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Analysis of the Transcriptional Activity of Immune Response Genes in the Blood of Patients with Acute Urticaria

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Abstract

OBJECTIVE: Alterations in the transcriptional activity of some immunoregulatory genes can play a key in the pathogenesis of acute urticarial (AU). Minimally-invasive markers of the transcriptional activity of immune response genes are essential not only for predicting the severity and activity of the disease but also as a potential target for therapy.

METHODS: In our research, we applied a pathway-specific polymerase chain reaction PCR array (Human Innate and Adaptive Immune Responses RT2 Profiler PCR Array, QIAGEN, Germany) to detect and verify innate and adaptive immune responses pathway-focused genes expression in patients suffering from AU and control group.

RESULTS: The AU development was accompanied by an increase in the transcriptional activity of genes for a number of costimulation molecules such as CD40, CD40LG, CD80 (B7-1), and C-reactive protein and myeloperoxidase genes either. Under AU conditions, transcriptional induction of genes of several cytokines was also observed: Interferon gamma, interleukin (IL4), IL5, IL17A, tumor necrosis factor, and also chemokine CXCL8. This process was also accompanied by an increase in the transcriptional activity of the RAR-related orphan receptor C Th17 differentiation regulator, the NLRP3 inflammasome genes, and the NFKB1 transcription factor. Such changes occurred against the background of transcriptional repression of the FOXP3 gene and the Treg-dependent suppressor cytokine IL10. The expression of other studied genes did not differ significantly from the controls.

CONCLUSIONS: The development of acute urticaria led to the transcriptional activation of pro-inflammatory signaling against the background of a deficiency of the suppressor link. Detected changes in gene expression can be important for targeted therapy.

Introduction

Urticaria is a skin disorder that manifests by the appearance of wheals and/or angioedema. The development of acute urticaria (AU) might be caused by triggers, such as infections, hypersensitivity to food or drug, physical triggers, insect bites, and have idiopathic origin [1]. The diagnosis of this disorder is made by detailed clinical history and physical examination. AU is defined as a skin condition lasting < 6 weeks, commonly self-limiting which disappears within 30 min–24 h. The pathophysiology of AU is not sufficiently comprehended. Still, it is obvious that derangement of both mast cell and basophil activation and degranulation plays a crucial role in the process. Two mechanisms are reported as significant concerning AU pathogenesis [2]. The first mechanism involves dysregulation of intracellular signaling pathways within mast cells and basophils that cause the impairment of trafficking or function of these cells. The second mechanism impacts the development of autoantibodies to FcεRIα or immunoglobulin E (IgE) on both, namely mast cells and basophils.

Two major groups of immune signals influence the production of IgE, which triggers an antigen response: (1) activation and differentiation of CD4 naive T cell to T helper type 2 (Th2) cells by the signals and (2) the cytokines and co-stimulatory molecules secreted by Th2 cells, which stimulate T follicular helper cells-induced immunoglobulin B cells class switch directed to IgE production [3]. Such antigen characteristics like concentration and localization of the encounter (namely, tissue, mucosa, circulation) can also impact Th2 cell induction. Interleukin (IL-4), IL-13, and STAT6 are critical mediators of Th2 responses and IgM class switch to IgE. IL-4 secretion and mast cell CD40 surface expression also promotes the IgM class switch to IgE at the site of hypersensitivity reaction to a particular allergen. Urticaria is marked by the presence of perivascular non-necrotizing cellular infiltrate observed around small venules of the skin. It consists mainly of CD4 (+) lymphocytes, the predominant T-helper (Th) 2 subtype, and also Th1 cells, with an elevation of Th17 cell-derived cytokines in plasma. Neutrophils, eosinophils, basophils, and monocytes are also observed. Chemokines originated from mast cells

and activated endothelial cells start the process [4].

Transcriptional induction or repression of genes is reported as an essential index of the functional activity of cells in various diseases [5], [6], [7], [8], [9] and to find new targets for therapy [10], [11], [12], [13], [14]. Changes in the transcriptional activity of several immunoregulatory genes can play a critical role in urticaria pathogenesis. Thus, Lin *et al*. [15] detected 5 considerably upregulated microRNAs (miRNAs) (2355-3p, 4264, 2355-5p, 29c-5p, and 361-3p), 8 considerably downregulated genes (e.g., CCNG2), and 5 considerably upregulated genes (e.g., THBS1, selectin E and CCL2). Moreover, the polymorphisms of genes associated with urticaria are important in predicting the population predisposition to diseases and drug responsiveness. For instance, ORAI1 and FCER1A polymorphisms can predict the potential therapeutic effect on patients treated with antihistamines [16]. The study reported that in patients with urticaria treated with omalizumab about 75% of lesional skin gene expression levels returned to normal levels. These genes are FCER1G, C3AR1, CD93, S100A8, S100A9, CYR61, KRT6A, and KRT16 [17]. Giménez-Arnau *et al*. [18] assessed the gene expression levels in patients with resistant responses to antihistamine therapy and found 130 abnormally expressed genes involved in epidermal cell differentiation, inflammation, blood coagulation, intracellular signal transduction, and other functions.

