BioNanoScience
https://doi.org/10.1007/s12668-022-01020-x

Mucosal Immunity After Novel COVID-19 Infection – Virus-Induced Immunosuppression: Preliminary Study

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Accepted: 28 July 2022

Abstract

In recovered COVID-19 patients, the state of mucosal immunity remains understudied. Cytological, functional, and metabolic characteristics of neutrophils and the interleukin status will help to correctly assess the need for immunorehabilitation measures. The study objective is to assess the state of mucosal immunity after COVID-19. A comprehensive study of mucosal immunity included the assessment of nasal mucosal neutrophils with the monitoring of destructive and apoptotic changes as well as examination of the functional and metabolic activity of neutrophils entering the nasal secretions. Phagocytic activity was assessed using microbial suspension of *Staphylococcus aureus*, as well as intracellular oxygen-dependent biocidity, the functions of capturing, absorbing, and killing pathogens. Study of the secretory component included assessment of interleukin levels (TNF-α, IL-10, IFN-γ) and the content of sCD95 (sAPO-1/FAS), membrane marker of apoptosis, in the nasal secretions. Cell wall neutrophils in recovered COVID-19 patients show enhanced destructive and apoptotic processes within the cells. Functional disorders due to inhibited phagocytosis of autoflora are recorded. Functionally defective cells are brought into the nasal secretions; they demonstrate severely inhibited oxygen-dependent biocidity, rapid depletion of reserves, incomplete phagocytosis, and limited ability to capture pathogens, which can contribute to the growth of various pathogenic viruses and bacteria. In the nasal secretions, the concentration of sCD95 (sAPO-1/FAS), the membrane marker of apoptosis, is increased. Elevated level of pro- and anti-inflammatory cytokines (TNF-α, IL-10) downregulates IFN-γ, thus directly contributing to the formation of functionally defective neutrophils. Compensatory increase in the IL-10 anti-inflammatory cytokine under the influence of SARS-CoV-2 virus proteins downregulates IFN-γ and is a cofactor of depression of intracellular biocidity of neutrophils. An increased level of the TNF-α pro-inflammatory cytokine increases apoptotic and destructive changes in neutrophils entering the nasal secretion. Virus-induced, functional, and metabolic impairment of neutrophils of the mucosal immunity system develop in recovered COVID-19 patients, thus providing a scientific rationale for immunomodulatory therapy.

Keywords Mucosal immunity · COVID-19 · Neutrophils · Cytokines · SARS-CoV-2

Potential dysfunctions of systemic immunity are currently a proven consequence of COVID-19 [1, 2]. It has been shown that the concentration of immune competent cells in persons with the history of this infection does not return to preinfection levels even a few weeks after recovery [2, 3]. Virus-induced immune response dysregulation leads to significant phenotypic changes in peripheral blood lymphocytes, which persist as long as 4–11 weeks after the disease [2–4]. After COVID-19, patients for a long time have an increased percentage of pro-inflammatory monocytes (CD14+, CD16+) secreting a number of cytokines, including MCP1, IP-10, and MIP1α, which contribute to the development of a "cytokine storm" [4, 5]. Cytotoxic lymphocytes (NK cells, CD8+T-cells) show overexpression of activation markers [4, 6].

The consequences of the "cytokine storm," which develops in some patients and leads to thrombosis, vascular inflammation, and other issues with vital organs, have also

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Published online: 02 September 2022

been the focus of vast research [7]. In patients with severe and moderately severe COVID-19, progressively high concentrations of IL-2, IL-7, IL-10, G-CSF, MCP1, and TNF-α can persist for a long time at the level of systemic and local immunity, thus causing long-term immune dysfunctions [8]. The study of the effects of SARS-CoV-2 on the cytokine profile shows that multiple structural and non-structural viral proteins counteract the interferon response for a long time [9]. This antagonism occurs at various stages of interferon signaling, e.g., by preventing the recognition of viral RNA by the pattern-recognizing receptor, inhibition of interferon signaling through STAT1, and the degradation of host mRNA [9, 10]. The negative effect of the viral proteins on the interferon response contributes to increased release of apoptosis products, which can later also cause aberrant inflammatory responses [9].

