Cytokine profile depending on titers of anti-SARS-CoV-2 IgG in the blood plasma of healthy volunteers

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Abstract

Post-COVID-19 conditions are the subject of extensive scientific study. It is known that system disorders may persist in people who recover from COVID-19 for 3 to 12 months (in most cases). The first line of systemic disorders is dysfunction of the immune system, especially the cytokine profile. The mechanism of the launch of immune complications is currently unknown. We hypothesized that anti-SARS-CoV-2 IgG might trigger the development of post-COVID-19 complications. Our research aimed to study the cytokine profile (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12β, TNF-α, IFN-γ) and the transcription factor NF-kB in the blood plasma of donor groups depending on various titers of anti-SARS-CoV-2 IgG. We determined the qualitative values of the cytokine parameters using ELISA (Enzyme Linked Immunosorbent Assay). We estimated the changes in the cytokine profiles in all donor groups. Differences between donor groups were established for each research parameter. We detected changes in NF-κB levels as a key transcription factor for the expression of inflammatory cytokines. The highest level was found in the donor group with a titer of anti-SARS-CoV-2 IgG 75±5 Index (S/C). We detected some differences between donor groups in the levels of pro-inflammatory cytokines compared to a reference point. IL-1β, IL-6, IL-8, INF-γ, and TNF-α levels changed in most donor groups with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C). In addition, we showed that the decrease in anti-inflammatory cytokine IL-4 in donor groups ranged from the titer of anti-SARS-CoV-2 IgG 55±5 Index (S/C) to 85±5 Index (S/C), but these changes for the anti-inflammatory cytokine IL-10 were not detected. We found that the cytokine profile may be affected during post-COVID-19 conditions. The cytokine profiles of the donor groups changed depending on the titers of anti-SARS-CoV-2 IgG. The obtained results testify to the fact that the antibody subpopulation may be a key reason for immune dysfunction, influencing the development of other pathological processes.

Introduction

Attacks of bacterial/viral pathogens on healthy human cells launch a cascade of immune reactions, including the production of various types of cytokines. There are cytokines categories such as interleukins (ILs), transforming growth factors (TGFs), colony-stimulating factors (CSFs), chemokines, and tumor necrosis factors (TNFs). Pro-inflammatory and anti-inflammatory cytokines are classified according to their functions.[1] It is necessary to remember that Pathogen-Associated Molecular Patterns /

Damage-Associated Molecular Patterns (PAMPs/DAMPs) are activated as a response to pathogens penetration into cells, then Pattern Recognition Receptors (PRRs) interact with them, leading to the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α.[2] The transcription factor NF-κB is a critical factor involved in pro-inflammatory cytokine synthesis pathways. Moreover, anti-inflammatory cytokines such as IL-4 and IL-10, participate in the physiological regulation of the inflammatory process, especially in the control of pro-inflammatory cytokine balance during the immune response.[3]

COVID-19 is a multiple disease that has been at the center of clinical and scientific research since December 2019. Most clinical cases are associated with a cytokine storm, which refers to the accumulation of pro-inflammatory cytokines in the focus of inflammation against pathogenic agents, such as SARS-CoV-2. The synthesis of various cytokines is a key component of the immune response during viral diseases, including COVID-19.[4-6]

Currently, the interest in science is in the behavior of the main immune components, such as NF-κB and pro- and anti-inflammatory cytokines, during post-COVID-19 conditions. People who recover from SARS-CoV-2 infection can suffer respiratory, digestive, cardiovascular, and nervous complications. According to Nalbandian *et al*.,[7] three prior immune causes could lead to the emergence of pathological conditions following COVID-19, such as virus-specific pathophysiologic changes; immunologic aberrations, and inflammatory damage in response to the acute infection; expected sequelae of post-critical illness. Additionally, post-COVID-19 immune mechanisms may be a trigger for the activation of autoimmune diseases. According to Mobasheri *et al.*,[8] cytokines play an essential role in the pathogenesis of autoimmune diseases. It was mentioned above that SARS-CoV-2 may cause the secretion of high concentrations of cytokines in the bloodstream due to a strong inflammatory reaction. This leads to an abnormal innate and acquired immune response that decreases tolerance to self-antigens (autoimmunity diseases) and impoverishes the process of immune reconstruction.[9] The reason for the persistence of such post-COVID-19 conditions for 3, 6, and 12 months has not yet been studied.[10] We suggest that one probable reason is the circulation of antibodies (IgG) against SARS-CoV-2, which is the basis of an imbalanced immune system. This leads to various systemic pathological processes, known as autoimmune diseases.

