IMPACT OF 21-DAY BED REST ON THE PHENOTYPIC FEATURES OF HUMAN PERIPHERAL BLOOD GRANULOCYTES

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Abstract. Nowadays, manned cosmonautics is faced with the task of carrying out a long-term space flight beyond the limits of low Earth orbit. Under the conditions of an orbital space flight, a person is exposed to a number of adverse effects on the body, among which microgravity is especially distinguished. Prolonged exposure to microgravity can lead to severe immune impairment. At present, immunological studies of cosmonauts can be conducted only after they return to Earth at the end of a space flight, as a result of which the use of ground-based models that imitate specific space factors seems to be an advanced direction. A well-studied microgravity model is bed rest, during which volunteers are kept in strict rest in bed. Granulocytes, as representatives of innate immunity, are the first among immune cells to respond to an altered state of the body; therefore, researches of the influence of bed rest on the granulocyte phenotypic characteristics can provide important information for the development of prophylaxis measures to the immune disorders' development when exposed to microgravity. The work used data obtained from six subjects. The impact of bed rest was determined at the end of the model, after 21 days. The following granulocyte clusters of differentiation (CD) were studied by flow cytometry: CD25, CD64, CD23, CD14, CD16, CD36, CD11b, CD18, CD286. Tendencies to a decrease in the percentage of $CD64^+$ subpopulation and an increase in the percentage of $CD23^+$, as well as $CD25^+$ subpopulation of granulocytes after 21 days of bed rest were revealed.

Keywords: bed rest, microgravity model, granulocytes, granulocyte phenotypic features.

Introduction

At present, manned cosmonautics is at the forefront of the development of world science and technology. A significant goal for cosmonautics is the implementation of a long-term space flight beyond the near-Earth orbit, primarily to the Moon and Mars. To realize such expeditions, we need to solve a number of complex problems related to maintaining the health and working efficiency of the crew throughout the mission and after its completion (Crucian *et al*., 2018). The adverse factors affecting the human body in the orbital space flight conditions are established to include microgravity, radiation, isolation from the outside world, artificial environment (Buchheim *et al*., 2019).

The long-term effect of microgravity on the human body can lead to serious disorders of different organ systems such as the cardiovascular, musculoskeletal, digestive and immune systems (Amirova *et al*., 2020; Gao & Chilibeck, 2020; Kehler *et al*., 2019; Crucian *et al*., 2018). Since the immune system has exceptional sensitivity to various adverse environmental conditions, it can be considered as an indicator system for monitoring the body's response to microgravity as one of the most serious factors in space flight (Ponomarev *et al*., 2016; Morukov *et al*., 2013).

To date, immunological studies of cosmonauts can be carried out only after their return to Earth at the end of the spaceflight. Consequently, the use of ground-based models that imitate specific spaceflight factors seems to be an advantageous direction (Hargens & Vico, 1985). Multiple-day anti-orthostatic hypokinesia, or bed rest, is a well-known model of weightlessness, which reproduces a variety of adverse impacts associated with the absence of gravity. The development of the special physiological status occasioned by both microgravity in space and hypokinesia in terrestrial conditions is based on such mechanisms as a change in the vector of the earth's gravity relative to the vertical axis of the body and a correlative decrease in the weight load on the musculoskeletal system and internal organs, a reduction in motor activity, and, finally, the abatement of the

dynamic force load on the skeleton elements (Sundblad *et al*., 2016). The results of immune system studies of healthy volunteers demonstrate that prolonged exposure to bed rest leads to a number of changes in the immunity (Konstantinova & Fuchs, 1991). Deviations of innate and adaptive immunity indicators suggest that under simulated microgravity conditions the work of the immune system alters both quantitatively and qualitatively. Notably, such changes as the inhibition of the natural killer and T-lymphocytes functional activity, as well as an increase in the B-lymphocytes and T-lymphocytes content in the peripheral blood were also observed in the majority of examined Russian crew members after long-term space expeditions in the early period of readaptation to earth conditions (Rykova, 2013; Morukov *et al*., 2010).