Non-invasive markers of the transcriptional activity of immune response genes are significant in a specific environment: (1) As a possible treatment target, (2) in the prediction of the disease severity and activity, and (3) in the evaluation of drug efficacy [19]. Different markers have been investigated in earlier studies where IgE is defined as clearly related to the severity of urticaria. Since mast cells can be activated in IgE or non-IgE-dependent manner and promote the release of histamine and leukotrienes, medications targeting IgE/FcεRI or histamine have been suggested for clinical purposes. Among such medications are omalizumab, non-sedating antihistamines, and montelukast, a leukotriene antagonist [20]. Therefore, our work aimed to study the transcriptional activity of the innate and adaptive immune system genes in the peripheral blood of patients with AU.

Materials and Methods

Participants and study design

Peripheral blood mononuclear cells (PBMC) from 12 patients with AU and 12 healthy individuals isolated. The control group included healthy subjects selected randomly without age and sex matching. AU was diagnosed with rashes that appeared on the skin. Since there are no established laboratory standards, the diagnostic procedure begins with a typical patient examination, such as detailed history taking, physical examination, and the exclusion of severe systemic disease using basic laboratory tests. To perform a physical examination of the patient, we also used a test for dermographism. Disease activity was determined using the urticaria activity score (UAS). This simple scoring system is based on the evaluation of main symptoms such as wheals and pruritus. The 2018 EAACI/GA2LEN/EDF/WAO diagnosis guideline advocates using the UAS suggested in the previous guideline version for measuring disease severity and observing treatment outcomes in daily practice. The UAS allocates a score from 0 to 3 for each of the 2 crucial urticaria symptoms, such as wheals and pruritus. The total scores display disease severity on a scale from 0 (minimum) to 6 (maximum).

RNA isolation and cDNA synthesis

Total RNA was isolated from PBMC using NucleoZOL (Macherey-Nagel, Germany) by protocol recommended by the manufacturer. The concentration and quality of the extracted RNA were defined by A260/A280 ratio in the range of 1.8-2.2 measured by NanoDrop spectrophotometer (Thermo Scientific™, USA). cDNA synthesis was performed using The RT2 HT First Strand Kit (Quigen, USA).

Polymerase chain reaction (PCR) array

cDNA was used for RT2 Profiler PCR Array (QIAGEN, Cat. no. PAHS-052ZA) combined with SYBR Green qPCR Mastermix (QIAGEN, Cat. no. 330504) following RT2 Profiler PCR Array procedure. Cycle threshold (CT) values were normalized to the panel of reference genes. CT values lower than 35 were considered as a negative hit. The PCR Array data analysis software was used to calculate expression changes using the ΔΔCt method. The software enables the determination of the best reference genes for normalization.

Statistical analysis of PCR array data

The student's t-test was used to define the difference between groups.

Ethical approval

The study was performed following the ethical principles of the Declaration of Human Rights adopted in Helsinki in 1975 and revised in 2008. Subjects completed and signed a written informed agreement. The local ethics committees of Ternopil National Medical University approved the study protocol.

Results

Using the RT2 Profiler PCR Array Human Innate and Adaptive Immune Responses, we studied the expression of 84 genes implicated in immune reactions. This array comprises genes associated with the IL-1R and Toll-like Receptor (TLR) signaling pathways, host defense to bacteria and viruses, innate immune response, and septic shock. Studied genes can be conventionally divided into several functional groups, which partially overlap with each other, due to that many of the presented genes simultaneously regulate both innate and adaptive immune responses.