SARS-CoV-2 is primarily spread through airborne transmission. Severe cases progress to acute respiratory distress syndrome, on average 8–9 days from illness onset [11]. The system of local immunity of the mucous membranes of the upper and lower respiratory tract is the main gate for SARS-CoV-2 introduction and replication and it is in the first turn exposed to massive cytopathic effects. Replication of the SARS-CoV-2 virus in the epithelial cells of the respiratory tract [3, 6, 8, 11] can cause severe destruction and virus-induced apoptosis, which is an inflammatory form of programmed cell death usually observed in cytopathic viral infections [12]. Using various pattern recognition receptors (PRRs) on epithelial and alveolar macrophage cells, the released pathogen-associated molecular patterns (PAMPs) trigger a massive local inflammatory process and stimulate increased secretion of pro-inflammatory cytokines and chemokines IL-6, IFN-γ, MCP1, and IL-10 [7, 8]. Extensive cytopathic effects, cytokine imbalance, and inflammatory cell infiltration at the level of local mucosal immunity can lead to acute damage to cellular structures due to excessive secretion of proteases and reactive oxygen intermediates in addition to direct damage caused by the virus. Also, some aspects of SARS-CoV-2 effect on the mucosal immunity parameters may be associated with COVID-19 treatment, e.g., the use of antibacterial and immunosuppressive drugs. The state of mucosal immunity after COVID-19 is of particular importance due to the fact that the antigen load during the activation of viral and bacterial pathogens and the vast majority of immune responses occur in barrier tissues [9, 12], and in particular, in the system of mucosa-associated lymphoid tissue (MALT). It has been proved that there is a single basis for the functioning of upper and lower respiratory tracts and the mucosal immunity system in general. The study of cyto-immunological and inflammatory shifts at the local level—in nasal secretions and induced sputum—can be considered promising both in theory and practice. Cytological characteristics and the interleukin status will make it possible to correctly estimate the need for immunorehabilitation measures after COVID-19. Despite the fact that literature contains enough information on changes in the systemic immune status of patients recovered from the novel coronavirus infection COVID-19, the state of mucosal immunity remains understudied.

1 Materials and Methods

1.1 Patients

Nasal swabs were collected from 37 COVID-19 convalescent healthcare professionals (HPs)—43.5 (32–55 years old; 17 male and 20 female). SARS-CoV-2 infection was confirmed by positive qPCR analysis as well as presence of virus specific IgM and IgG. Also, nasal swabs were collected from 30 controls—43 (31–55 years old; 14 male and 16 female) which had no history of COVID-19 and lack of serum anti-SARS-CoV-2 antibodies.

Inclusion Criteria Each participant completed the questionnaire on clinical signs and clinical test results to establish the COVID-19 convalescence status. The levels of IgG and IgM to the SARS-CoV-2 virus were analyzed in all participants Inclusion criteria were (1) history of COVID-19 symptoms (fever, dry cough with scanty sputum, muscle pain, fatigue, shortness of breath, chest congestion); (2) positive PCR results and/or positive specific serum IgG and IgM; and (3) absence of chronic or acute diseases at the time of examination. Out of 37 HPs, 15 (40.5%) were diagnosed with a mild and 22 (59.5%) with a moderate form of COVID-19. The work was approved by the local ethics committee of the Federal state-financed scientific institution.

1.2 COVID-19 ELISA

The "SARS-CoV-2 IgG-IFA-BEST" and "SARS-CoV-2 IgM-IFA-BEST" kits (Russia, "Vector-Best," Novocibirsk) were used to determine SARS-CoV-2-specific antibody titers as per manufacturer's instructions. Briefly, control or COVID-19 convalescent serum was diluted 1:100 with PBS and incubated for 60 min at 37 °C in a 96-well plate with pre-adsorbed SARS-CoV-2 antigens. Following washes (3×; 0.5% Tween20 in PBS, PBS-T), wells were incubated with anti-human-IgG-HRP conjugated antibodies (1:10,000 in PBS-T, Amerixan Qualex Technologies, USA) for 30 min at 37 °C. Post-incubation and washes (3×; PBS-T), wells were incubated with 3,3′,5,5′ tetramethylbenzidine (Chema Medica, Moscow, Russia). The reaction was stopped by adding an equal amount of 10% phosphoric acid (TatKhimProduct, Kazan, Russia). Data were measured using a Tecan 200 microplate reader (Tecan, Switzerland) at OD450 with reference OD650.

OD450 values higher than 0.5 were considered positive results, according to the manufacturer's protocol.

1. Cellular composition of nasal swab

   To prepare smears of prints from the mucous membrane of the nasal cavity, the material was taken with a cotton swab from the walls of the nasal cavity with rotational movements without pressure, applied to a glass slide free of ethanol, the smears were dried in air, fixed, and stained with eosin methylene blue according to May-Grunwald (3 min), counterstained with hematoxylin–eosin. Microscopic at least 500 cells. The percentage of neutrophils, eosinophils, lymphocytes, and epithelial cells was assessed.
2. The functional and metabolic activity of neutrophils of nasal swab

   The functional and metabolic activity of neutrophils was assessed as described by Malanicheva et al. with modifications [13].
3. In the population of neutrophils, the different degree of damage was identified where neutrophils 0 was cells without signs of damage; neutrophils 1 were cells with minimum damage; neutrophils 2 was cells with pronounced signs of cytoplasm destruction; neutrophils 3 was cells with signs of pronounced cytoplasm destruction; and neutrophils 4 was cells with signs of nuclear destruction.

The cell damage index was calculated as

$$\mathrm{CDI} = \text{neutrophils } 1 + \text{neutrophils } 2 + \text{neutrophils } 3 + \text{neutrophils } 4/100$$

The average damage index was calculated as

$$\mathrm{ADI} = \text{neutrophils } 1 \times 1 + \text{neutrophils } 2 \times 2 + \text{neutrophils } 3 \times 3 + \text{neutrophils } 4 \times 4$$

The cell cytolysis index was calculated as

$$\mathrm{CCI} = 4/(\text{neutrophils } 1 + \text{neutrophils } 2 + \text{neutrophils } 3 + \text{neutrophils } 4)$$

b. In a smear imprint from the nasal mucosa, the phagocytic activity of cell wall neutrophils was assessed in relation to autoflora.