The development of measures for the prevention and prompt averting of the immune dysfunction progression under the influence of microgravity requires attention to both innate and adaptive immunity (Crucian *et al*., 2018). Given that the system of natural resistance is the first to react to the penetration of microorganisms into the body and, accordingly, further contributes to the formation of a specific adaptive immune response, the study of the mechanisms of changes in innate immunity within the framework of space medicine seems to be an attractive direction. However, studies on quantitative shifts, as well as phenotypic and functional changes in granulocytes, as the main cells of the innate immune system, under the influence of space flight factors are extremely few. There are data on the effect of a long-term space flight (more than 140 days) on surface adhesion molecules and the level of production of reactive oxygen species by polymorphonuclear neutrophilic granulocytes of 12 astronauts (Buchheim *et al*., 2019), as well as on the expression of signal pattern-recognition receptor genes by cells of the innate immune system (Ponomarev *et al*., 2016). However, granulocytes have a wide range of protective functions, such as phagocytosis, degranulation, NETosis, apoptosis, and most of them have yet to be studied (Liew & Kubes, 2019).

The purpose of our research is to study the influence of 21-day bed rest as a ground-based microgravity model on the phenotypic features of human peripheral blood granulocytes.

Materials and Methods

Microgravity simulation experimental model

The experiment «The state of the physiological systems of the human body when modeling distinct factors of space flight under conditions of 21-day anti-orthostatic hypokinesia» was conducted with the participation of 6 healthy male volunteers aged 25–40 years (height was no more than 195 cm, weight was no more than 85 kg), who received permission from the medical expert commission and signed an informed consent to participate in the experiment in accordance with the Declaration of Helsinki. The program of the experiment was approved by the Commission on Biomedical Ethics at the State Scientific Center of the Russian Federation-IMBP RAS and maintained by the section of the Academic Council. For 21 days, the subjects were in an anti-orthostatic position on a medical bed with a body inclination angle of -6 ° relative to the horizon without physical exercises and with moderate limitation of movements.

Materials

The research material was venous blood taken on an empty stomach in the morning in the background period (2 days before the start) and on the 21st day of the experiment. Blood sampling was carried out in line with the standard method under aseptic conditions. The samples were placed in vacuum tubes from Greiner Bio-One (Austria) with a standard content of K3-EDTA anticoagulant.

Flow cytometry for granulocyte receptor profile analysis

Analysis of granulocyte receptors is carried out by flow cytometry using antibodies conjugated with fluorescent dyes to detect clusters of differentiation (CD11b, CD18, CD64, CD16, CD23, CD14, CD36, CD25, CD286).

Whole blood samples (50 μl) were incubated in the dark at 4 °C for 30 minutes with the fol-

lowing monoclonal antibodies linked to a fluorescent label: CD11b-FITC (Cl. Bear1), CD18-PE (Cl. 7E4), CD64-FITC (Cl. 22), CD16-FITC (Cl. 3G8), CD23 (Cl. 9P25), CD14-PE (Cl. RMO52), CD36 (Cl. FA6.152), CD25 (Cl. B1.49.9) – Beckman Coulter (USA), TLR6-PE (cl. TLR6.127) – Hycult Biotech (Netherlands). After that, 250 µl of OptiLyse C Lysing Solution containing a fixing agent (1.5% formaldehyde) was added to each sample to perform erythrocyte lysis, and the cells were incubated for 15 minutes in the dark at 4 °C, according to the Beckman Coulter manufacturer's protocol. The cells were then washed twice from the lysing solution by adding 3 ml of phosphate-buffered saline followed by centrifugation at 1500 g at 4 °C for 10 minutes. Finally, 250 µl of phosphate-buffered saline was added to the cell pellet, resuspended, and placed in a flow cytometer for analysis.

Results

To study the phenotypic features of granulocytes in simulated microgravity, we chose the following surface markers: CD11b and CD18 adhesion molecules, receptors for the Fc fragment of immunoglobulins (CD64 and CD16 for IgG, CD23 for IgE), CD14 receptor for lipopolysaccharide (LPS), TLR-6 as receptor for lipoproteins of gram-positive bacteria, the CD36 receptor for thrombospondin as a marker of cell clearance and CD25, the ligand of which is interleukin-2 (IL-2).