Our study describes the cytokine profile of healthy volunteers who recovered from COVID-19 and had various titers of anti-SARS-CoV-2 IgG in their blood plasma. The key scientific task is the detection of any changes in the cytokine profiles of people depending on titers of anti-SARS-CoV-2 IgG, which will be useful for future confirmation or refutation of the influence of IgG on the development of post-COVID-19 complications, including immune dysregulation and autoimmune conditions. All the obtained results are presented as graphs and statistical plots.

Materials and Methods

Participants and study design

Healthy volunteers who had suffered from COVID-19 agreed to be donors of blood plasma for biotechnological purposes at "BIOPHARMA-PLASMA" (Kyiv, Ukraine). At the time of blood sampling, volunteers were free from COVID-19 and metabolic diseases. The donors ranged in age from 25 to 45 years old. Blood plasma was collected from donors who had recovered from COVID-19 3-6 months prior the beginning of the study. Donors were checked by screening tests before blood plasma was used to produce targeted biotechnological drugs. The blood plasma of donors was given to us with determined anti-SARS-CoV-2 IgG titers for scientific research.

The titers of anti-SARS-CoV-2 IgG in the blood plasma were determined using a chemiluminescent microparticle immunoassay (Abbott Laboratories, USA) according to the manufacturer's instructions. All donors were selected based on their anti-SARS-CoV-2 IgG titers. We had donor groups with anti-SARS-CoV-2 IgG titers: 0 (n = 20), 10±3 (n=20), 55±5 (n=20), 65±5 (n=20), 75±5 (n=20), 85±5 (n=20), 95±5 (n=20), 125±5 (n=20) and 175±5 (n=20) Index (S/C). The unit of measurement of titers of anti-SARS-CoV-2 IgG is Index (S/C) which means dividing the sample result by the stored calibrator result (S/C), according to the manufacturer's instruction. People with severe cardiovascular and cerebrovascular diseases, vitamin K deficiency, traumatic coagulation disorders, people taking anticoagulant therapy, and people with other disorders that can affect the hemostasis were excluded from the study.

All donors voluntarily agreed to participate in the study and provided written informed consent.

We described changes in the cytokine profile in the blood plasma of the donor groups. The main feature of this research was the separation of groups of donors depending on the titers of anti-SARS-CoV-2 antibodies. All donors were healthy; therefore, the presence of various titers of anti-SARS-CoV-2 IgG in blood plasma is the most significant factor for initiating an imbalance in physiological functions. We estimated the differences in the components of the cytokine profile between all donor groups and a donor group with a titer of anti-SARS-CoV-2 IgG 0 Index (S/C) as a reference point.

Collection of data and blood samples

Blood was collected by puncture from the patient's ulnar vein between 8 and 9 a.m. before breakfast, placed in a plastic tube containing sodium citrate (38 g/L) at a final ratio of 9:1, and stirred carefully and slowly. The contents of the tube were centrifuged for 15 minutes at 2500 rpm, and the supernatant was transferred to a plastic tube with a laboratory dispenser. The obtained blood plasma was frozen at -20°C in Eppendorf tubes. The frozen blood plasma was used for research. The plasma was thawed by heating in a water bath (37°C) for 20 min, mixed by gently inverting the tube, and immediately transferred to ice.

Cytokine analysis

Plasma levels of transcription factor NF-κB and cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ) were determined by enzyme-linked immunosorbent assay (Biotrak ELISA System, Healthcare, USA) following standard instruction. Studied samples of plasma (100 µl each) were previously 100 times diluted with Tris-HCl buffer, at pH 7.4, and incubated in 96-hole ELISA plates for 12 hours at 4°C. After being washed, plates were blocked with 5% nonfat dry milk for 1 h at 37°C with specific primary polyclonal antibodies (Santa Cruz, USA) against transcription factor NF-κB and cytokines IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ. Plates were washed and incubated for 1 h at 37°C with corresponding secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, USA); o-phenylenediamine/hydrogen peroxide (Sigma, USA) was used as a substrate of peroxidase reaction. The reaction was stopped by the addition of 2.5 N sulfuric acid (100 µl each). The absorbance was measured using a spectrophotometer

microplate reader (μQuantTM, BioTek Instruments, Inc, Winooski, USA) at 492 nm wavelength.[11,12]

Statistical analysis

Statistical analysis of the results was performed using the computer program STATISTICA. The arithmetic mean and mean squared error indicators were calculated. The hypothesis of normal distribution was checked using the Shapiro-Wilk and Kolmogorov-Smirnov tests. All donor groups showed a normal distribution. Therefore, the differences between the samples were determined using a one-way analysis of variance (ANOVA) with Tukey's criterion. A statistically significant test result ($p < 0.05$) was considered reliable.