Phagocytic activity to autoflora was determined by calculation of:

PNR: the percentage of neutrophil phagocytosis.
PIR: the percentage of killed neutrophils containing intact microorganisms in their cytoplasm.
To identify apoptosis of neutrophils (NA), we used light microscopy of imprint smears stained with Giemsa stain. This method is mainly helpful for identifying apoptosis based on reduced size, condensed cytoplasm, and condensed unstructured chromatin, often shown as several rounded bodies [12].

Neutrophils evacuated into secret
Obtaining a suspension of neutrophils evacuated into a secret

The lavage fluid containing neutrophils evacuated into the nasal secretion was obtained by rinsing the nasal cavity through a transnasal catheter with sterile saline in a volume of 5 ml. The washing liquid was placed in a test tube and centrifuged at 1000 g for 10 min. The supernatant was removed, the cell sediment was resuspended in 1 ml of physiological solution (pH 7.2–7.4), washed 2 times with physiological solution, and brought to a concentration of neutrophils in the suspension of 1.0×10^6 per ml.

b. Determination of the viability of neutrophils evacuated into the nasal secretion

0.2 ml of a suspension of a cell suspension in physiological solution (concentration of neutrophils 1×10^6 cells/ml) was mixed with 0.02 ml of a 1% solution of trypan blue; the cells were resuspended and placed in a Goryaev chamber, where they were examined under a light microscope. Trypanonegative live (clear) and trypanopositive nonviable (purple) NGs were evaluated. The count was made per 100 neutrophils. The result is 98% of viable cells. Subsequently, the cell suspension was used to assess the functional activity of neutrophils.

iii. Determination of the phagocytic activity of neutrophils evacuated into the nasal secretion

In the wells of a flat-bottomed 96-well plate, 200 µl of leukocyte suspension (concentration of neutrophils 1×10^6 cells/ml) was added, mixed with 50 µl of bacterial suspension of *Staphylococcus aureus* (laboratory strain "Sakharov") in a dilution of 2×10^6 microbial bodies in 1 ml of physiological solution. All ingredients were mixed by gentle rocking of the plate and incubated at 37 °C for 30 min. A leukocyte suspension was applied to a glass slide, defatted with ethanol; the smears were dried in air, fixed, and stained with eosin with methylene blue according to May-Grunwald (3 min) and counterstained with hematoxylin–eosin. Microscopic at least 200 cells.

To assess the phagocytic activity of neutrophils evacuated into the secret, we calculated.

Phagocytic index (PIA) represented the percentage of "active" neutrophils that contained microorganisms in cytoplasm.
Phagocytic number (PN) represented the average number of bacteria found in single neutrophils.
Percentage of digestion (%D) was calculated as ratio of the number of killed bacteria to the total number of phagocytized bacteria.

Digestion index (DI) was calculated as an average number of killed bacteria per single neutrophils.

Determination of intracellular oxygen-dependent metabolism of neutrophils evacuated into the nasal secretion using opsonized zymosan

An integral indicator of the biocidal capacity of neutrophils, which makes it possible to assess the activity of the oxygen-dependent NADPH oxidase system of a cell, is the nitroblue tetrazolium reduction test (NBT test). Under the influence of the superoxide anion formed in the NADPH oxidase reaction, the soluble dye nitroblue tetrazolium is reduced to insoluble diformazan. The NBT test is carried out in two versions—spontaneous and induced, which makes it possible not only to determine the reactivity of non-stimulated neutrophils, but also to assess their functional reserve, readiness to complete phagocytosis. Opsonized zymosan-Zimosan A is from *Saccharomyces cerevisiae*, Sigma-Aldrich, Merck (Millipore, Sigma-Aldrich, Supelco) Z 4250-1G-pack/1 g; Dia-M.

e. Obtaining opsonized zymosan

Zymosan (20 mg) was thoroughly mixed with pool donor serum AB (IV) Rh (-) at a concentration of 20 mg zymosan per 1 ml of serum. Incubated at 37 °C for 30 min with continuous stirring, after incubation, centrifuged at 250 g for 10 min, the supernatant was removed. The settled zymosan was resuspended in 10 ml of buffered saline (PH 7.2–7.4), washed three times at 250 g for 10 min and diluted to a concentration of 20 mg/ml. It was poured into aliquots of 0.5–1.0 ml and stored at − 70 °C, thawed once immediately before use.

f. Conducting the NBT test using neutrophils evacuated into the nasal secretion

In the wells of a flat-bottomed 96-well plate, 100 µl of leukocyte suspension (concentration of neutrophils in the suspension 1.0×106 in ml) was added (BioChemica, Applichem-A1243,0001, Diaem). In the stimulated version (NST stim.), we add 20 µl of opsonized zymosan (concentration 20 mg/ml) and 20 µl of 0.1% nitro blue tetrazolium solution (BioChemica, Applichem-A1243,0001, Diaem). All ingredients were mixed by gentle rocking of the plate and incubated at 37 °C for 30 min. After incubation, the plate was stirred in a circular motion, smears were prepared from the leucoconcentrate, dried, fixed with methanol, and stained with a 0.5% solution of neutral red. At least 200 neutrophils were counted, and percentage of cells with diformazan-positive granules stained in blue-black color in the spontaneous (NBT sp.%) and stimulated (NBT stim.%) versions of the NBT test were recorded.

