Original Article

Peripheral Blood Mononuclear Cell Cytokine mRNA Profiles in Acute Respiratory Infection Patients

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Abstract

Introduction: Respiratory infections, collectively, are one of the World's most common and serious illness groups. As recent observations have shown, the most severe courses of acute respiratory infection, often leading to death, are due to uncontrolled cytokine production (hypercytokinemia). **Methods:** The study involved 364 patients with respiratory illness being treated in clinics in St. Petersburg (Russia) in 2018–2019 and 30 healthy controls. Cytokine analysis was carried out in the acute phase of illness (2–3 days from onset of initial symptoms) and in the stage of recovery (days 9–10). The research presented is devoted to the assessment of mRNA expression of specific cytokines (interleukin [IL]-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, tumor necrosis factor-α [TNF-α], and interferon-λ) and MxA in whole blood leukocytes, by means of real-time polymerase chain reaction. **Results:** In 70% of patients, bacterial or viral pathogens were identified, with influenza viral infections (types A and B) prevailing. Significant increases in the expression of IL-18, TNF, and IL-10 were observed, relative to controls, only with influenza viral infections. We have shown a difference in IL-6 mRNA expression in patients with bacterial or viral pathogens. No statistically significant difference was found in white blood cells IL-4 expression levels between patients and healthy controls. **Conclusion:** Investigation of the nuances of systemic cytokine production, in response to specific viral and bacterial pathogens, makes it possible to assess the risks of developing hypercytokinemia during respiratory infection with agents circulating in the human population and to predict the pathogenicity and virulence of circulating threats.

Keywords: Cytokines, immune response, influenza virus, respiratory infections

Introduction

Influenza and influenza-like infections (ILIs), both viral and bacterial in origin, are the most common group of acute illnesses. ILIs are cyclic (seasonal), affect a large proportion of the population, and feature huge epidemic or pandemic potential. Various respiratory pathogens elicit similar symptoms, which can range from mild to severe. The primary cellular target of respiratory viruses is airway epithelium. These cells, in response to infection, secrete excessive levels of interferons (IFNs) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1b, IL-6, IL-8, and chemokines (CCL2, CCL5, CXCL8, and CXCL10).[1]

Cytokines are produced by the local cellular environment at sites of infection to promote antiviral activity and to recruit innate immune cells. The recruitment of immune cells to the area of infection triggers secondary cytokine production by blood leukocytes. Systemic cytokine production by white blood cells (WBCs) is of key importance in the development of immunopathological conditions with ILI. Hypercytokinemia (cytokine storm) of some blood cytokines

Access this article online

Quick Response Code:

Website: www.jgid.org

DOI: 10.4103/jgid.jgid_301_21

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How to cite this article: Plotnikova MA, Klotchenko SA, Lozhkov AA, Lebedev KI, Taraskin AS, Baranovskaya IL, *et al*. Peripheral blood mononuclear cell cytokine mRNA profiles in acute respiratory infection patients. J Global Infect Dis 2022;14:147-53.

Received: 15 November 2021 **Revised:** 08 April 2022
Accepted: 18 October 2022 **Published:** 30 November 2022

is positively correlated with illness severity measures in outpatients; it is also associated with fatal outcomes. Cytokine storms are associated with a wide variety of infectious and noninfectious diseases.[2,3] Infectious agents that cause hypercytokinemia include Epstein–Barr virus, cytomegalovirus, and group A streptococcus. Respiratory pathogens and infections are especially associated with hypercytokinemia, such as H5N1 influenza,[4] MERS, SARS,[5] and SARS-CoV-2.[6] Sustained, elevated cytokine levels have been implicated as a sign of poor COVID-19 prognosis.[7] High serum levels of pro-inflammatory cytokines (IFN-γ, IL-1, IL-6, IL-12, and TGF-β) and chemokines (CCL2, CXCL10, CXCL9, and IL-8) have been noted in SARS patients with severe illness compared to individuals with uncomplicated SARS.