Results

First of all, we described the NF-κB levels in donor groups (Figure 1, A) and estimated the statistical significance of the obtained results, respectively. All donor groups, except the group with a titer of anti-SARS-CoV-2 IgG 10±3, 95±5, and 125±5 Index (S/C), showed significant statistical differences compared to the reference point regarding the levels of NF-κB in blood plasma. All donors were compared with each other. Figure 1, B demonstrates the statistical differences in NF-κB levels between the donor groups.

The next stage of our research was to measure the quantitative composition of pro-inflammatory interleukins IL-1β, IL-6, IL-8, TNF-α, and INF-γ. Figure 2 (A.1, B.1, C.1) shows the changes in IL-1β, IL-6, and IL-8 levels depending on the titers of the donor groups. We estimated statistically significant differences in IL levels between donor groups with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C). Besides, the levels of IL-1β, IL-6, and IL-8 in each donor group with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C) were compared to the reference point. The difference in IL-1β level was statistically significant in all donor groups with titers of anti-SARS-CoV-2 IgG 10±3, 65±5, 75±5, 85±5, 95±5, 125±5, and 175±5 Index (S/C) compared to the reference point. The level of IL-6 differed in donor groups with titers of anti-SARS-CoV-2 IgG 10±3, 55±5, 65±5, 75±5, 85±5, 95±5, and 175±5 Index (S/C) from the reference point. The level of IL-6 showed significant differences in donor groups with titers of anti-SARS-CoV-2 IgG 55±5, 65±5, 75±5, and 95±5 Index (S/C) compared to the reference point. More detailed information about the statistical analyses between donor groups is described in Figure 2 (A.2, B.2, C.2).

The measurement of TNF-α and IFN-γ levels is an important part of our research. Similarly to the previously described parameters, we established the TNF-α and INF-γ levels in each donor group (Figure 3 A.1, B.1) and compared them in two ways (Figure 3 A.2, B.2): between donor groups with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C) and reference point, respectively. Regarding the reference point, we found statistically significant differences in INF-γ levels for donor groups with titers of anti-SARS-CoV-2 IgG 10±3, 55±5, 65±5, 75±5, 85±5, 95±5 and 175±5 Index (S/C). TNF-α level statistically changed compared to reference point in donor groups with titers of anti-SARS-CoV-2 IgG 10±3, 55±5, 65±5, 75±5 and 95±5 Index (S/C).

The final stage of our research was to measure anti-inflammatory interleukins IL-4 and IL-10. The results are shown in Figure 4 (A.1, B.1). The statistical matrices are presented in Figure 4 (A.2, B.2). We determined that IL-4 and IL-10 had statistically significant changes in all donors with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C) compared to the reference point.

Discussion

We put forward the theoretical prediction that anti-SARS-CoV-2 IgG is the trigger of pathological conditions, including immune disorders involving transcription factors as regulators of inflammatory processes through cytokine expression, such as IL of various types, TNF-α, and IFN-γ. Additionally, the cytokine profile changed depending on the titer of anti-SARS-CoV-2 IgG. We emphasize that donors had a single distinguishing criterion: anti-SARS-CoV-2 IgG titer. Each group is characterized by unique antibody subpopulations, which may lead to the development of atypical biochemical processes in various ways.