4. The study of the content of cytokines and the marker of apoptosis in the nasal secretion
5. Obtaining nasal secretions

To obtain nasal secretions, cotton swabs with a well-defined weight (1 mg) moistened with sterile saline were used. The swabs were placed under the middle turbinate of each half of the nose and held for 30 s, then placed in an Eppendorf tube with saline in a volume of 0.75 ml. Centrifuged 1000 g for 10 min, the supernatant was taken, poured into aliquots of 0.2 ml, stored until the study at t-70° for no more than 1 month.

b. Cytokine ELISA

The content of interleukins was determined in the nasal secretion—tumor necrosis factor (TNF-α), interleukin 10 (IL-10), and gamma-interferon (IFN-γ) were determined by using "gamma-interferon-IFA-BEST," "interleukin-10-IFA-BEST," and "tumor necrosis factor α-IFA-BEST" ELISA kits ("Vector-Best," Novosibirsk, Russia). Concentration of sCD95 (sAPO-1/FAS) membrane marker of apoptosis in the nasal secretions was measured using enzyme immunoassay (ELISA) commercial kits "Human sAPO-1/FAS ELISA" (Bender MedSystems GmbH, Austria).

1.3 Statistical Analysis

Statistical analysis was done using the Statistica 10 software package (StatSoft, USA). Statistically significant differences between comparison groups were accepted as $p < 0.05$, assessed by the Kruskal–Wallis test with

Table 1 Characteristics of imprint smears in recovered COVID-19 patients and in the comparison group

Group	Neutrophils % M ± m	Lymphocytes % M ± m	Eosinophils % M ± m	Epithelial cells % M ± m
Total ($n = 37$)	58.5 ± 4.6	3.5 ± 0.5*	1.5 ± 0.1*	36.5 ± 3.1
Type 1 – "neutrophilic" ($n = 19$)	79.4 ± 6.9*	4.5 ± 0.6*	1.8 ± 0.2*	14.2 ± 1.8*
Type 2 – "epithelial" ($n = 18$)	37.3 ± 4.6*	2.3 ± 0.3*	1.2 ± 0.1*	59.2 ± 7.5*
Comparison group ($n = 30$)	54.6 ± 6.9	8.8 ± 2.7	4.3 ± 0.3	32.3 ± 4.1

*The difference with the control is significant, $p < 0.05$

Table 2 Cytomorphological characteristics of neutrophils in individuals after COVID-19 and in the comparison group

Group	Number of cells with different destruction classes %					NA Number/% M ± m	CDI M ± m	ADI M ± m	CCI M ± m
	n 0 Number/% M ± m	n 1 Number/% M ± m	n 2 Number/% M ± m	n 3 Number/% M ± m	n 4 Number/% M ± m				
Recovered COVID-19 patients ($n = 37$)	14.0/24.1 ± 3.9*	19.0/32.4 ± 3.9*	12.5/21.4 ± 3.9*	9.5/16.2 ± 3.9*	3.5/5.9 ± 0.9*	6.5/11.1 ± 0.6*	0.44 ± 0.05*	0.9 ± 0.06*	0.06 ± 0.02*
Comparison group ($n = 30$)	35.5/65.0 ± 7.5	10.0/18.3 ± 1.5	5.0/9.1 ± 1.5	2.5/4.6 ± 0.5	1.5/2.8 ± 0.1	3.5/2.8 ± 0.1	0.19 ± 0.1	0.34 ± 0.5	0.03 ± 0.02

*The difference with the control is significant, $p < 0.05$

Table 3 Functional activity of neutrophils in patients after COVID-19 and in the comparison group

Groups of children	PNR M ± m	PIR M ± m	PIA M ± m	PN M ± m	% D M ± m	DI M ± m	NBT sp. % M ± m	NBT stim., % M ± m
Recovered COVID-19 patients ($n = 37$)	14.7 ± 1.7*	12.7 ± 0.9*	22.7 ± 1.6*	2.71 ± 0.8*	2.8 ± 0.01*	0.3 ± 0.04*	6.91 ± 0.5*	33.9 ± 2.8*
Comparison group ($n = 30$)	32.7 ± 3.3	1.6 ± 0.2	67.6 ± 5.2	7.6 ± 0.8	8.7 ± 0.7	0.9 ± 0.1	19.9 ± 2.1	78.2 ± 5.2

*The difference with the control is significant, $p < 0.05$

Table 4 Cytokine profile in patients after COVID-19 and in the comparison group

Groups	TNF-α, pg/ml M ± m	IFN-γ, pg/ml M ± m	IL-10, pg/ml M ± m	sCD95 (sAPO-1/FAS), pg/ml M ± m
Recovered COVID-19 patients ($n=37$)	6.44 ± 0.4*	1.16 ± 0.22*	10.82 ± 1.06*	79.7 ± 6.2*
Comparison group ($n=30$)	1.65 ± 0.2	3.52 ± 0.18	2.75 ± 0.31	19.7 ± 1.22

*The difference with the control is significant, $p<0.05$

Benjamini–Hochberg adjustment for multiple comparisons. Correlations were analyzed using the R psych package (based on Spearman's rank correlation coefficient, *p*-values were adjusted with the Benjamini–Hochberg method).