This study aimed at analyzing cytokine expression in WBCs of patients with viral or bacterial respiratory infections. Characterization of systemic cytokine responses among ILI patients facilitates our understanding of the host immune response. It may also provide prognostic parameters useful in community-acquired pneumonia diagnostics.

Methods

Patient information and selection criteria

The study involved 364 patients with the respiratory illness being treated at clinics in St. Petersburg (Russia) in 2018–2019. Inclusion of patients in the noncontrol group was based on the presence of the following signs of acute respiratory illness: fever, intoxication syndrome (weakness, headache, and muscle pain); and/or catarrhal syndrome (nasal congestion, rhinorrhea, sore throat, cough, and chest pain). On the 2nd or 3rd day after the onset of clinical symptoms, samples (nasal and throat swabs, blood samples for WBC isolation) were collected from patients. Following recovery (10–14 days after onset), blood was again taken from patients for analysis.

The control group consisted of donors, aged 25–60 years without diagnosed chronic illness, who were healthy at the time of sampling.

Diagnosis of pathogens

Laboratory diagnosis of pathogens in selected swabs was performed by reverse transcription-polymerase chain reaction (RT-PCR) using certified AmpliSens Biotechnologies kits.[8]

Isolation of white blood cells

Blood for WBC isolation was collected in vacuum tubes with sodium heparin. Eight milliliters of blood, diluted with DPBS to a volume of 12 ml, was introduced (avoiding mixing) into a tube containing 9 ml of Lymphosep (BioWest). Tubes were then centrifuged at 400 g for 20 min; resulting, WBC layers were taken and washed twice with DPBS containing 2% FBS. Before analysis, frozen cells were stored in liquid nitrogen vapor (RPMI storage medium containing 10% DMSO, 50% FBS).

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA preparations were extracted using the RNeasy mini kit (QIAGEN). Following RNA extraction, samples were reverse transcribed using M-MLV reverse transcriptase (M-MLV RT) (Promega, USA). A mixture of 1–2 μg total RNA and 0.5 μg oligo (dT) $_{16}$ primers (DNA-Synthesis, Russia), adjusted with ultrapure water to a final volume of 15 μl, was incubated at 70°C for 10 min for preannealing. Tubes were immediately cooled on ice, followed by the addition of the final reaction component mix (all Promega): 4 μl 5x MMLV Reaction Buffer; 0.5 μl 5 mM dNTPs; 200 u M-MLV RT; 25 u RNase inhibitor; and ultrapure water to 10 μl. Complementary DNA synthesis was carried out at 42°C for 60 min; products were stored at −20°C until use. qPCR was performed using the ×2 BioMaster HS-qPCR reagent (BioLabMix) and previously-developed primers.[9] Absolute expression values were calculated by the ΔCt method using GAPDH and β-actin as normalization genes.

Statistical analysis

Because variables were not normally distributed, a nonparametric Kruskal–Wallis test was used to identify multiple differences between groups. Dunn's multiple comparisons test was used for the pairwise comparison of patient groups with the healthy volunteer group. A comparison of paired groups was performed using the Wilcoxon matched-pairs signed rank test. A Spearman's test was used for correlation analysis. Statistical significance was considered based on *P* value: $P < 0.05$, two-tailed, were considered statistically significant. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, USA).

Results and Discussion

General patient characteristics

In total, 364 patients, St. Petersburg (Russia) residents aged 18–90, were examined during the 2018–2019 epidemic season. The study was initiated before the appearance and spread of COVID-19 disease, caused by the SARS-CoV-2 virus, in Russia. All patients had moderate-to-severe symptoms typical of influenza-like respiratory infection (ILI), such as runny or congested nose, moderate fever (over to 39°C), myalgia, and/or sore throat. Patient swabs were examined for the presence of: Influenza A or B viruses (IVA, IVB), human orthopneumovirus (RSV), human Metapneumovirus (HMPV), human parainfluenza virus types 1–4 (HPIVs); human Coronaviruses (HCoV) that cause common cold (not SARS or MERS), human Rhinovirus (HRV), human Adenovirus serotypes B, C, or E (HAdV), and human Bocavirus. Samples were also analyzed for the presence of the bacterial pathogens *Neisseria meningitidis*, *Haemophilus influenza*, and *Streptococcus pneumoniae*. RT-PCR analysis of nasal and throat swabs identified a pathogen in 62.91% of patients. Most of the infections we identified in patients were of a viral nature and were directly caused by influenza