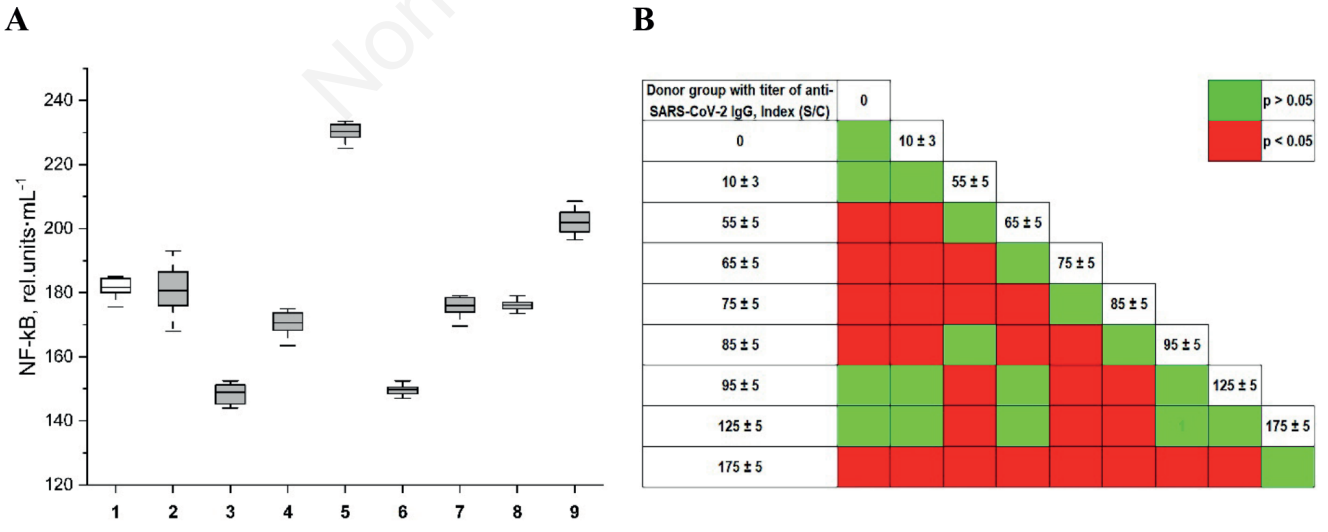

Figure 1. A) NF-κB levels in blood plasma of donor groups with titers of anti-SARS-CoV-2 IgG, Index (S/C): 1: 0; 2: 10±3; 3: 55±5; 4: 65±5; 5: 75±5; 6: 85±5; 7: 95±5; 8: 125±5; 9: 175±5. B) Significance plot of data are compared using one-way analysis of variance with Tukey's honestly significant difference post-hoc test to determine p values.