2 Results and Discussion

Table 1 shows the distribution of the main cell elements in imprint smears from the nasal mucosa of persons recovered from the novel coronavirus infection COVID-19 as compared to the comparison group. An upward trend was registered for two cell populations, of neutrophils and epithelial cells, but there were no statistically significant differences with the control group. A statistically significant decrease was observed for the populations of lymphocytes (3.5%; $p<0.05$) and eosinophils (1.5%; $p<0.05$). The analysis of imprint smears according to an individual profile revealed 2 different types of abnormalities: the prevalence of neutrophils population (neutrophilic) or the prevalence of epithelial cells (epithelial). Neutrophil are an active component in the system of humoral and cellular mucosal immunity, which makes them a universal target and a unique indicator of various homeostasis disorders. We also included data on population as a whole for the group (the first message). Cytomorphological assessment of neutrophils in recovered COVID-19 patients showed a significant increase in destructive and apoptotic processes in the cell. In the study group, there was an increase in neutrophils with signs of damage: n 1, by a factor of 1.9 ($p<0.05$); n 2, by a factor of 2.5 ($p<0.05$); n 3, by a factor of 3.8 ($p<0.05$); and n 4, by a factor of 2.4 ($p<0.05$) (Tables 2 and 3). The number of cells without destructive processes decreased sharply (14.1%; $p<0.05$), and there was an increase in the indices characterizing destructive disorders in the cell, i.e., in CDI (2.3 times; $p<0.001$), ADI (2.6 times; $p<0.05$), and CCI (2 times; $p<0.05$). Pronounced cytodestructive processes at the neutrophils level, as well as a low lymphocytes content, were associated with an imbalance of key cytokines involved in the regulation of cellular and humoral adaptive immune responses at the level of the MALT system (Table 4). In the neutrophils population, an increasing neutrophil apoptosis was revealed (Table 2) (11.1%; $p<0.05$), manifesting with cell shrinkage, rearrangement of membrane structures, decreased volume, nuclear condensation, and nuclear DNA strand breaks with nucleorrhexis. Phagocytic potential of the nasal mucosa neutrophils as representatives of mucosal immunity mainly depends on subpopulations without or with minimal signs of destructive changes [12, 13]. In recovered COVID-19 patients, there were marked changes in the features of autoflora phagocytosis: decreased PNR (14.7%; $p<0.05$) and increased PIR (12.7; $p<0.05$). PNR demonstrated correlations with n 2 ($r=-0.47$; $p<0.05$), n 3 ($r=-0.49$; $p<0.05$), and n 4 ($r=-0.51$; $p<0.05$), whereas PIR demonstrated correlations with n 3 ($r=0.53$; $p<0.05$) and n 4 (r = 0.57; $p<0.05$). Also, in post-COVID-19 patients, a change in the phagocytic activity of neutrophils migrating into the secretions is seen: the content of "active" neutrophils decreases (PIA 22.7%; $p<0.05$), as does the amount of captured pathogens (PN 2.71; $p<0.05$). PIA showed correlations with n 2 ($r=-0.49$; $p<0.05$), n 3 ($r=-0.53$; $p<0.05$), n 4 ($r=-0.44$; $p<0.05$), whereas PN – with n 2 ($r=-0.47$; $p<0.05$), n 3 ($r=-0.49$; $p<0.05$), and n 4 ($r=-0.61$; $p<0.05$). At the level of neutrophils migrating into the secretions, reduced pathogen killing is revealed in the form of decreased % D (2.8%; $p<0.05$) and DI (0.3; $p<0.05$). Correlations are also shown for these indicators: for % D—with n 2 ($r=-0.55$; $p<0.05$), n 3 ($r=-0.47$; $p<0.05$), and n 4 ($r=-0.63$; $p<0.05$); for DI—with n 2 ($r=-0.53$; $p<0.05$), n 3 ($r=-0.62$; $p<0.05$), n 4 ($r=-0.51$; $p<0.05$). The outcomes of bacterial and viral processes during pathogen activation are largely determined by the state of oxygen-dependent microbicidal activity of neutrophils migrating into the secretions and its functional potential. In recovered COVID-19 patients, severe inhibition of both spontaneous (6.91%; $p<0.001$) and induced (33.9%; $p<0.001$) intracellular biocidicity of neutrophils entering the nasal cavity was found. Negative correlations of NBT sp. with n 2 ($r=-0.52$; $p<0.05$), n 3 ($r=-0.61$; $p<0.05$), and n 4 ($r=-0.56$; $p<0.05$), and negative correlations NBT stim. with n 2 ($r=-0.52$; $p<0.05$), n 3 ($r=-0.61$; $p<0.05$), and n 4 ($r=-0.56$; $p<0.05$) were revealed. Thus, our data strongly suggest that cytodestructive processes in the population of mucosal immunity neutrophils in recovered COVID-19 patients downregulate the cell phagocytic activity. Functional disorders in the population of the cell