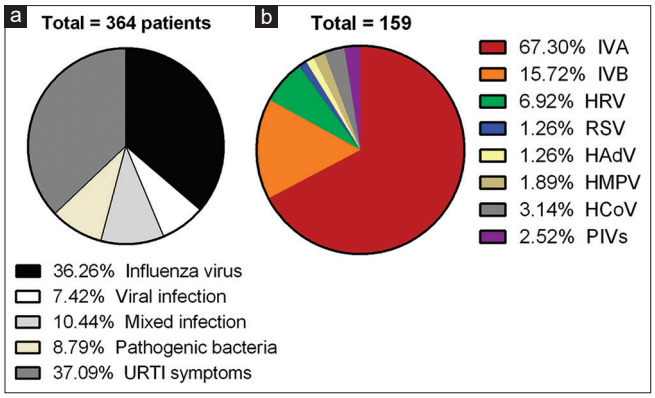

Figure 1: Patients with ILI, St. Petersburg 2018-2019: (a) Confirmed infections in patients: (b) Identified cases with virus pathogen. ILI: Influenza-like infection

viruses (A or B) [Figure 1a]. Of 32 laboratory-confirmed cases of bacterial monoinfection, 25 cases were caused by *S. pneumoniae*, 2 cases were caused by *H. influenzae,* and 1 case was N. meningitis.

The predominant viral agents, after influenza viruses, were: HRV (6.92%), HCoV (3.14%), and HPIVs (2.52%) [Figure 1b]. In 37% of patients with ILI symptoms, a pathogen could not be detected. Presumably, these patients were ill with whooping cough, *Bordetella bronchiseptica* infection, diphtheria (Corynebacterium), or pneumonia caused by Mycoplasma pneumoniae or Chlamydia pneumoniae. Those specific pathogens were not analyzed. Within 2–3 days from the onset of symptoms, blood was collected from all patients, and WBC-expressed cytokine levels were studied. Some patients were additionally (blood) sampled for postrecovery studies (10–14 days from disease onset). As a control group, 32 volunteers (men and women) without chronic illness were selected; they were free of infectious illness at the time of sampling (and in the month prior).

Increased MxA and cytokine mRNA levels in peripheral white blood cells

To investigate the role of WBC cytokine production in various ILI etiologies, we analyzed the mRNA expression levels of selected pro-inflammatory (IL-1b, TNF-α, IL-6, and IL-1b) and anti-inflammatory (IL-4, IL-10) cytokines. Peripheral WBCs of patients and healthy donors were used. We found that mRNA levels of MxA, IL-1b, TNF-α, IL-8, and IL-10 were significantly higher in the WBCs from all ILI patient groups compared with healthy controls [Table 1]. Expression of IL-18 was significantly different in all patient groups, compared to the control group, with the exception of the heterogeneous group of patients with ILI symptoms of undetermined origin.