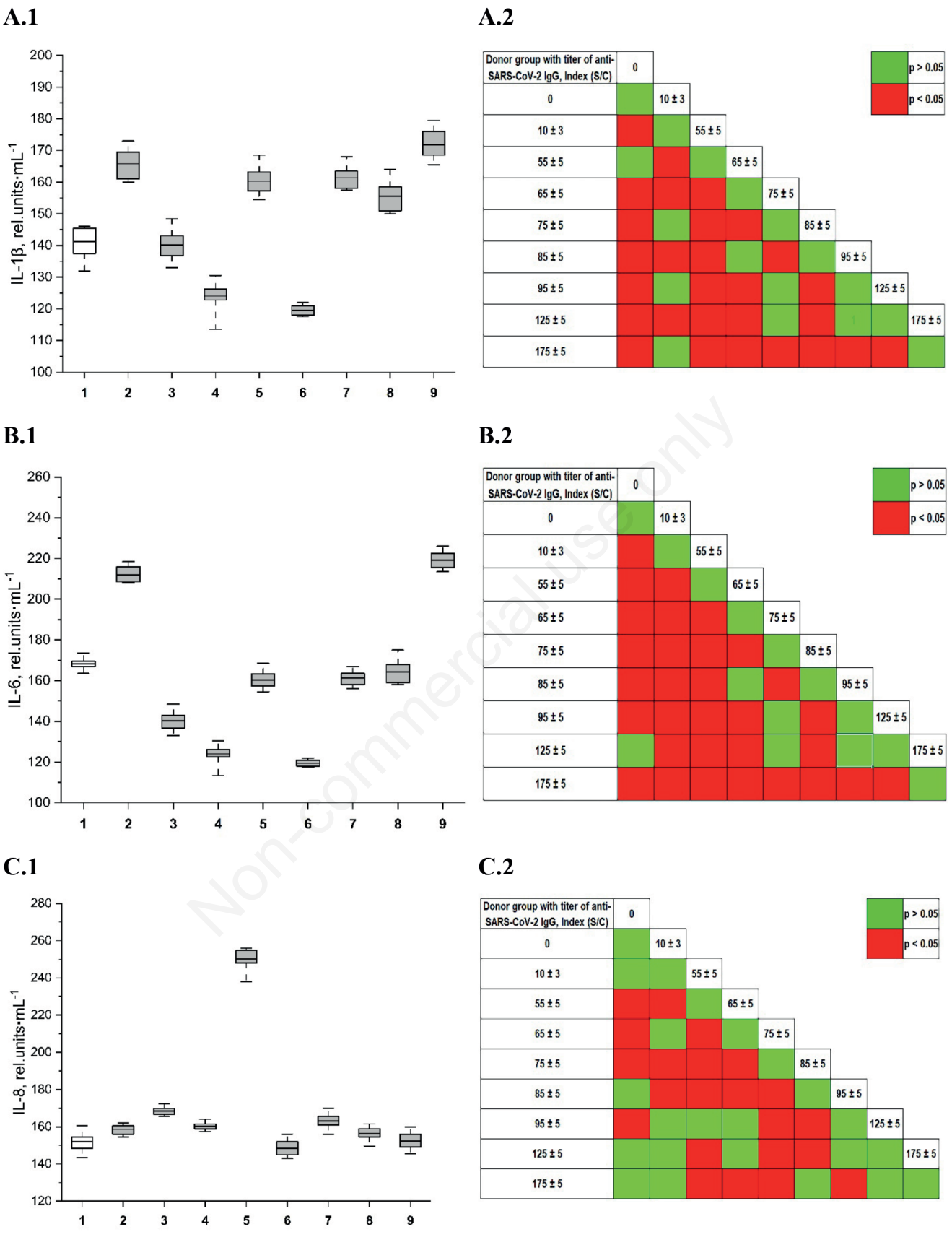

Figure 2. Cytokine levels of A.1: IL-1β; B.1: IL-6; C.1: IL-8 depending on titers of anti-SARS-CoV-2 IgG, Index (S/C): 1: 0; 2: 10±3; 3: 55±5; 4: 65±5; 5: 75±5; 6: 85±5; 7: 95±5; 8: 125±5; 9: 175±5. Significance plot of data A.2: IL-1β; B.2: IL-6; C.2: IL-8 are compared using one-way analysis of variance with Tukey's honestly significant difference post-hoc test to determine p values.

First, the central part of our results belongs to the transcription factor NF-κB, which acts as an initiator of pro-inflammatory cytokine expression. The scientific data on NF-κB level changes in the post-COVID-19 period are too little today, but some information is accumulated about the participation of NF-κB during COVID-19. According to Gudowska-Sawczuk and Mroczko,[13] NF-κB activity increases in SARS-CoV-2 infection by modulating powerful adaptive immune responses, including stimulation of pro-inflammatory cytokine synthesis. The results of the NF-κB levels between the donor groups are ambiguous. Depending on the anti-SARS-CoV-2 IgG titer, we detected different changes, such as increases and decreases in NF-κB levels compared to the reference. We emphasize that statistical analyses also confirmed changes in NF-κB levels between donor groups with titers of anti-SARS-CoV-2 IgG ≥ 10±3 Index (S/C). The most significant difference in NF-κB levels among all donor groups was in the donor group with a titer of anti-SARS-CoV-2 IgG 75±5 Index (S/C), namely the highest level of NF-κB. We have given attention to the fact that this donor group is characterized by high levels of pro-inflammatory cytokines such as IL-1β, IL-8, and TNF-α. Concerning this point, the literature reports that NF-κB takes part in some classical pro-inflammatory signal cytokine pathways, which are responsible for COVID-19.[14,15] Scientific research has established that the transcription factor NF-κB is a key parameter for prolonged transcription changes that may continue in the post-COVID-19 period.[16] Based on the obtained results, we propose that antibody subpopulations against SARS-CoV-2 may contribute to the preservation of NF-κB activity and progression of inflammatory status post-COVID-19.