Innate Immunity genes included Pattern Recognition Receptors (DDX58, NLRP3, NOD1 (CARD4), NOD2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9), cytokines (CCL2 (MCP-1), CCL5 (RANTES), CSF2 (GM-CSF), CXCL10, CXCL8 (IL8), IFNA1, IFNB1, IL18, IL1A, IL1B, IL2, tumor necrosis factor [TNF]) and other Innate Immunity Genes (APCS, C3, CASP1, CD14, CD4, CD40 (TNFRSF5), CD40LG, CD8A, C-reactive protein (CRP), HLA-A, HLA-E, IL1R1, IRAK1, IRF3, IRF7, ITGAM, LY96 (MD-2), LYZ, MAPK1 (ERK2), MAPK8 (JNK1), MBL2, myeloperoxidase (MPO), MX1, MYD88, NFKB1, NFKBIA (IκBα, MAD3), STAT1, TICAM1 (TRIF), TRAF6). Adaptive Immunity genes included Th1 cells Markers and Immune Response (CCR5, CD80, CXCR3, Interferon gamma [IFNG], IL18, IL23A, SLC11A1, STAT4, TBX21, TLR4, TLR6), Th2 cells Markers and Immune Response (CCR4, CCR8, CD86, GATA3, IFNB1, IL10, IL13, IL18, IL4, IL5, IL6, NOD2, STAT6), Th17 cell Markers (CCR6, IL17A, RAR-related orphan receptor C [RORC], STAT3), Treg Markers (CCR4, CCR8, FOXP3, IL10), T-Cell Activation, costimulation molecules and transcriptional factors (CD80, CD86, ICAM1, IFNG, IL23A, IL6, SLC11A1, CD4, CD40 (TNFRSF5), CD40LG, CD8A, CRP, FASLG (TNFSF6), HLA-A, IFNAR1, IFNGR1, IL1B, IL1R1, IRF3, IRF7, ITGAM, JAK2, MAPK8 (JNK1), MBL2, MX1, NFKB1, RAG1, STAT1), T-cells and B-cells dependent Cytokines (CCL2 (MCP-1), CCL5, CSF2 (GM-CSF), CXCL10 (INP10), CXCL8 (IL8), IFNA1, IFNG, IL10, IL13, IL17A, IL18, IL2, IL23A, IL4, IL5, IL6, TNF), Humoral Immunity genes (C3, CCL2 (MCP-1), CCR6, CRP, IFNB1, IFNG, IL6, MBL2, NOD2, TNF), and Inflammatory Response genes (APCS, C3, CCL5, CRP, FOXP3, IL1A, IL1B, IL4, IL6, MBL2, STAT3, TNF).

We found that AU was accompanied by changes in transcriptional activity of certain genes (Figure 1). The mRNA levels of the two genes were significantly decreased and of 14 genes increased.

Figure 2 shows the significant changes in PBMCs of AU patients. mRNA levels of CD40 (or TNFRSF5, TNF receptor superfamily member 5) - costimulatory protein of antigen-presenting cells CD154 (TNFSF5) located on T-helpers was increased

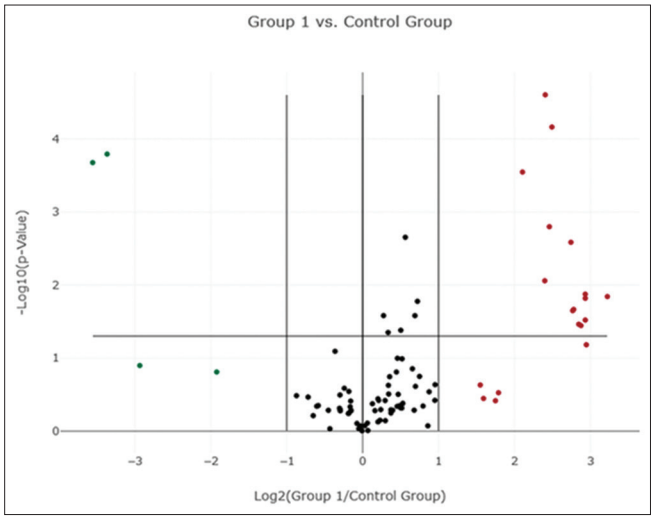

Figure 1: Transcriptional changes of 84 studied genes. The center vertical line indicates unchanged gene expression, while the two outer vertical lines indicate the selected fold regulation threshold. The horizontal line indicates the selected p-value threshold. Genes with data points in the far upper left (down-regulated and green) and far upper right (up-regulated and red) sections meet the selected fold regulation and p-value thresholds

by 6.7-fold (p = 0.002), its direct ligand CD40 ligand (CD154/TNFSF5) - by 7.6-fold (p = 0.030), the molecule CD80 (B7-1) capable of interacting with molecules CD28 and CTLA-4, providing respectively positive and negative costimulation - by 6.9-fold (p = 0.022).