Interestingly, WBCs also showed increased IFN-λ expression, but only in patients with influenza (approximately 14-fold) or bacterial infection (4-fold). Current literature indicates that: IFN-λ is expressed in DCs, respiratory epithelial cells, keratinocytes, hepatocytes, and others; it largely depends on IRF3 and NF-κB.[10]

Increased WBC IL-6 expression may be a sign of the transition of the infectious process to the systemic level and the development of a cytokine storm. We found that significantly increased WBC mRNA IL-6 levels were seen only with infections caused by viral pathogens or in the "infection of undetermined etiology" group. Bacterial infections did not cause significant changes in IL-6 expression relative to the healthy volunteer group. Interestingly, our results are consistent with those obtained by other researchers: respiratory viral infections in obstructive pulmonary disease induce increased serum IL-6 production compared to bacterial pathogens.[11]

The absence of significant changes in WBC IL-6 expression in patients with bacterial ILI can be explained by the fact that the IL-6 measured is a secondary response, with production stimulated primarily by TNF and IL-1b (not respiratory pathogens). In the analyzed bacterial infections, cytokine storm is not a typical phenomenon, which is probably why the IL-6 level did not change.

All patient groups had increased systemic production of IL-10, which probably provides a negative regulation of systemic response to local ILI.

Expression of the IFN-inducible MxA protein, which possesses specific antiviral activities (especially with influenza virus infection), was also increased in patients with bacterial ILI. Presumably, this may be because the bacterial infections analyzed were secondary in nature, the trigger for which was a primary respiratory viral infection. However, MxA mRNA levels in patients with influenza virus (both type A and type B) were twice as high as those with bacterial ILI ($P < 0.0001$). It is possible that MxA expression is further regulated at the translational level with bacterial infection. Since the analyzed patient groups were quite heterogeneous in terms of pathogens, we next compared cytokines in patients with the only viral infection.

Cytokine changes, depending on viral pathogen

The WBC cytokine statuses of patients with ILI caused by different viral pathogens (IVA, IVB, HRV, HCoV, and HPIVs) were studied separately [Figure 2]. Other viruses caused too few illnesses to form a statistically reliable group. Unfortunately, all the pathogens represented in the groups were RNA-containing viruses. DNA-containing viruses, such as HAdV and human Bocavirus, were found in just two people, with Bocavirus occurring in patients with only a concomitant bacterial infection. Analysis of variance analysis, using the Kruskal–Wallis test for nonparametric samples, showed no significant differences (WBC expression) in any patient group for: IL-2 versus control ($P = 0.2369$); or IL-4 versus control ($P = 0.1868$).

Influenza-related infections, both IVA and IVB, caused significant increases in the levels of MxA, IL-6, IL-8, IL-1b, TNF, IL-10, and IL-18 compared to the control. Interestingly, a significant increase in the expression of IL-18, TNF, and IL-10 was observed only in the case of influenza infection relative to control. Other pathogens caused random changes in these cytokines' mRNA levels, and differences (comparing these

Table 1: MxA and cytokine differential expression (delta Ct) in patient white blood cells