The literature separates the multiple factors responsible for the development of long-term post-COVID-19 conditions, including oxidative stress, alterations in the immune-inflammatory response, and the spread of micro-/macro-thrombotic complications. However, these factors are linked to a single origin named the inflammatory process.[17] Recent research has demonstrated abnormal levels of pro-inflammatory mediators, such as IFN-β,

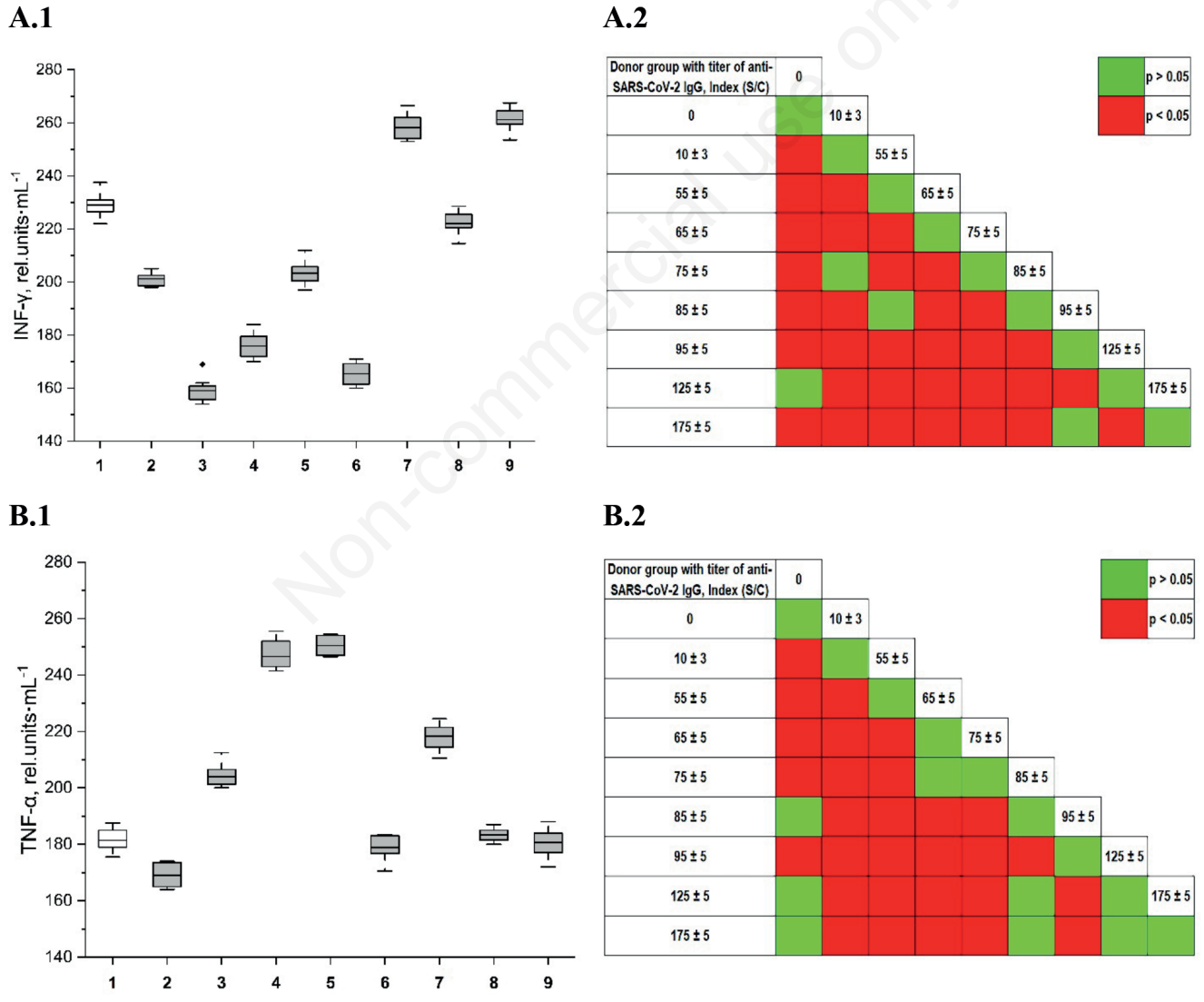

Figure 3. Levels of A.1: INF-γ; B.1: TNF-α depending on titers of anti-SARS-CoV-2 IgG, Index (S/C): 1: 0; 2: 10±3; 3: 55±5; 4: 65±5; 5: 75±5; 6: 85±5; 7: 95±5; 8: 125±5; 9: 175±5. Significance plot of data A.2: INF-γ; B.2: TNF-α are compared using one-way analysis of variance with Tukey's honestly significant difference post-hoc test to determine p values.