Levels of mRNA of CRP (CRP), which belongs to the group of pentraxins and is one of the main opsonins and complement activators were increased by 7.6-fold (p = 0.013); MPO, an enzyme of neutrophil lysosomes that generates hypochlorite anion, capable of damaging tissues - by 7.6-fold (p = 0.015). In AU, we also observed transcriptional induction of genes of a number of cytokines such as Th1-mediated IFNG (IFNG, Interferon, gamma) - by 5.3-fold (p < 0.001), Th2-mediated IL4 and IL5 - 7.2- fold (p = 0.034) and 6.8 folds (p = 0.022), respectively, Th17-mediated IL17A - 7.3-fold (p = 0.036), as well as chemokine CXCL8 (CXC motif ligand 8, IL8) - 9.3-fold (p = 0.014) and systemic proinflammatory cytokine TNF - 5.6-fold (p < 0.001). AU was also accompanied by an increase in mRNA levels of the regulator of differentiation of Th17 cells RORC - by 5.5-fold (p = 0.002), the gene NLRP3-inflammasome, belonging to the group of NOD-like cytoplasmic sensors - by 5.3-fold (p = 0.008) and the transcription factor NFKB1- by 4.3-fold (p < 0.001). Interestingly, PBMCs from AU patients had decreased mRNA levels of the FOXP3 gene by 11.8-fold (p < 0.001) and Treg-mediated suppressor cytokine IL10 by 10.3-fold (p < 0.001) (Figure 2).

Therefore, the development of AU resulted in transcriptional activation of pro-inflammatory signaling with simultaneous suppressor deficiency. Using obtained data on Innate and Adaptive Immune Responses genes, we have created a gene network using the GeneMANIA software (http://genemania.org)

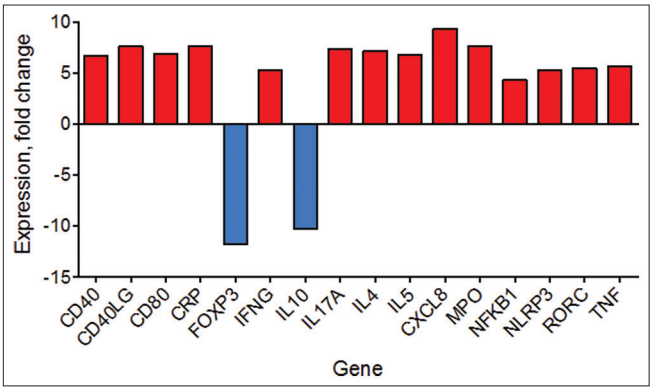

Figure 2: Changes in the transcriptional activity of immune response genes in patients with acute urticaria

(Figure 3). The most significant functional relationships are detected among the genes CD40, CD40LG, CD80, FOXP3, IFNG, IL10, IL17A, IL4, IL5, CXCL8, MPO, NFKB1, NLRP3, RORC, and TNF. These relationships mainly regulate the nature of the inflammatory response, innate and adaptive immunity, the activation and proliferation of T-lymphocytes, leukocytes migration, and cytokine receptor binding.

Discussion

Changes in the transcriptional activity of genes of the immune system in the peripheral blood and skin of patients with urticaria have been evaluated in various studies, mainly concerning chronic urticaria (CU). Thus, Wang (2014) found increased mRNA levels of TLR2, TLR7, TLR9, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) mRNA in PBMCs in patients with CU [21]. The DC-SIGN mRNA and protein levels were significantly reduced in PBMCs from patients with CU. The activation of genes for systemic pro-inflammatory cytokines has been addressed in various studies [18], [22]. IL-6 was shown to be elevated in the sera or plasma of patients with CU but we have found no changes in mRNA. Moreover, IL-6 levels correlate with disease activity and have been considered as a biomarker [23]. Moreover, we have observed no connections with autologous serum skin test results.

