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Transcriptional activity analysis of the immune response genes in the peripheral blood of patients with comorbid acute urticaria and lyme borreliosis

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Acute urticaria (AU) and Lyme borreliosis (LB) are known to alter the transcriptional profile of blood cells. The nature of changes in the transcriptional activity of genes of the innate and adaptive immune system in the peripheral blood in patients with comorbid AU and LB is unknown. In our study, we applied a pathway-specific PCR array (Human Innate & Adaptive Immune Responses RT2 Profiler PCR Array, QIAGEN, Germany) to detect and verify the innate and adaptive immune responses of pathway-focused genes expression in the blood of patients with a comorbid course of these pathologies. It was found that in patients with comorbid AU and Lyme disease, transcriptional induction of a number of genes of the innate immune system in PBMC was observed, in particular: TLR2, NOD2, NLRP3, APCS, complement component 3 (C3), CD14, CD86 compared with patients suffering from acute urticaria. These changes were accompanied by an increased transcriptional activity of systemic proinflammatory cytokines IL1B, IL6, IFNG and its receptor IFNGR1, TNF, ligands, and chemokine receptors CXCL10, CXCR3, CCR5, tyrosine kinase JAK2 and transcription factors STAT1 and TBX21. At the same time, the comorbid course of acute urticaria and Lyme disease led to the repression of the transcriptional activity of the CD80, IL4, and CXCL8 genes. The comorbid course of acute urticaria and Lyme borreliosis is accompanied by activation of the transcriptional activity of genes of the innate immune system and proinflammatory cytokines.
Key words: acute urticaria; Lyme borreliosis; mRNA; innate and adaptive immune responses.

INTRODUCTION

Lyme borreliosis (LB) is one of the most common arthropod-borne infectious diseases [1]. LB has local and systemic manifestations, and, as a result, some patients may experience persistent complications after treatment [2]. It is considered that innate and acquired immunity-related inflammation plays a crucial role in host defense against Borrelia burgdorferi, and impacts the severe course of disease [3]. Recently, large-scale analytical approaches have emerged to quantify gene expression in LB, which can contribute to the development of disease biomarkers in different stages of the disease, namely the early, disseminated, and post-treatment ones [4]. The use and expansion of the transcriptomics approaches provide researchers with large datasets of the cellular and systemic environments, which can identify new pathways that may be related to a specific biosignature or biomarker of various diseases [5–8]. Transcriptome profiling is becoming more common and expounds on the mechanisms regulating phenotypic changes [9–12]. In addition, it provides information for finding effective targeted therapies [13].

It is a common knowledge that in the initial stages of Lyme disease, cutaneous manifestations in the form of migrating erythema (EM) are the most common and pronounced symptoms, which are present in 70% of infected people and appear within 3 to 30 days after infection [15]. At the same time, urticaria may be one of the initial clinical manifestations of LB [16]. However, the development of urticaria on its own is also accompanied by significant changes in the transcription and methylation profiles in whole

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blood [17]. In this aspect, it is of considerable interest to compare the transcriptional activity of genes of innate and adaptive immunity in the peripheral blood in patients with acute urticaria and in patients with comorbid acute urticaria and Lyme borreliosis, which was the purpose of this study.

METHODS

Participants and study design. For our study, we collected peripheral blood mononuclear cells (PBMC) from 12 patients with comorbid acute urticaria (AU) and Lyme borreliosis (test group) and 12 patients with acute urticaria (control group). The diagnosis of AU is based on the appearance of skin lesions. No laboratory reference standard is available. The diagnosis was started with a routine patient evaluation, comprising a thorough history and physical examination. Severe systemic disease were ruled out by basic laboratory tests. Physical examination of the patient included a test for dermographism. The disease activity was determined using urticaria activity score (UAS). This simple scoring system is based on the assessment of key urticaria symptoms (wheals and pruritus). The 2018 EAACI/GA2LEN/EDF/WAO diagnosis guideline recommends using the UAS proposed in the previous version of the guideline to measure the disease severity and monitor treatment results in daily practice [18]. The UAS assigns a score from 0 (no disease activity) to 3 (intense activity) for each of the 2 key urticaria symptoms, wheals and pruritus. The sum of the scores represents the disease severity on a scale from 0 (minimum) to 6 (maximum). We excluded patients meeting the following criteria: angioedema, anaphylaxis, the use of antihistamines or glucocorticoids within 7 days before the emergency department visit, chronic urticaria. The ELISA method (enzyme-linked immunosorbent assay) was applied using Euroimmun AG test systems (Germany): antibodies of the class M-Anti-Borrelia burgdorferi ELISA (IgM), immunoglobulins of class G Anti-Borrelia plus VIsE ELISA (IgG) and immunoblot test system from Euroimmun AG to diagnose Lyme borreliosis.