	ILI symptoms	Influenza viruses	ILI other viruses	Bacterial ILI	HD
MxA					
Mean (low 95% CI, upper 95% CI)	0.8480 (0.514, 1.182)	1.793 (1.098, 2.488)	0.396 (0.224, 0.568)	0.2101 (0.129, 0.291)	0.054 (0.040, 0.068)
SEM	0.1690	0.3512	10.08365	0.0392	0.006748
Dunn's multiple comparisons test (vs. HD)	Significance****	Significance****	Significance***	Significance*	
IFN-λ					
Mean (low 95% CI, upper 95% CI)	3.067e-6 (7.9e-7, 5.3e-6)	1.389e-5 (−5.3e-6, 3.3e-5)	3.299e-6 (1.1e-6, 5.5e-6)	4.421e-6 (1.8e-6, 7.1e-6)	9.956e-7 (4.0e-7, 1.6e-6)
SEM	1.034e-006	9.216e-006	9.708e-007	1.098e-006	2.710e-007
Dunn's multiple comparisons test (vs. HD)	NS	Significance**	NS	Significance*	
IL-1b					
Mean (low 95% CI, upper 95% CI)	1.366 (0.348, 3.080)	0.4988 (0.361, 0.636)	0.7273 (0.169, 1.286)	0.3579 (0.181, 0.535)	0.1083 (0.075, 0.142)
SEM	0.8665	0.06952	0.2722	0.08664	0.01629
Dunn's multiple comparisons test (vs. HD)	Significance***	Significance****	Significance***	Significance*	
TNF-α					
Mean (low 95% CI, upper 95% CI)	0.1939 (0.158, 0.230)	0.2517 (0.193, 0.310)	0.1792 (0.107, 0.252)	0.1539 (0.106, 0.202)	0.06406 (0.0374, 0.0908)
SEM	0.01829	0.02942	0.03538	0.02339	0.01312
Dunn's multiple comparisons test (vs. HD)	Significance****	Significance****	Significance***	Significance***	
IL-8					
Mean (low 95% CI, upper 95% CI)	0.03525 (0.011, 0.060)	0.01275 (0.009, 0.017)	0.02232 (0.0006, 0.044)	0.02282 (0.0089, 0.055)	0.00117 (0.0005, 0.0018)
SEM	0.01228	0.001975	0.01058	0.01552	0.00031
Dunn's multiple comparisons test (vs. HD)	Significance****	Significance****	Significance**	Significance*	
IL-18					
Mean (low 95% CI, upper 95% CI)	0.1606 (0.132, 0.189)	0.2236 (0.076, 0.371)	0.1726 (0.054, 0.291)	0.1856 (0.104, 0.267)	0.0762 (0.066, 0.086)
SEM	0.01444	0.07452	0.0576	0.0397	0.004937
Dunn's multiple comparisons test (vs. HD)	Significance****	Significance***	NS	Significance**	
IL-6					
Mean (low 95% CI, upper 95% CI)	0.009678 (0.0061, 0.1324)	0.01586 (0.0102, 0.0216)	0.007023 (0.0042, 0.0098)	0.007121 (0.0025, 0.0118)	0.002107 (0.0016, 0.0027)
SEM	0.001800	0.002887	0.001361	0.002255	0.000273
Dunn's multiple comparisons test (vs. HD)	Significance**	Significance****	Significance*	NS	
IL-10					
Mean (low 95% CI, upper 95% CI)	0.009692 (0.0074, 0.012)	0.01566 (0.0125, 0.0189)	0.008771 (0.005, 0.0126)	0.01117 (0.007, 0.015)	0.00159 (0.0013, 0.0021)
SEM	0.001152	0.001617	0.001853	0.002015	0.0002734
Dunn's multiple comparisons test (vs. HD)	Significance****	Significance****	Significance****	Significance****	
IL-4					
Mean (low 95% CI, upper 95% CI)	0.01901 (0.012, 0.026)	0.03781 (0.0101, 0.0656)	0.03129 (0.0073, 0.07)	0.02465 (0.008, 0.041)	0.01187 (0.008, 0.0157)
SEM	0.003729	0.01402	0.01881	0.007978	0.001869
Dunn's multiple comparisons test (vs. HD)	NS	NS	NS	NS	
IL-2					
Mean (low 95% CI, upper 95% CI)	0.001147 (0.0007, 0.0016)	0.001386 (0.0001, 0.0029)	0.001186 (0.00044, 0.0019)	0.001658 (0.0001, 0.0032)	0.0003497 (0.0003, 0.0004)
SEM	0.000239	0.0007586	0.0003615	0.0007474	0.00004052
Dunn's multiple comparisons test (vs. HD)	NS	NS	NS	NS	

NS: Not significance, INFs: Interferons, IL: Interleukin, ILI: Influenza-like infection, HD: Healthy donors, SEM: Standard error of the mean, CI: Confidence interval, MxA: Human myxovirus resistance protein 1, TNF-α: Tumor necrosis factor-α. **** - Adjusted P < 0.0001; *** - Adjusted P < 0.001; ** - Adjusted P < 0.01; * - Adjusted P < 0.05