Pentraxin 3 (PTX3), IFN-γ, IFN-λ2/3, and IL-6, in people after 8 months of COVID-19 recovery. The authors have selected reasons for these long-lasting inflammatory conditions, such as antigen persistence, autoimmunity, or reflection of damage repair.[18] Other research has represented the triad of cytokines (IL-1ß, IL-6, and TNF-α), which play the most critical role in long-term post-COVID-19 conditions.[19] Here, we confirmed the primary influence of IL-1ß, IL-6, and TNF-α on the clinical symptoms of post-COVID-19 complications. In some cases, patients have a high level of autoimmune antibodies, but no direct correlation with changes in the cytokine profile has been established.[20] Some studies have reported the influence of TNF-α and IFN-γ on the death of cells infected by SARS-CoV-2.[21] According to the results described in our paper, we detected that the levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, and INF-γ) varied in the blood plasma of donors depending on the titers of anti-SARS-CoV-2 IgG. It should be noted that IL-8 levels were within the normal range (compared with the reference point) in most donor groups. As follows, we may conclude various changes in pro-inflammatory cytokine levels depending on the anti-SARS-CoV-2 IgG titer. Depending on the titer of anti-SARS-CoV-2 IgG, the levels of pro-inflammatory cytokines can either increase or decrease.

The necessary part of our discovery was the study of anti-inflammatory cytokine levels, such as IL-4 and IL-10, in donor groups. Recent research has confirmed that increased levels of IL-4 and IL-10 in patients who recovered from COVID-19 are indicative of the absence of post-COVID-19 complications. This suggests better control of the inflammatory process via the anti-inflammatory cytokine synthesis pathway. In addition, patients with clinical complications after COVID-19 had decreased IL-4 and IL-10 levels.[22] Our results have described the decrease in IL-4 among donor groups from the titer of anti-SARS-CoV-2 IgG 55±5