Experimental procedures. RNA isolation. Total RNA was isolated from white blood cells using NucleoZOL (“Macherey-Nagel”, Germany) according to the manufacturer’s instructions. NucleoZOL is designed for the isolation of total RNA (small and large RNA) in a single or separate fraction from a variety of sample materials, such as cells, tissue, and liquids of human or animal origin. White blood cells were lysed and homogenized in NucleoZOL reagent based on guanidinium thiocyanate and phenol.

cDNA synthesis. The RNA quality was determined and it was reverse transcribed. The concentration and quality of the isolated total RNA were determined on a NanoDrop spectrophotometer (“Thermo Scientific™”, USA). For the reverse transcription procedure with a cDNA conversion RT² First Strand Kit (“QIAGEN”, Germany, Cat. No. 330401), RNA samples with the following parameters were selected: ratio A260/A280 within the range of 1.8-2.2. The RT2 HT First Strand Kit procedure comprises 2 steps: elimination of genomic DNA contamination, and reverse transcription, which enable fast and easy handling of 96 RNA samples simultaneously. After genomic DNA elimination, the RNA sample undergoes reverse transcription with an RT master mix, as well as random hexamers and oligo-dT prime reverse transcription to capture more difficult-to-detect genes.

PCR Array. The cDNA was then used with RT2 Profiler PCR Array (“QIAGEN”, Germany, Cat. No. PAHS-052ZA) in combination with RTI SYBR® Green qPCR Mastermix (QIAGEN, Germany, Cat. No. 330504), following the complete RT2 Profiler PCR Array procedure (www.qiagen.com). Samples were assigned to the control and study groups. CT values were

normalized based on an automatic selection from the full panel of reference genes. Any Ct value >35 was considered to be a negative call. The RT2 Profiler PCR Array data analysis software calculates the fold change based on the widely used and agreed upon ΔΔCt method. The data analysis web portal calculates fold change/regulation using the delta-delta CT method, in which delta CT is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta CT calculations (delta CT (Test Group)-delta CT (Control Group)). Fold change is then calculated using a 2^ (-delta-delta CT) formula. This data analysis report was exported from the QIAGEN web portal at GeneGlobe. The software allows defining the best reference genes for normalization.

Statistical analysis of PCR array data. The RT2 Profiler PCR Array Data Analysis software does not perform any statistical analysis beyond the calculation of P-values using a Student's t-test (two-tail distribution and equal variances between the two samples) based on the triplicate 2^(-ΔCT) values for each gene in the experimental group compared to the control group. The Microarray Quality Control (MAQC) published results indicating that a ranked list of genes based on a fold-change and such a P-value calculation was sufficient to demonstrate reproducible results across multiple microarrays and PCR Arrays including the RT2 Profiler PCR Arrays.

Ethical approval. The ethical principles contained in the Declaration of Human Rights adopted in Helsinki, in 1975, and revised in 2008, were fully respected in our study. The subjects enrolled were voluntarily participated in this study and completed and signed written informed consent. The protocol of the study was approved by the local ethics committees of I. Horbachevsky Ternopil National Medical University.