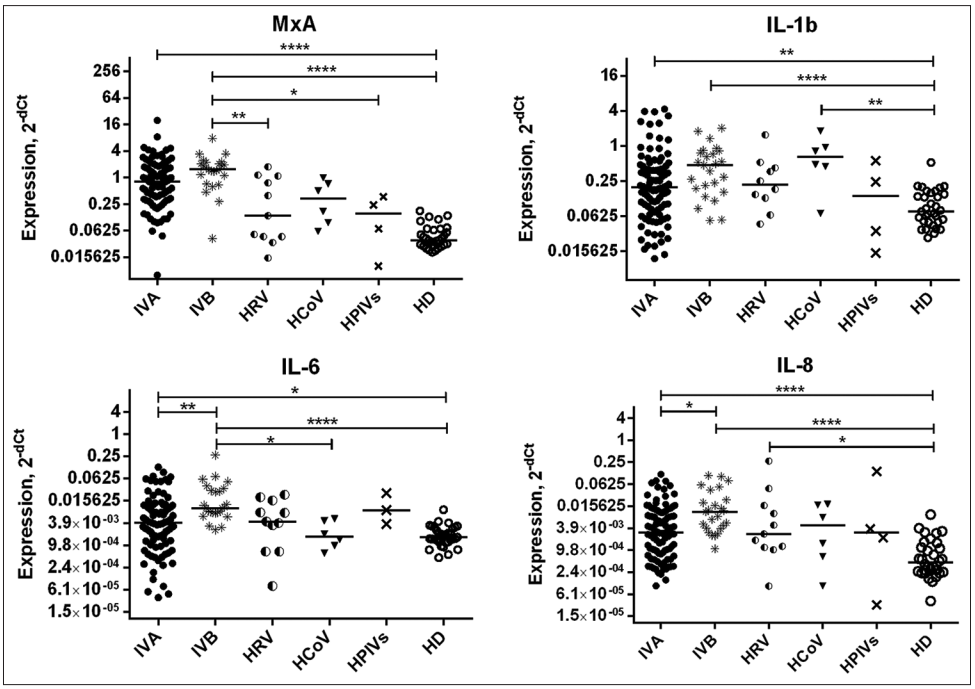

Figure 2: MxA and cytokine mRNA expression in patient WBCs, by viral pathogen. Values are marked as scatter dot plot with median as short vertical lines. Long bars show the statistical differences estimated using Kruskal-Wallis with Dunn's test for multiple comparisons. **** — Adjusted $P < 0.0001$; *** — Adjusted $P < 0.001$; ** — Adjusted $P < 0.01$; * — Adjusted $P < 0.05$. WBCs: White blood cells

groups of viral infection with the control group or with each other) were unreliable. Further, we showed earlier that cytokine expression in the heterogeneous ILI group was increased approximately 3-fold (TNF) and 6-fold (IL-10), compared to the control group. Pathogens not included in statistical analysis as discrete groups (HAdV, HMPV, RSV, human Bocavirus) appear to have made a large contribution to these changes. IL-18 mRNA levels in the "noninfluenza viral pathogen" group did not differ from the control group, which is consistent with the previously obtained results for the heterogeneous ILI group. It is interesting to note that IVB increased IL-6 and IL-8 expression by about 2-fold compared to IVA. In addition, IVB caused statistically significant differences in MxA expression compared to HRV (3.7-fold) and HPIVs (10.5-fold). IVB also increased IL-6 expression, compared to HCoV (12-fold). IFN-λ expression increased only in response to IVB infection. IL-b levels were also significantly increased in patients with HCoV infection, as was IL-8 in patients with HRV infection.

Cytokine comparison, acute phase versus recovery phase

To assess the formation of a systemic immune response, a cytokine study was also performed 10–14 days after the onset of illness. Interestingly, on days 10–14 of the study, IL-6 mRNA expression levels in patient WBCs were similar to those measured at the first point. Like early samples (days 2–3), late samples (days 10–14) showed increased IL-6 levels (compared with controls) in all patients, with the exception of the "bacterial infection" group. IL-6 probably manifests its regulatory properties in the recovery phase analyzed: suppressing TNF and IL-1b secretion; eliciting increased IL-2 production by T-helper cells; and facilitating switching from immunoglobulin (Ig) M to IgG. This is also evidenced by a significant decrease in the levels of MxA and certain pro-inflammatory cytokines (IL-1b, TNF) by 10–14 days of infection with influenza and heterogeneous ILIs [Figure 3].

