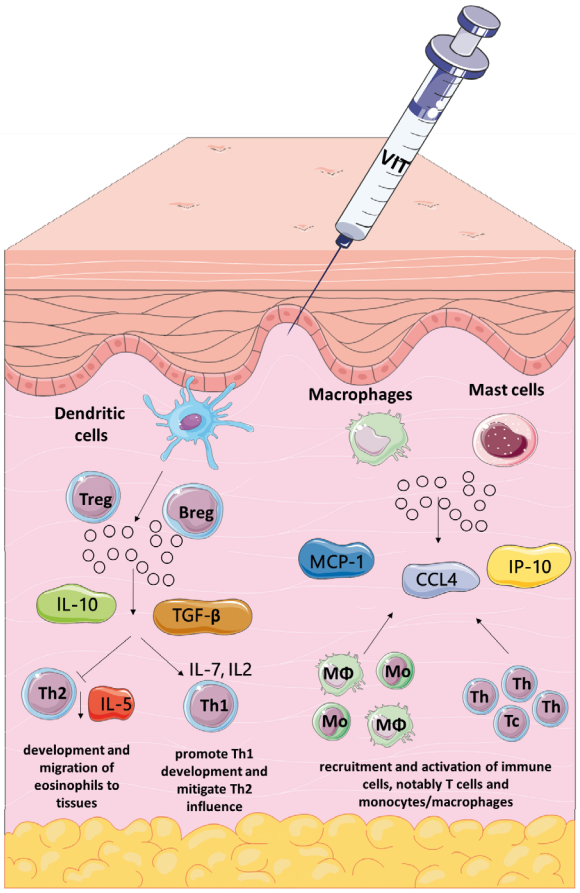

FIGURE 2. Schematic representation of potential functions of selected immune system parameters, including IL-10, IL-5, IP-10, CCL4, and MCP-1, within the venom allergic process

Article information and declarations

Data availability statement

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of Military Institute of Medicine, Warsaw Poland, resolution No. 130/WIM/2018.

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Conflict of interest
The authors declare no competing interests.

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