RESULT

By applying the RT^2 Profiler PCR Array Human Innate & Adaptive Immune Responses, we studied the expression of 84 genes implicated in the immune reactions (Table; Figs. 1-3). It was found that in patients suffering from acute urticaria with Lyme disease, transcriptional induction of a number of innate immune system genes in PBMC was observed. In particular, membrane sensors of peptidoglycan components and bacterial lipoproteins, including OspA (Outer surface protein A) from the Lyme disease spirochete Borrelia burgdorferi - Toll-like receptor 2 (TLR2) - 10.84-fold (P = 0.014829), cytoplasmic sensors of bacterial molecules, which possess the muramyl dipeptide (MDP) - nucleotide-binding oligomerization domain-containing protein 2 (NOD2) - 6.14-fold (P = 0.010411), NLRP3 inflammasome - 2.74-fold (P = 0.027355), member of the pentraxins family APCS (Serum amyloid P component) - 5.22-fold (P = 0.002716), complement component 3 (C3) - 6.09-fold (P = 0.000306), CD14 molecule, which acts as a co-receptor (in addition to the Toll-like receptor TLR 4 and MD-2) for revealing bacterial lipopolysaccharide (LPS) - 4.94-fold (P = 0.006223), costimulatory molecule CD86 - 8.55- fold (P = 0.004637), compared with patients with acute urticaria.

These changes were accompanied by an increase in the transcriptional activity of systemic proinflammatory cytokine genes IL1B - 7.94-fold (P = 0.018963), IL6 - 6.63-fold (P = 0.016980), IFNG - 4.22-fold (P = 0.000014) and its receptor IFNGR1- 14.34-fold (P = 0.000001), TNF - 3.98-fold (P = 0.000024), ligands and chemokine receptors CXCL10 - 6.36-fold (P = 0.026997), CXCR3 - 4.42-fold (P = 0.023611), CCR5 - 6.72-fold (P = 0.035054), tyrosine kinase JAK2- 4.32-fold (P = 0.031630) and transcription factors STAT1 - 5.56-fold (P = 0.001355) and TBX21 - 6.09-fold (P = 0.000373). At the same time, the course of comorbid acute urticaria and Lyme disease led to repression of the transcriptional activity of

the genes of the costimulatory molecule CD80 - 21.38-fold (P = 0.027429) and Th2-dependent cytokines IL4 - 16.43-fold (P = 0.017277) and CXCL8 - 34.33 (P = 0.006605) compared with patients suffering from acute urticaria.

Fold-Change (2^(-Delta Delta CT)) is the normalized gene expression (2^(-Delta CT)) in the Test Sample (Acute urticaria + Lyme borreliosis, AU + LB) divided by the normalized gene expression (2^(-Delta CT)) in the Control Sample (Acute urticaria, AU). Fold-Regulation represents fold-change results in a biologically meaningful way

DISCUSSION

The use of transcriptome analysis assists in comprehending the development of the host response to Lyme disease. Owing to easy accessibility, availability, and being one of the predominant classes of immune cells in the blood, peripheral blood mononuclear cells (PBMCs) are extensively used in *ex vivo* and *in vivo* analysis of Lyme borreliosis. Analysis of the transcription profile of macrophages and peripheral blood monocytes using microarray

Changes in the transcriptional activity of immune response genes in patients with comorbid acute urticaria and Lyme borreliosis compared with patients suffering from acute urticaria

Gene symbol	Description	Fold change	T-test	Fold regulation
		AU+LB/Control group (AU)	P value	AU+LB/Control group (AU)
APCS	Amyloid P component, serum	5.22	0.002716	5.22
C3	Complement component 3	6.09	0.000306	6.09
CCR5	Chemokine (C-C motif) receptor 5	6.72	0.035054	6.72
CD14	CD14 molecule	4.94	0.006223	4.94
CD80	CD80 molecule	0.05	0.027429	-21.38
CD86	CD86 molecule	8.55	0.004637	8.55
CXCL10	Chemokine (C-X-C motif) ligand 10	6.36	0.026997	6.36
CXCR3	Chemokine (C-X-C motif) receptor 3	4.42	0.023611	4.42
IFNG	Interferon, gamma	4.22	0.000014	4.22
IFNGR1	Interferon gamma receptor 1	14.34	0.000001	14.34
IL1B	Interleukin 1, beta	7.94	0.018963	7.94
IL4	Interleukin 4	0.06	0.017277	-16.43
IL6	Interleukin 6	6.63	0.016980	6.63
CXCL8	Interleukin 8	0.03	0.006605	-34.33
JAK2	Janus kinase 2	4.32	0.031630	4.32
NLRP3	NLR family, pyrin domain containing 3	2.74	0.027355	2.74
NOD2	Nucleotide-binding oligomerization domain containing 2	6.14	0.010411	6.14
STAT1	Signal transducer and activator of transcription 1	5.56	0.001355	5.56
TBX21	T-box 21	6.09	0.000373	6.09
TLR2	Toll-like receptor 2	10.84	0.014829	10.84
TNF	Tumor necrosis factor	3.98	0.000024	3.98