IL-17 mediates adverse effects in urticaria. Neutrophils, which are observed in urticarial wheals, and IL-17 can advance this neutrophil infiltration. The transcriptional induction of the IL-17 gene in peripheral blood was found to be accompanied by an increase in the expression of the RORC transcription factor, directing the differentiation of naive lymphocytes toward Th17 cells. The crucial role of Th17 cells in urticaria pathogenesis has been reported [24], [25]. Unfortunately, in CU, serum levels of IL-17 cannot be compliant with positive or negative correlation with

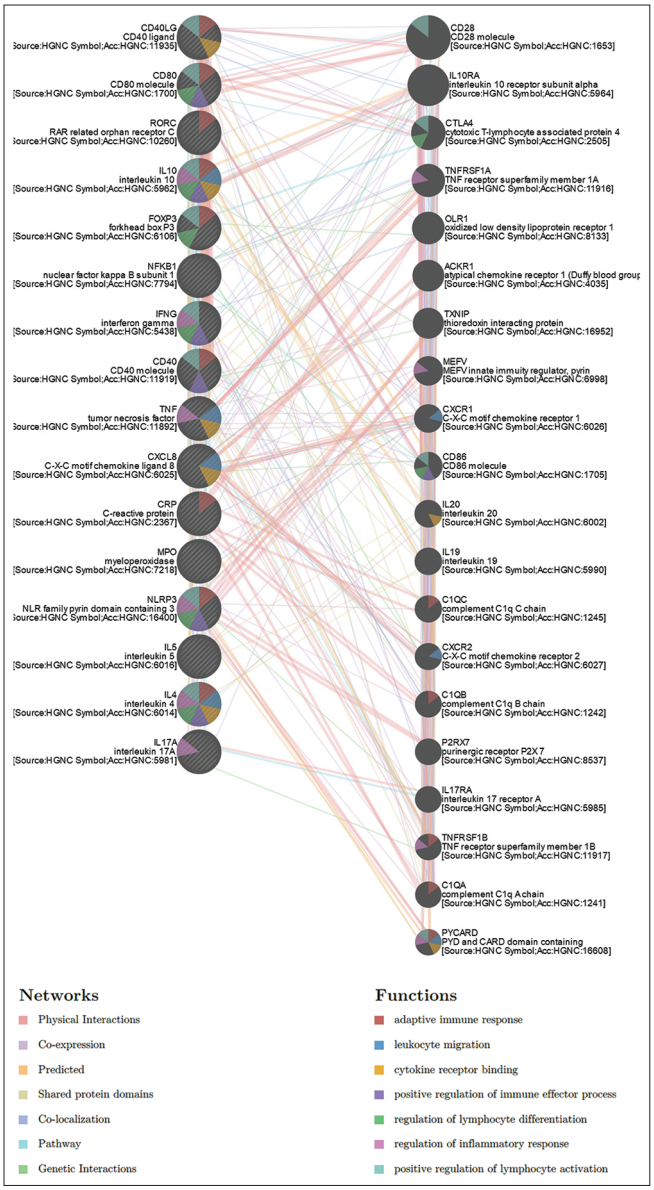

Figure 3: Functional relationships between the genes of innate and adaptive immune responses

disease activity [26]. The positive influence of anti-IL-17A (secukinumab) should be investigated as a new therapeutic strategy, especially in CU patients treated with high dose antihistamines, recurrent course of steroids, and omalizumab, and have no reasonable treatment response [27].

Increased levels of TNFα and IL-1β in serum often correlate with CU progression. Increased levels of TNFα were found in spontaneous wheals and uninvolved skin of patients with CU compared with healthy skin [28]. Our results also show an increase in TNFα expression in the absence of changes in IL-1β. Some case reports and small controlled studies demonstrated the efficacy of TNFa inhibitors in urticaria [29].

We found transcriptional induction of the IFN-γ gene; the main cytokine produced by Th1 cells. However, data regarding IFN-γ in serum upon urticaria are contradictory and unproven [30]. Elevated IFN-γ expression was observed in spontaneous wheals

compared to healthy skin, with fewer cells expressing IFN-γ as compared with cells expressing IL-4 or IL-5 [31]. Contradictory results were also found when nonlesional skin patients suffer from CU versus spontaneous wheals or normal skin.

In our study, we found increased expression of the main Th2-dependent cytokines IL-4 and IL-5. This finding confirms the critical role of Th2 cells in the mechanisms of urticaria development. IL-4 is produced by Th2 cells, basophils, MCs and promotes IgE synthesis induced by activated CD4+ or CD8+ T cells. IL-4 stimulated the expression of high-affinity IgE receptors (FcεRI). Levels of circulating IL-4, as well as IL-4 produced by PBMC, were not affected in patients with urticaria that is comparable to our results [32]. IL-5 stimulates the eosinophil responses to chemokines and is vital for their development and survival. IL-5 serum levels were shown to be affected in patients with CU differ and IL-5 expression was increased in wheals compared with nonlesional skin and normal skin. The study by Chen (2018) suggests that Th1-/Th2- and Th17-associated cytokines are considerably increased and correlate with disease activity in urticaria than in healthy people [33]. This study demonstrates that AU causes a more marked Th2 immune response than CU. The different cytokines profiles between AU and CU give the possibility to suggest that patients have generally activated Th1/Th2 and Th17 cytokines instead of dominant Th2 cytokine pattern will change upon CU. A time-course analysis of the cytokine changes in patients with CU will possibly allow us to identify the patients who tend to develop CU in the future. Recently evidence strengthens the theory that T cells are a proper candidate to be targeted in urticaria. Drugs such affects levels of IL-5 (benralizumab and mepolizumab), IL-4 (Dupilumab), or IL-1 (Canakinumab) are currently under development for the treatment of CU [34].