Observed IL-8 expression values were higher than normal in all patient groups during acute phase illness, regardless of the pathogen. This indicates the presence of a pronounced inflammatory response. Only with type A influenza, however, was there a tendency toward increasing IL-8 in the early recovery period, relative to the acute phase. The significance of such changes was low ($P < 0.05$); presumably, a significant contribution to these changes was made by cases of complicated influenza.

In almost all noncontrol study groups (with the exception of the viral groups "Influenza Viruses" and "ILI Other Viruses"), decreases in the anti-inflammatory cytokine IL-10 were also observed by days 9–10 [Table 1]. IL-10 limits inflammatory reaction duration and provides a system of negative regulation of the inflammatory response. It is also interesting that by 10–14 days after the onset of illness, almost all patient groups showed decreased IL-10 expression, which probably reduces the risk of immunoparalysis or the development of opportunistic infection.[12] It is known that with an adequate acute immune response, induction of IL-10 does not affect viral clearance. However, sustained expression during primary or secondary immune responses may contribute to virus persistence or the development of chronic infection.[13]

It is noteworthy that, in all patient groups, there was a significant increase in IL-2 by days 10–14 of illness compared with the acute phase of infection. Earlier, on days 3–4 of illness, patient

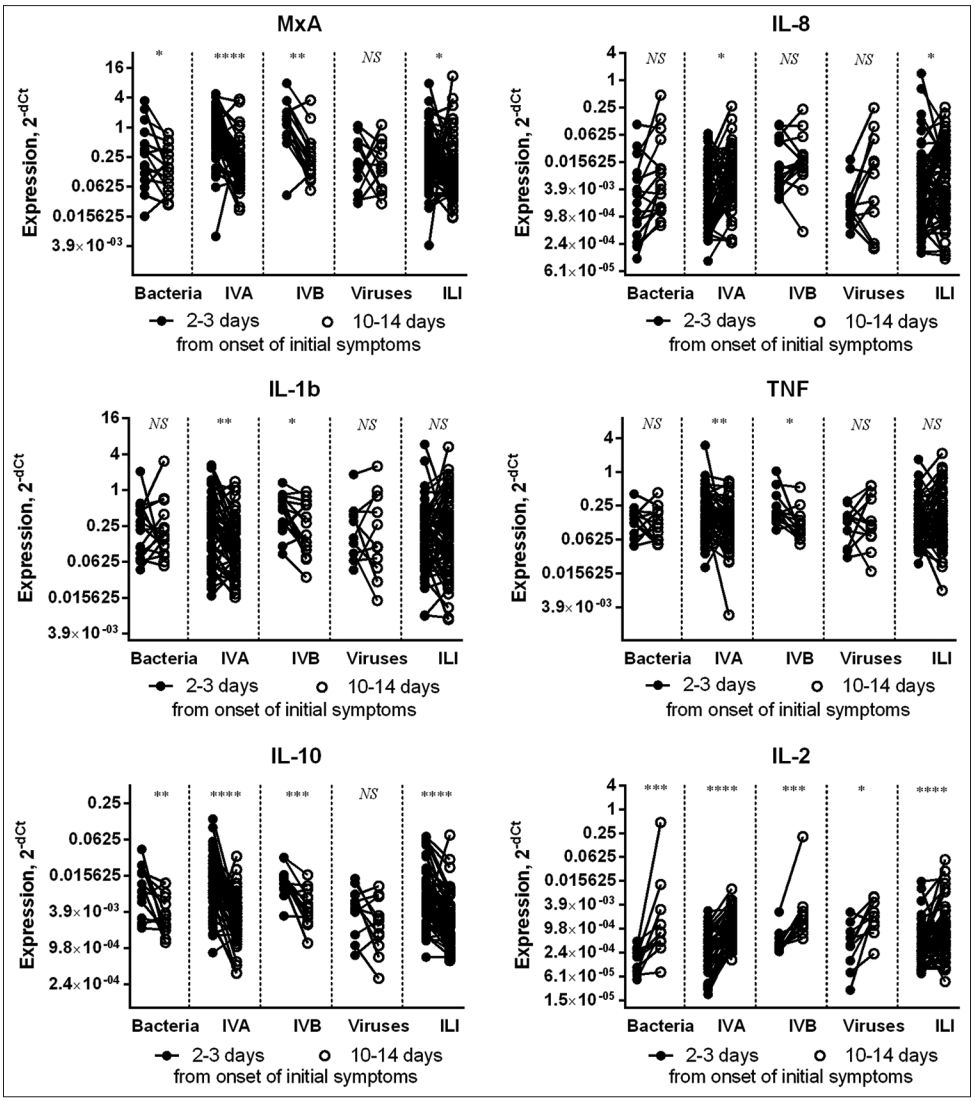

Figure 3: Cytokine comparison, acute phase of infection and recovery. Paired *t*-test was performed using nonparametric Wilcoxon test. **** — Adjusted $P < 0.0001$; *** — Adjusted $P < 0.001$; ** — Adjusted $P < 0.01$; * — Adjusted $P < 0.05$; NS – no significant difference

IL-2 expression levels did not differ from the healthy volunteer group. Numerous studies have shown that IL-2 is responsible for the clonal expansion of antigen-selected $CD4^+$ and $CD8^+$ T cells.[14,15] It also enhances B-cell growth and synthesis of immunoglobulins.[16,17] With a high degree of probability, it can be assumed that induction of IL-2 production by WBCs on days 10–14 after illness is due to the development of an adaptive immune response and the formation of immunological memory in patients.