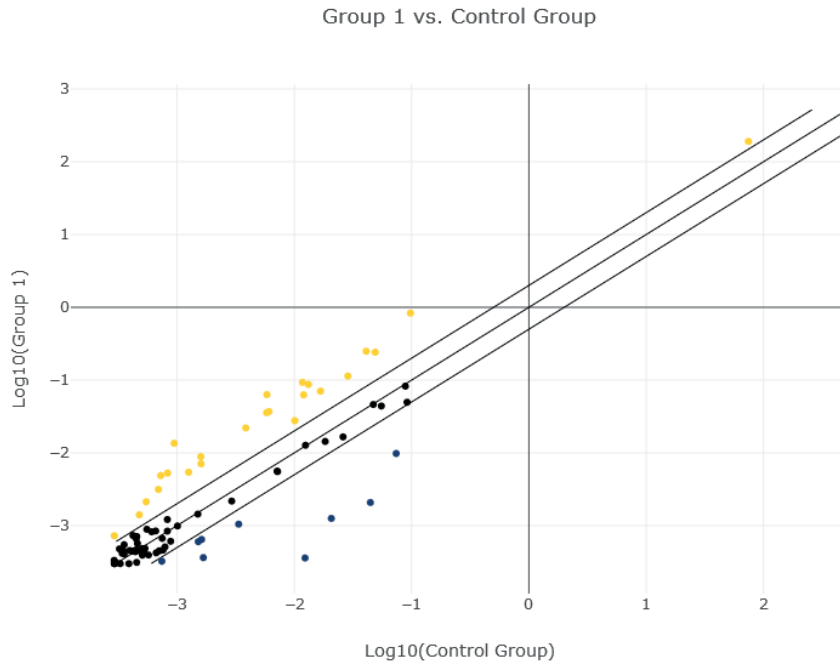

Fig. 1. The Scatter Plot compares the normalized expression of every gene on the PCR Array between the two selected groups by plotting them against one another to quickly visualize large gene expression changes. The center diagonal line indicates unchanged gene expression, while the outer diagonal lines indicate the selected fold regulation threshold. Genes with data points beyond the outer lines in the upper left and lower right corners are up-regulated or down-regulated, respectively, by more than the fold regulation threshold in the y-axis Group relative to the x-axis Group. Group 1 – acute urticaria + Lyme borreliosis. Control group – acute urticaria

analysis performed by Carreras-González et al. [19] had shown that 1962 genes were upregulated and 2096 downregulated. B. burgdorferi stimulation of TLR-dependent and independent signaling in host cells causes transcriptional activation, the release of inflammatory mediators, and anti-microbial responses.