wall neutrophils, manifesting as inhibition of autoflora phagocytosis, can contribute to exacerbation of chronic diseases. Functionally defective neutrophils are brought into the nasal secretions, showing pronounced inhibition of oxygen-dependent biocidity, rapid depletion of the cell reserve capabilities, incomplete phagocytosis, and limited ability to pathogen capture, which can contribute to the activation of various viral and bacterial agents after COVID-19. In the nasal secretions of recovered COVID-19 patients, an increased content of sCD95 (sAPO-1/FAS) membrane marker of apoptosis was found as compared to the group of healthy donors (79.7 pg/ml; $p < 0.05$). Correlations of the sAPO-1/FAS level with the level of TNF-α ($r = 0.47$; $p < 0.05$), NA ($r = 0.47$; $p < 0.05$), and lymphocytes ($r = 0.47$; $p < 0.05$) were found. Induction of apoptosis in recovered COVID-19 patients at the level of mucosal immunity is due to dysregulation mediated by TNF-α and Fas receptors [14]. Elevated sAPO-1/FAS in the nasal secretions of recovered COVID-19 patients stimulates apoptosis of neutrophils and lymphocytes cell populations, which, along with the suppression of the functional characteristics of cells, leads to the development of immunological insufficiency in the mucosal immunity system, requiring immunomodulatory therapy.

COVID-19 pathogenesis results largely from the synthesis dysregulation of a wide range of cytokines ("pro-inflammatory," immunoregulatory, and "anti-inflammatory"), which reflects the pathological activation of innate and acquired (type Th1- and Th17-) immunity [7–9]. The cytokines include IL-1, IL-2, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-17, IL-18, G-CSF, TNF-α, macrophage inflammatory protein 1α (MIP1α), and chemokines (CCL1, CCL3, CCL5, CXCL8, CXCL9, CXCL10, etc.). It has been shown that in COVID-19 infection, the concentrations of these cytokines increase to various extents and in different combinations, which is especially typical of severe and critical forms of the disease [8, 9]. During the recovery period, both cytokine profile changes and impairment of pleiotropic regulatory mechanisms of cytokines, as well as impaired susceptibility to signals through cellular receptors, can be expected [9]. Research into the cytokine profile of the nasal secretions in recovered COVID-19 patients revealed a stark imbalance between the key pro- and anti-inflammatory cytokines (Table 4). The level of IL-10 was almost 3.9 times higher than in healthy donors (10.82 pg/ml; $p < 0.05$). For IL-10, correlations were found with the level of IFN-γ ($r = -0.57$; $p < 0.0$), spontaneous ($r = -0.58$; $p < 0.05$), and induced neutrophils microbiocidicity ($r = -0.61$; $p < 0.05$). IL-10 is one of the most important anti-inflammatory cytokines [15] with primarily anti-inflammatory and anti-cytokine effects. Type 2 T-helpers are the source of IL-10, which has its effects through a receptor complex that is expressed on the surface of many cells. It has been shown that the synthesis of the IL-10 anti-inflammatory cytokine begins early in the course of COVID-19 [16], and its high level along with IL-1RA (the so-called prohibiting intermediaries) restricts the production of pro-inflammatory cytokines, prevents the development of a "cytokine storm," and is associated with mild and moderately severe forms of the infection. Lower level of IL-10 is associated with a more severe course of COVID-19 [16]. It seems that the increased concentration of IL-10 in the moderately severe and mild forms in our study persisted during the recovery period against the backdrop of the high concentration of TNF-α pro-inflammatory cytokine. Moreover, high level of IL-10 is due to the high level of the T-reg subpopulation, which is one of the main producers of IL-10 (adaptive suppressors) in moderately severe and mild cases [17]. However, the duality of pleiotropic cytokine influences leads to the fact that the compensatory increase in IL-10, which limits the "cytokine storm" in the acute period, when persisting during the recovery period contributes to the formation of a neutrophils pool of mucosal immunity with low functional characteristics [18]. In recovered COVID-19 patients, we noted an increased level of the TNF-α pro-inflammatory cytokine (6.44 pg/ml; $p < 0.05$). TNF-α is a multifunctional cytokine with pronounced pleiotropy: it controls the immune inflammation and cell apoptosis, and promotes the utilization of destructive material [19]. There are numerous reports in the literature demonstrating changes in TNF-α production in viral infections [19]. TNF-α hyperproduction was revealed during exacerbations of chronic infections induced by the viruses of hepatitis B and C, HIV, herpes simplex, Epstein-Barr, cytomegaly, influenza, polio, and tick-borne encephalitis. In recovered COVID-19 patients, correlations of the TNF-α level with IFN-γ ($r = -0.47$; $p < 0.05$), with N a ($r = -0.57$; $p < 0.05$), n 3 ($r = -0.67$; $p < 0.05$), and n 4 ($r = -0.51$; $p < 0.05$) were found. One of the crucial biological functions of TNF-α is its role in the regulation of apoptosis, including that of virus-damaged cells [19]. For instance, it is known that increased production of TNF-α, which induces programmed cell death in chronic viral hepatitis B and C at an early stage of the infectious process, can mediate increased apoptosis of hepatocytes, thus contributing to the destruction of liver tissue. According to our data, chronically elevated TNF-α in recovered COVID-19 patients downregulates destructive and apoptotic neutrophils processes in the mucosal immunity system. Our data are also consistent with the results of studies of the important role of TNF-α in the regulation of neutrophils functions at the level of local immunity [20]. In recovered COVID-19 patients, we found a severe decrease in the content of IFN-γ (1.16 pg/ml, $p < 0.05$). It has been shown that the most important aspects of IFN-γ pleiotropic effects are a dramatic increase in the antimicrobial and anti-inflammatory activity of Ns due to increased production of superoxide radicals by cells, an increase in immune phagocytosis and antibody-mediated cytotoxicity of phagocytes, which is due to increased expression of