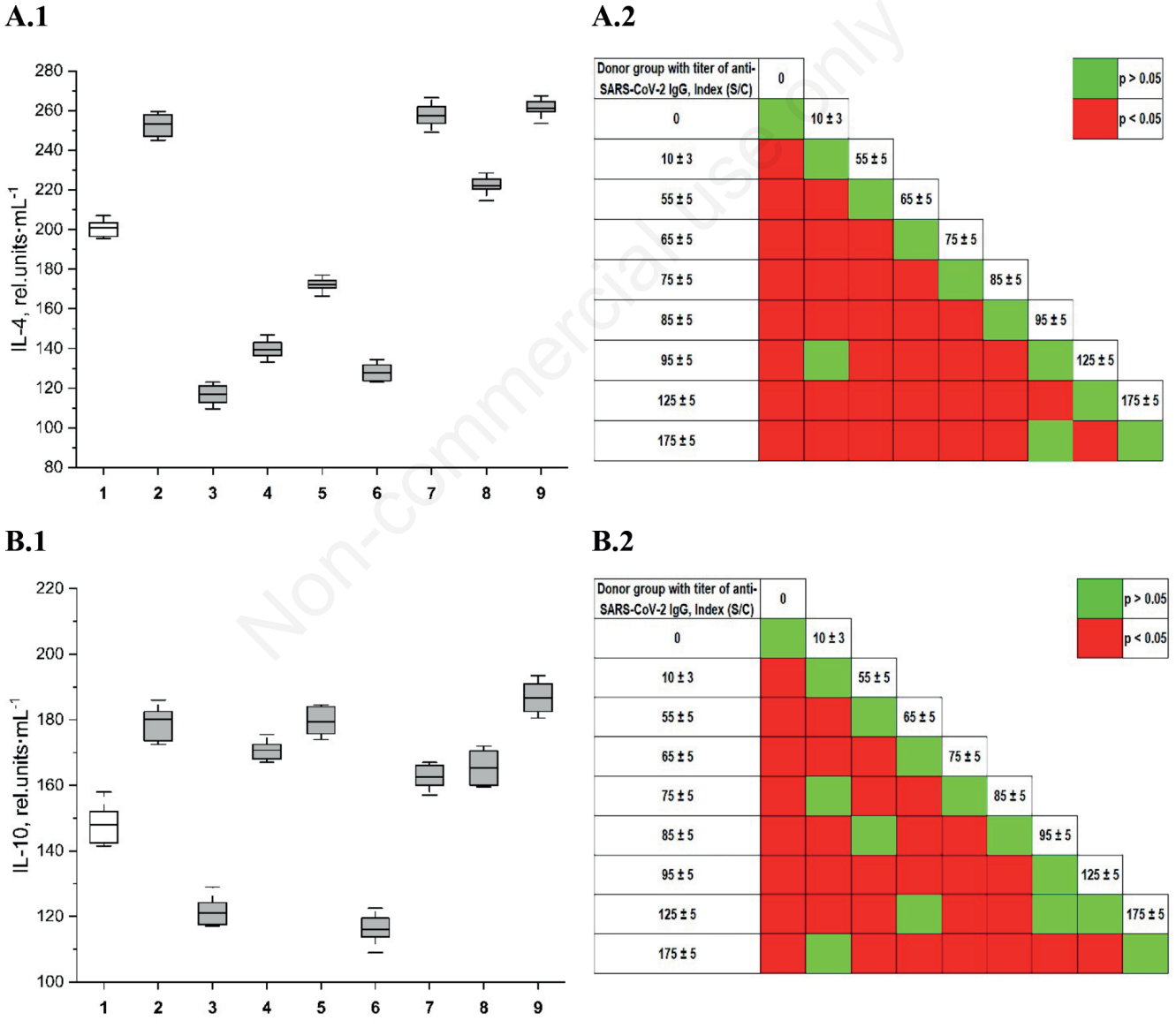

Figure 4. Levels of A.1: IL-4; B.1: IL-10 depending on titers of anti-SARS-CoV-2 IgG, Index (S/C): 1: 0; 2: 10±3; 3: 55±5; 4: 65±5; 5: 75±5; 6: 85±5; 7: 95±5; 8: 125±5; 9: 175±5. Significance plot of data A.2: IL-4; B.2: IL-10 are compared using one-way analysis of variance with Tukey's honestly significant difference post-hoc test to determine p values.

Index (S/C) to 85±5 Index (S/C). We predicted that these titers of anti-SARS-CoV-2 IgG might lead to the most significant pathological changes for 3-6 months after COVID-19 recovery, but it needs additional research for confirmation. At the same time, an increase in IL-10 levels was observed in most donor groups. This may be linked to attempts to stabilize immune activity in the post-COVID-19 period.

According to the cited literature and the obtained results, we predicted the critical reason for inflammatory complications is the occurrence of antibody subpopulations. Based on literature reports on autoantibody imbalance during the post-COVID-19 period, we predict that each group in our research had specific types of antibodies, including autoantibodies. Powerful immune activity during COVID-19 (for instance, a cytokine storm) may cause dysregulation of immune control.[23] This leads to a deviation in autoimmune tolerance during the post-COVID-19 period. Moreover, the actual presence of anti-SARS-CoV-2 IgG may trigger prolonged immune destabilization. We demonstrated the dependence of inflammatory profile changes on anti-SARS-CoV-2 IgG titers. This is a key reason for the abnormal activity of transcription and inflammatory factors, which may cause pathological conditions during the post-COVID-19 period.

In addition, we would like to give attention to our published results regarding changes in the hemostasis system in donor groups depending on the titers of anti-SARS-CoV-2 IgG.[24] The immune response is closely related to blood coagulation. We predict that the changes in the cytokine profile observed in this study may also be one of the reasons for coagulation dysfunction. However, we emphasize that the initial link to the development of disorders may be antibody subpopulations, including autoantibodies. Conclusions regarding the potential activation of autoantibodies were made based on previous studies concerning the detection of autoantibodies and their direct impact on functional status in various diseases, including systemic sclerosis,[25] Epstein-Barr infection,[26] and ischemic stroke.[27]

Conclusions

We determined the key changes in cytokine profiles between the donor groups depending on the titers of anti-SARS-CoV-2 IgG. The results confirmed the influence of anti-SARS-CoV-2 IgG on cytokine lines, including the transcription factor NF-κB, pro-inflammatory cytokines, and anti-inflammatory cytokines. We propose a scientific hypothesis regarding the main role of the produced antibody subpopulations (during the inflammatory response against SARS-CoV-2) in the persistence of post-COVID-19 complications. It means the presence of cytokines profile changes depending on various titers of anti-SARS-CoV-2 IgG which may take part in the immune dysregulation.

The findings of analyses of cytokine profiles in donor groups based on anti-SARS-CoV-2 IgG titers are presented in this paper. We emphasize that titers of anti-SARS-CoV-2 IgG are unique for each group because they have different functional manifestations in inflammatory processes, including changes in cytokine profiles. Currently, we are working to select and purify antibody fractions from the blood plasma of donors. We plan to carry out *in vivo* and *in vitro* experiments using antibody fractions.

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