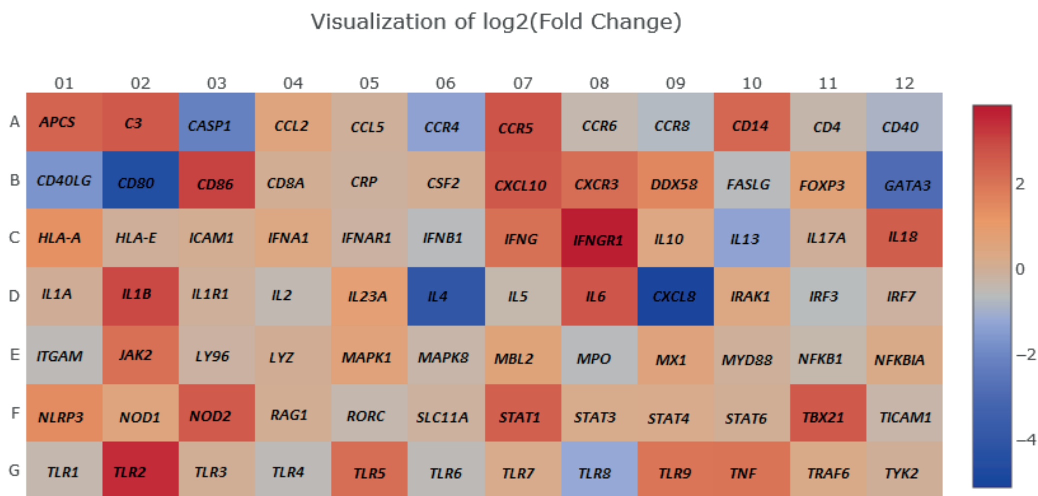

Fig. 2. Heat Map plot. Visualization of log2 (Fold Change) gene expression. Acute urticaria + Lyme borreliosis vs Acute urticaria (control)

In part, our results are proved by the transcriptome of peripheral blood mononuclear cells (PBMCs) by Salazar et al. [20], which contain an environment of peripheral immune cells including monocytes. Their study, performed on human PBMCs infected with B. burgdorferi, clarified that PBMCs caused a greater and more expanded inflammatory and immune response to live bacteria compared to lysates, and the phagocytosis of live B. burgdorferi influenced this immune response. Within these PBMCs, human monocytes were established as a significant driver of this gene expression profile through the upregulation and secretion of pro-inflammatory cytokines, comprising IL-1β, TNF-α, and IL-6. Moreover, collected monocytes were found to be a source of a substantial type I interferon response through the upregulation of IFN-β and several types I interferon-inducible genes [20].

To identify the global and temporal transcription pathways implicated in infecting a human with B. burgdorferi, Bouquet et al. [21] carried out a longitudinal transcriptional analysis (RNA-seq) of PBMCs isolated from patients with acute Lyme disease at three-time points: time of diagnosis / pre-treatment, completion of treatment (3 weeks), and 6 months post-treatment to detect transcriptional symptom profiles after treatment. The transcriptional analysis demonstrated that several TLRs upregulated comprise TLR-1, -2, -7, and -8. B. burgdorferi does not comprise lipopolysaccharides (LPS) and does not activate TLR4. On the other hand, it contains ligands that activate different TLRs. In particular, B. burgdorferi activates TLR2/1 heterodimers by recognizing the triacylated lipid moiety on its cell surface localized lipopeptides, as shown by our results. The interaction of TLR2 with B. burgdorferi outer surface protein A (Osps A) is crucial in the early stages of LB pathogenesis [22] and is believed to mediate short-term and long-term disease outcomes. Toll-like receptor 2 (TLR2) is a transmembrane signal transducer for tripalmitoyl-S-glyceryl-cysteine (Pam3Cys)-modified lipoproteins, comprising OspA from spirochete B. burgdorferi in Lyme disease. The Pam3Cys modification contributes to adjuvant activity for the induction of humoral responses, suggesting that TLR2 may function as an adjuvant receptor for the OspA vaccine. The importance of TLR2 in the humoral response to OspA was proved by the observation that the total levels of immunoglobulin G (IgG) were decreased in TLR2-deficient mice compared to those in wild-type mice. Nevertheless, the production levels of IgG1 were similar in both mouse strains, and the induction levels of protective immunity were compared. Unlipidated OspA was not immunogenic in wild-type mice or TLR2-deficient mice, specifying that lipid modification was active without TLR2. These results show that the Pam3Cys modification of bacterial lipoprotein has adjuvant properties, which are independent of TLR2 signaling [23].

Recently, several single-nucleotide polymorphisms (SNPs) in TLR genes and their downstream factors have been suggested to modulate the host response to infection [24]. After the initial recognition of B. burgdorferi by TLR2/TLR1 heterodimers, phagocytosis is considered the first stage of inflammation associated with innate immunity. This recognition leads to the sustained synthesis of pro-inflammatory cytokines such as IL-6, IL-12, TNF-α, and pro-IL1β. TLRs are known to be involved in recognition nucleic acids (e.g., TLR7, 8, and 9) and can also recognize B. burgdorferi RNA or DNA. This would lead to creating a Type I IFN signature, a process to which NLRs can contribute. In general, Bouquet et al. [21] has found that the transcription profile of patients with acute Lyme disease before and after treatment has many common characteristics. Moreover, many overlapping differentially expressed genes (DEGs) are implicated in inflammatory and immune processes, but the activation of inflammatory T cell apoptosis and B-cell developmental pathways has been inhibited compared to other acute infectious diseases. Many of the immune and inflammatory responses detected by Bouquet et al. [21] were