Adhesion molecules

CD11b is the heavy α subunit of the β2 integrin and CD18 is the light β2 subunit of the integrins. Together, CD11b and CD18 form the CR3 complex, which is a receptor for the iC3b component of the complement system, which is necessary for phagocytosis of particles opsonized by iC3b (Fukuda & Schmid-Schonbein, 2003; Metelitsa *et al*., 2002). In addition, CD11b/CD18 is directly involved in the adhesion of neutrophilic granulocytes to endothelial cells of blood vessels, which occurs at the first stages of granulocyte migration to the inflammation site (Berton *et al*., 1996).

When comparing the baseline values with the values on day 21 of bed rest, no statistically significant differences were found between the two groups in the percentage of granulocytes carrying CD11b and CD18 on their surface (Fig. 1). The number of $CD11b^+$ granulocytes, as well as the number of $CD18^+$ granulocytes among the total number of granulocytes taken as 100% was about 99% and did not alter on day 21 of bed rest (Fig. 1A, B). Data are also retained when analyzing the percentage of granulocytes carrying the CD11b/CD18 complex, that is, the number of $CD11b^+/CD18^+$ granulocytes is 99% both before the start of the experiment and after 21 days (Fig. 1B).

Receptors for Fc fragments of immunoglobulins

Receptors for Fc fragments of immunoglobulins (Ig) are necessary for immune cells to carry out such functions as phagocytosis of opsonized microorganisms and antibody-dependent cellular cytotoxicity for the purpose of microorganism lysis (Trefers *et al*., 2019). CD64 (FcγRI) is a high affinity receptor for IgG. CD64 is a marker of activated granulocytes, since at rest there are few copies of this receptor on the surface of granulocytes, and when these cells are activated during inflammation, the number of CD64 copies on the cytoplasmic membrane increases significantly (Li *et al*., 2013). Expression of the CD16 gene (FcγRIII), a low-affinity IgG receptor, is very high even in resting granulocytes. CD16 interacts with other FcγRs, including CD64, which is necessary for phagocytosis (Trefers *et al*., 2019). CD23 (FcεRII), a low-affinity IgE receptor, has been shown to be found not only in eusinophilic but also in neutrophilic granulocytes in allergic patients (Monteseirin *et al*., 2003). Quantitative changes in CD64, CD16 and CD23 on the surface of granulocytes can serve as early markers of infectious or allergic disorders in real or simulated microgravity.

When studying the effect of bed rest on changes in the percentage of granulocytes containing CD64 on the surface as a high-affinity receptor for IgG, a tendency ($p = 0.063$) was found to decrease the percentage of $CD64^+$ sub-