Patient WBC IL-18 levels remained stably elevated, even on days 10–14, in patients diagnosed with influenza, with levels similar to those measured on days 2–3 of illness. IL-4 levels on days 10–14 of illness did not change relative to those on days 2–3. Moreover, when comparing IL-4 over time with the healthy volunteer group, no significant differences were noted. Thus, it can be assumed that our study did not reveal any significant differences in WBC IL-4 expression in pathological conditions caused by ILIs.

Conclusion

The vast majority of epidemics in the 21st century, including the current COVID-19 pandemic, have been driven by respiratory viruses. Obviously, a key factor for the development of severe pathology can be not so much the pathogen itself but the nature of the host organism's immune response to it. Current research points to cellular and molecular contributions to cytokine storm phenomena in various disease states. Analysis of the nuances of systemic cytokine production provides several benefits. This and further data, specific for certain viral and bacterial pathogens, will likely make it possible to assess the risks of developing hypercytokinemia during ILI with agents circulating in the human population and to rationally predict the pathogenicity and virulence of circulating threats.

Research quality and ethics statement

This study was approved by the Institutional Review Board/ Ethics Committee (Smorodintsev Research Institute of

Influenza institutional Ethics Committee, protocol No 108, dated September 03, 2018). The authors followed applicable EQUATOR Network (http://www. equator-network. org/) guidelines during the conduct of this research project.

Serum specimens used for the isolation of WBC (control group) were provided by the Blood Transfusion Center, Research Institute of Hematology and Transfusion Science (contract number 128_15092017). Informed consent was obtained from all donors and patients who provided research materials. All biological experiments were performed in accordance with the relevant guidelines and regulations.

Financial support and sponsorship

This research work was supported by a Russian State Assignment for Fundamental Research, 0784-2020-0023 (Andrey V Vasin).

Conflicts of interest

There are no conflicts of interest.

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