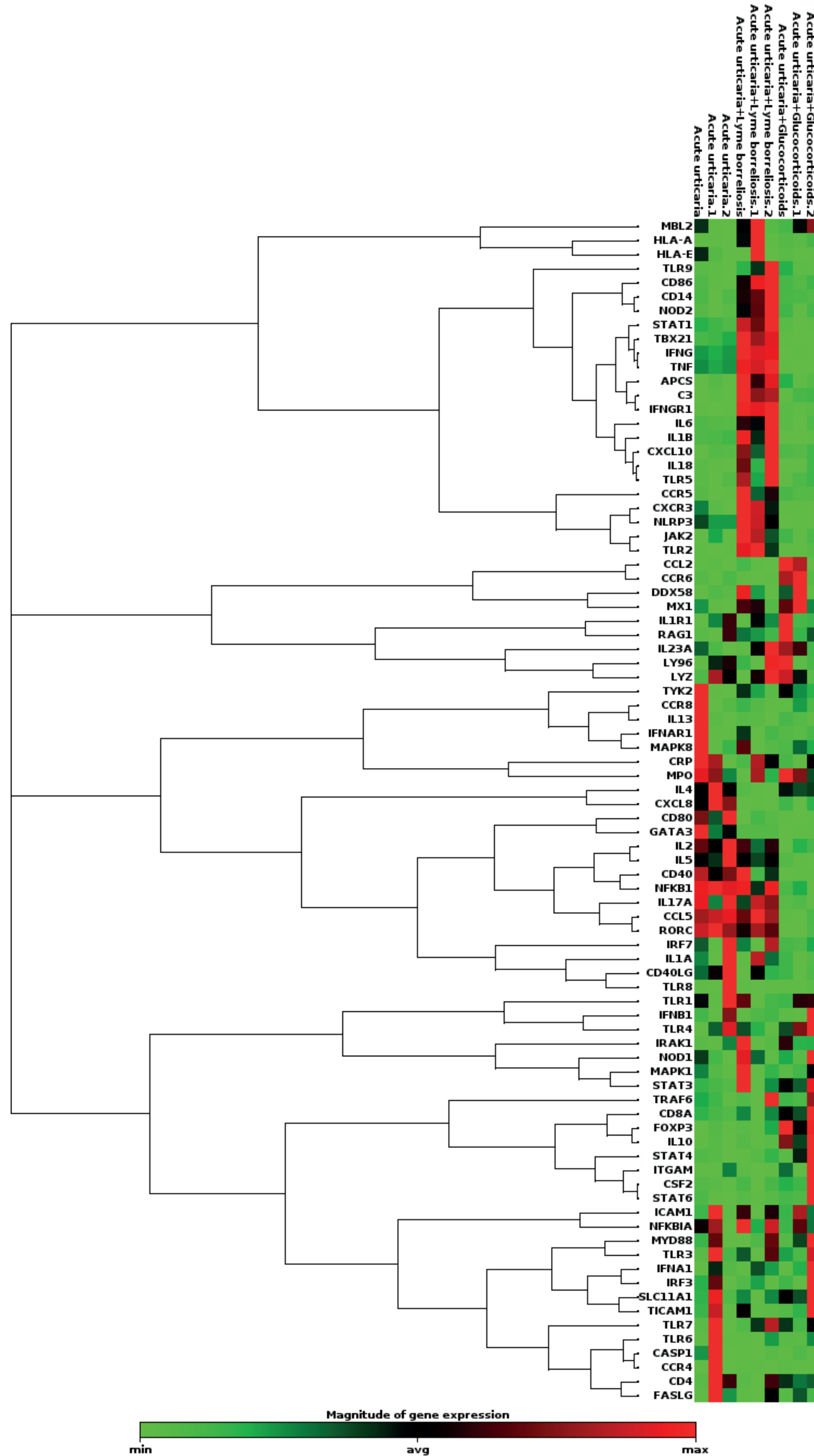

Fig. 3. Expression clustergram. Magnitude of gene expression in acute urticaria + LB vs control (AU)

confirmed by Petzke et al. [25] in previous studies, where RVMS cultures ex vivo were used. In fact, 44% of DEG were distributed between the two studies.