We found decreased levels of mRNA for FOXP3 and IL10 genes. Earlier it was shown that circulating FOXP3+ Tregs are reduced and/or defective under CU [35]. Arshi and colleagues (2014) demonstrated a significant reduction of circulating CD4 + CD25 + FOXP3 + T cells in patients with CU compared to control individuals [36]. However, there was no remarkable difference in serum levels of IL-10, TGF-β, and IL-17 in patients with CU. IL-10 also has inhibitory effects on eosinophil survival. However, in CU, the expression of IL-10 was not affected [37]. There is no systemic link between data on the expression of IL-10 in the experiment and patients with CU compared with control or mRNA levels in PBMCs [38].

Changes in the transcriptional activity of genes involved in a variety of biological functions such as epidermal differentiation, intracellular signal function, transcriptional factors, cell cycle differentiation, inflammation, or coagulation, were described earlier [18]. Differentially expressed genes in the LS-CSU (skin lesions resulting from chronic spontaneous urticaria) with a pathogenic interest were associated with intracellular transcriptional factors (ATF3, EGR1, FOSL1, MYC, NR4A2, CSRNP1), intracellular signals (AKR1B10, DDX, FPR1, MT2A, SLC25A25, STEAP4, TUBB2A), lipid metabolism (LDLR, CH25H), innate immunity (LILRB4, TLR2), metalloproteinase (ADAMTS4), T lymphocytes activation (CD69), growth factor (AREG-amphiregulin, CSF3R), or other chemokines (CCL2, CCL4, CXCL2).

Transcriptomic analysis of biopsies of damaged skin in patients with urticaria showed notable upregulation of 506 genes and decreased expression of 51 genes [39]. Most upregulated genes were implicated in cell adhesion (selectin E), cell activation (CD69), and chemotaxis (CCL2). Changes in canonical pathways were detected (comprising intracellular kinase pathways (Ras-related nuclear protein and Janus activated kinase/interferon), cytokine signaling pathways (IL-9, IL10, and IFN), a robust inflammatory response (iNOS and glucocorticoid pathways), and increased cell proliferation (cell cycle control and tRNA charging). Two important pathways in the pathophysiology of urticaria were associated with mast cells and the complement system. Multiple upregulated genes in mast cells after activation through the high-affinity receptor for IgE were detected, including CSF1, IL1R1, CCL4, CD69, TNFAIP6, NFKB1, MYC, and MAP3K14. It was earlier shown that C1QBP and ITGAX genes associated with the complement system were not affected [39]. These results correlate with our data on the detected transcriptional activation of the NFKB1 and CRP genes.

The disturbed cytokine-chemokine network could play an important role in the onset of diseases associated with inflammatory processes such as chronic idiopathic urticaria (CIU). Santos and coauthors (2012) detected considerably elevated serum levels of the CXCL8, CXCL9, CXCL10, and CCL2 in CIU patients compared to the healthy subjects [40]. The basal secretion of CCL2 by PBMC or induced by *Staphylococcus aureus* enterotoxin A (SEA) was elevated in CIU patients. In addition, increased levels of CCL2 and CXCL8 mRNA were found in monocytes of patients upon SEA stimulation. These findings showed high responsiveness of monocytes through CCL2/CXCL8 expression, contributing to the formation of a pro-inflammatory environment in CIU [40]. Upregulation of CXCL8 genes in our case indicates high responsiveness by monocytes that contribute to the inflammatory upon urticaria. These findings confirm that the innate immune system through chemokines and monocytes might induce immune activation to stimulate the pathogenesis of urticaria.

Institutional Review Board Statement

The study was conducted following the Declaration of Helsinki, and approved by the Ethics Committee of Ternopil National Medical University for studies involving humans.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data are available from corresponding authors upon request

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