IgG-Fc receptors [21]. In recovered COVID-19 patients, correlation between the level of IFN-γ and almost all parameters of the functional activity of neutrophils entering the nasal secretions was found, including pathogen capture, i.e., PIA ($r = -0.53$; $p < 0.05$), PNR ($r = -0.57$; $p < 0.05$), PN ($r = -0.57$; $p < 0.05$), and pathogen killing – % D ($r = -0.57$; $p < 0.05$) and DI ($r = -0.57$; $p < 0.05$), the level of intracellular biocidity – spontaneous NBT ($r = -0.57$; $p < 0.05$), and stimulated NBT ($r = -0.57$; $p < 0.05$). According to the literature, in COVID-19, a successful activation of the cascade of interferon-producing reactions should lead to virus replication control and suppression of SARS-CoV-2 dissemination [9, 10]. At the same time, it should be noted that, along with the activation of immune cells, SARS-CoV-2 expresses proteins that suppress the synthesis of interferons (IFN-α, IFN-β, IFN-γ), thereby weakening the antiviral immune response. A pronounced decrease in IFN-γ in recovered COVID-19 patients seems to be the result of two immunopathological processes [21], i.e., pleiotropic effects of increased levels of pro- and anti-inflammatory cytokines (TNF-α/IL-10), as well as depletion of the mechanisms of IFN-γ synthesis in the acute period of disease, which have not been not restored by the time of early COVID-19 recovery. A low level of IFN-γ during the recovery period is a cofactor of inhibition of almost all functional capabilities of neutrophil, i.e., oxygen-dependent microbicidal activity, functions of pathogen uptake, capture, and killing.

Etiopathogenesis of immune disorders at the level of mucosal immunity in recovered COVID-19 patients can be explained schematically as follows: impaired balance of the cytokine profile, which started during the acute stage, persists during the recovery period. Increased content of pro- and anti-inflammatory cytokines (TNF-α/IL-10) downregulates IFN-γ, which directly contributes to the development of functionally inferior neutrophils of the mucosal immunity system in terms of oxygen-dependent microbicidal activity, functions of pathogen uptake, capture, and killing. Compensatory increase of the anti-inflammatory cytokine IL-10 under the influence of COVID-19 proteins downregulates the level of IFN-γ and is a cofactor of depression of intracellular biocidity and mucosal immunity. Increased pro-inflammatory cytokine TNF-α results in enhanced apoptotic and destructive changes in neutrophil entering the nasal secretions, thus contributing to the suppression of cell functional and metabolic activity, as well as the development of lymphopenia and eosinopenia at the level of the cellular elements of the MALT system.

Therefore, virus-induced functional and metabolic disorders at the level of Ns of the mucosal immunity system develop after COVID-19, requiring lengthy rehabilitation and immunomodulatory therapy.

3 Conclusions

1. In recovered COVID-19 patients, the cell-bound component of mucosal immunity reveals lympho- and eosinopenia. The neutrophils population shows cytodestruction, increased apoptosis, and decreased functional and metabolic activity of the cell.
2. Impaired cytokine profile at the level of the MALT system in recovered COVID-19 patients shows an imbalance of pro-inflammatory cytokines (TNF-α/IFN-γ) and a pronounced increase in the level of IL-10, anti-inflammatory cytokine.
3. Pleiotropic effects of cytokine imbalance on the neutrophils systems of mucosal immunity are associated with two types of changes, i.e., cytodestructive and apoptotic changes causing the inhibition of functional and metabolic activity of the cell, as well as direct effects on oxygen-dependent microbicidal activity, functions of pathogen uptake, capture, and killing.
4. Virus-induced functional and metabolic disorders at the neutrophils level require lengthy rehabilitation and provide a scientific rationale for immunomodulatory therapy in recovered COVID-19 patients.

Funding This work is part of Kazan Federal University Strategic Academic Leadership Program (PRIORITY-2030).

Declarations

Ethical Approval The ethics committee of the Kazan Research Institute of Epidemiology and Microbiology of Rospotrebnadzor approved this study, and signed informed consent was obtained from each COVID-19 convalescent and control subjects according to the guidelines adopted under this protocol (Protocol 4/09 of the meeting of the ethics committee of the KSMA dated September 26, 2019).