During the early stages of B. burgdorferi infection, monocytes/macrophages produce IL-1β in high concentrations [26], a synthesis that is activated mainly by the peptidoglycan molecules of the bacterial cell wall. IL-17/Th17 response amplifies the immune activation upon microbial recognition with IL-1β controlling the production of IL-17A, IL-17F, IL-17AF, and IL-22. The production of various cytokines is crucial for the pathogenesis of LB, for instance, IL-1β, IFN-γ, and IL-17 are eventually induced. Notably, IL-1β was shown to be related to the acute and chronic inflammatory processes observed in LB.

In the study performed by Soloski et al. [27], the levels of 58 immune mediators and 7 acute phase markers from sera of patients diagnosed with acute LB and matched controls were assessed. Patients with acute LB were observed to have increased levels of monocyte-derived chemokines (CCL19, CXCL9, CXCL10), acute phase inflammatory reactants, in particular, CRP and serum amyloid A (SAA), several IL-1 cytokine family members (IL-1Ra, IL-18, IL-33), inflammatory cytokines (TNF-α and IL-6) and the T-cell cytokine IL-2.

Using gene arrays, Marques et al. [28] defined the global transcriptional alterations in skin biopsy samples of erythema migrans (EM) skin lesions taken from untreated adult patients with Lyme disease and compared them to controls. The transcriptional pattern in EM biopsies constituted 254 differentially regulated genes (180 induced and 74 repressed) specified by the induction of chemokines, cytokines, Toll-like receptors, antimicrobial peptides, monocytoid cell activation markers, and multiple genes annotated as interferon (IFN)-inducible. The IFN-inducible genes are comprised of 3 transcripts implicated in tryptophan catabolism (IDO1, KMO, KYNU) that play a significant role in immune evasion by some other microbial pathogens by driving the differentiation of regulatory T-cells [28].

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ТРАНСКРИПЦІЙНА АКТИВНІСТЬ ГЕНІВ ІМУННОЇ ВІДПОВІДІ В ПЕРИФЕРИЧНІЙ КРОВІ ПАЦІЄНТІВ З КОМОРБІДНИМ ПЕРЕБІГОМ ГОСТРОЇ КРОПИВ'ЯНКИ ТА ЛАЙМ-БОРЕЛІОЗУ

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Гостра кропив'янка та Лайм-бореліоз змінюють транскрипційний профіль клітин крові. Ми вивчали характер змін транскрипційної активності генів вродженої та адаптивної імунної системи в периферичній крові у пацієнтів з коморбідним перебігом цих захворювань. Застосовували специфічний ПЛР-array (Human Innate & Adaptive Immune Responses RT2 Profiler PCR Array, «Qiagen», Німеччина) для виявлення та перевірки експресії генів, орієнтованих на шляхи вродженої та адаптивної імунної відповіді, у крові пацієнтів. Виявилося, що у пацієнтів із поєднаними гострою кропив'янкою та хворобою Лайма спостерігається транскрипційна індукція низки генів вродженої імунної системи в мононуклеарних клітинах периферичної крові, зокрема: TLR2, NOD2, NLRP3, APCS, C3, CD14, CD86 порівняно з особами, які страждають на гостру кропив'янку. Ці зміни супроводжувалися підвищенням транскрипційної активності генів системних прозапальних цитокінів IL-1B, IL-6, IFNG та його рецептора IFNGR1, TNF, хемокінових рецепторів і їх лігандів CXCL10, CXCR3, CCR5, тирозинкінази JAK2 та транскрипційних факторів STAT1 та TBX21. Водночас коморбідний перебіг гострої кропив'янки та хвороби Лайма призводив до пригнічення транскрипційної активності генів CD80, IL-4 та CXCL8. Отже, коморбідний перебіг цих хвороб супроводжується активацією транскрипційної активності генів вродженої імунної системи та прозапальних цитокінів.
Ключові слова: гостра кропив'янка; Лайм-бореліоз; мРНК; вроджені та адаптивні імунні реакції.

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