Conflict of Interest The authors declare no competing interests.

References

1. Liu, J., Li, S., Liu, J., et al. (2020). Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. *EBioMedicine, 55*, 102763. https://doi.org/10.1016/j.ebiom.2020.102763
2. Qin, C., Zhou, L., Hu, Z., et al. (2020). Dysregulation of immune response in patients with COVID-19 in Wuhan. *China. Clinical Infectious Diseases, 12*(pii), ciaa248. https://doi.org/10.1093/cid/ciaa248
3. Shi, Y., Tan, M., Chen, X., et al. (2020). Immunopathological characteristics of coronavirus disease 2019 cases in Guangzhou, China. *medRxiv, 03*(12), 20034736. https://doi.org/10.1101/2020.03.12.20034736

4. Zhou, Y., Fu, B., Zheng, X., et al. (2020) Pathogenic T cells and inflammatory monocytes incite inflammatory storm in severe COVID-19 patients. Available at: https://academic.oup.com/nsr/article-abstract/doi/10.1093/nsr/nwaa041/5804736
5. Zhou, Y., Fu, B., Zheng, X., et al. (2020) Abberant pathogenic GM-CSF+T cells and inflammatory CD14+CD16+ monocyte in severe pulmonary syndrome patients of a new coronavirus. bioRxiv https://doi.org/10.1101/2020.02.12.945576
6. Xu, B., Fan, C. Y., Wang, A. L., Zou, Y. L., Yu, Y. H., et al. (2020). Suppressed T cell-mediated immunity in patients with COVID-19: A clinical retrospective study in Wuhan, China. *Journal of Infection, S0163–4453*(20), 30223–1. https://doi.org/10.1016/j.jinf.2020.04.012
7. Jose, R. J., & Manuel, A. (2020). COVID-19 cytokine storm: The interplay between inflammation and coagulation. *The Lancet Respiratory Medicine, 8*, e46–e47.
8. Mangalmurti, N., & Hunter, C. A. (2020). Cytokine storms: Understanding COVID-19. *Immunity, 53*, 19–25.
9. Mehta, P., McAuley, D. F., Brown, M., Sanchez, E., Tattersall, R. S., Manson, J. J., Hlh Across Speciality Collaboration, U.K. (2020). COVID-19: Consider cytokine storm syndromes and immunosuppression. *The Lancet, 395*, 1033–1034.
10. Huang, K., Su, I.-J., Theron, M., et al. (2005). An interferon-gamma-related cytokine storm in SARS patients. *Journal of Medical Virology, 75*(2), 185–194. https://doi.org/10.1002/jmv.20255
11. Zhou, P., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature, 579*, 270–273.
12. Zhou, X., Jiang, W., Liu, Z., Liu, S., & Liang, X. (2017). Virus infection and death receptor-mediated apoptosis. *Viruses, 9*, 316.
13. Malanicheva T. G., Agafonova E. V., Mozhgina S. S. (2016) Osobennosti mukozal'nogo immuniteta u detej doshkol'nogo vozrasta s vnebol'nichnoj pnevmoniej, protekayushchej na fone rekurrentnyh respiratornyh zabolevanij. Prakticheskaya Medicina 7: 68–73. (in. Rus) [Malanicheva T. G., Agafonova E. V., Moshkina S. S. Features of mucosal immunity in preschool children with community-acquired pneumonia occurring against the background of recurrent respiratory diseases. Practical medicine 2016; 7: 68–73]. (in. Rus)
14. Zheng, C., Zhou, X. W., & Wang, J. Z. (2016). The dual roles of cytokines in disease: Update on interleukins, TNF-alpha, TGF-beta and IFN-gamma. *Translational Neurodegeneration, 5*, 7.
15. Tinsley, J. H., South, S., Chiasson, V. L., et al. (2010). Interleukin-10 reduces inflammation, endothelial dysfunction, and blood pressure in hypertensive pregnant rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 298*(3), 713–7192010.
16. Zhao, Y., Qin, L., Zhang, P., et al. (2020). Longitudinal COVID-19 profiling associates IL-1RA and IL-10 with disease severity and RANTES with mild disease. *CI Insight, 5*(13), e139834. https://doi.org/10.1172/jci.insight.139834
17. Lan, R. Y., Ansari, A. A., Lian, Z.-X., et al. (2005). Regulatory T cells: Development, function and role in autoimmunity. *Autoimmunity Reviews, 4*(6), 351–363.
18. Zheng, C., Zhou, X. W., & Wang, J. Z. (2016). The dual roles of cytokines in disease: Update on interleukins, TNF-alpha, TGF-beta and IFN-gamma. *Translational Neurodegeneration, 5*, 7.
19. Bradley, J. R. (2008). TNF-mediated inflammatory disease. *The Journal of Pathology, 214*(2), 149–60.
20. Yee, J., & Christou, N. V. (2004). The local role of tumor necrosis factor alpha in the modulation of neutrophil- function at sites of inflammation. *Archives of Surgery, 129*, 1249–1255.
21. Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-gamma: An overview of signals, mechanisms and functions. *Journal of Leukocyte Biology, 75*, 163–189.