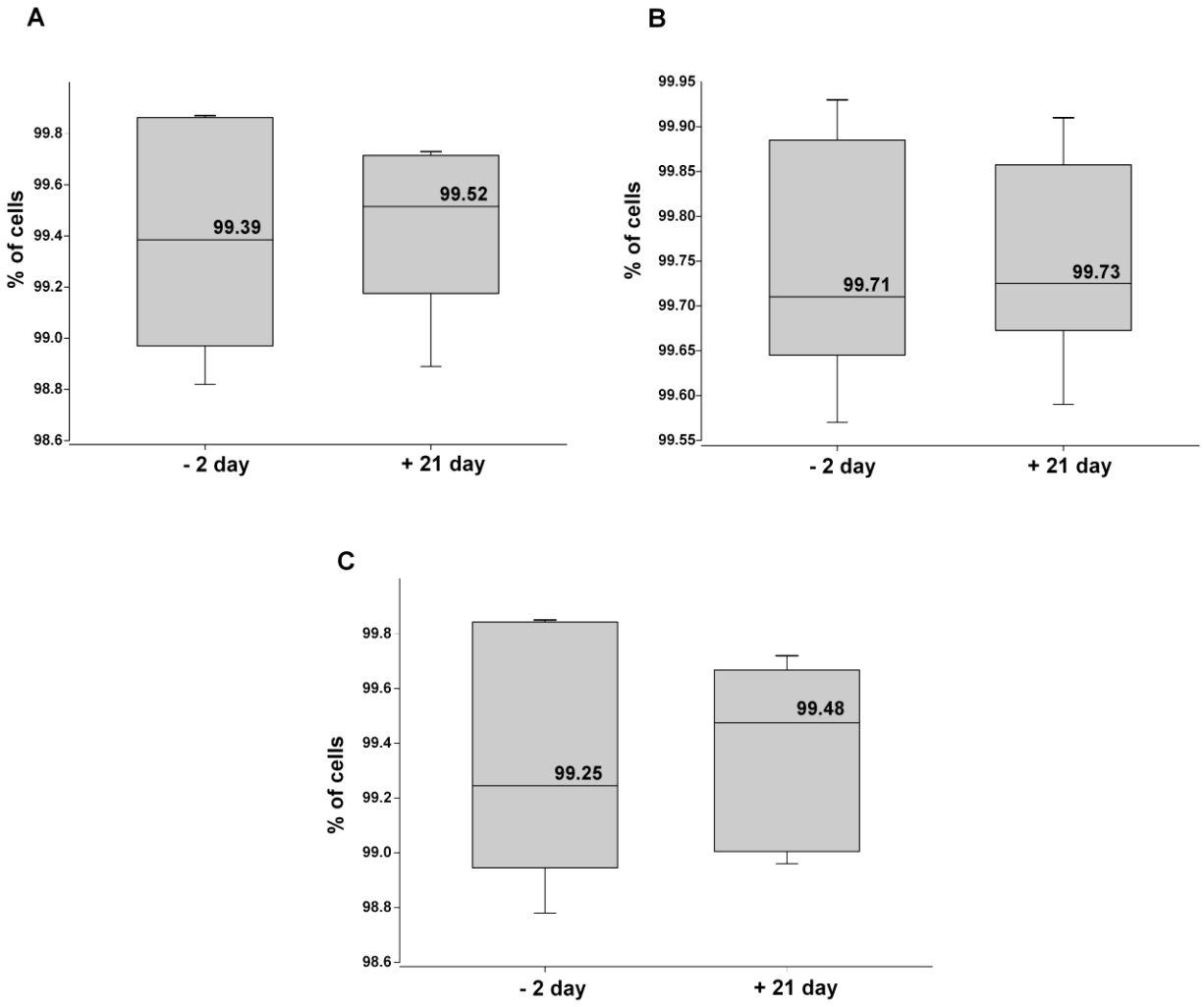

Fig. 1. The effect of 21-day bed rest on the subpopulation composition of peripheral blood granulocytes bearing on their surface clusters of differentiation responsible for granulocyte adhesion. A – $CD11b^+$ granulocyte subpopulation; B – $CD18^+$ granulocyte subpopulation; C – $CD11b^+CD18^+$ granulocyte subpopulation

population of granulocytes on day 21 of bed rest compared with baseline values (Fig. 2A). However, when assessing CD16 as a low-affinity receptor for IgG, no statistically significant differences or trends to change were found between the baseline values and the indicators on the 21st day of the experiment (Fig. 2B). The study of the IgE receptor revealed that exposure to 21-day bed rest can lead to an increase ($p = 0.063$) in the number of $CD23^+$ subpopulation of granulocytes (Fig. 2C).

Signal pattern recognition receptors

To study the effect of simulated microgravity on the subpopulations of granulocytes carrying signal pattern-recognition receptors of the Toll-like family on their surface, we chose the CD286 receptor, known as Toll-like receptor 6 (TLR 6), the ligands of which can be lipoproteins of gram-positive bacteria and saccharides of the fungi cell wall (Choteau *et al.*, 2017).

The study $TLR6^+$ subpopulation of human peripheral blood granulocytes did not reveal statistically significant differences between the values before and after the microgravity simulation experiment (Fig. 3A). These data are consistent with the results that we obtained in the study of $CD64^+$ and $CD16^+$ granulocyte subpopulations.

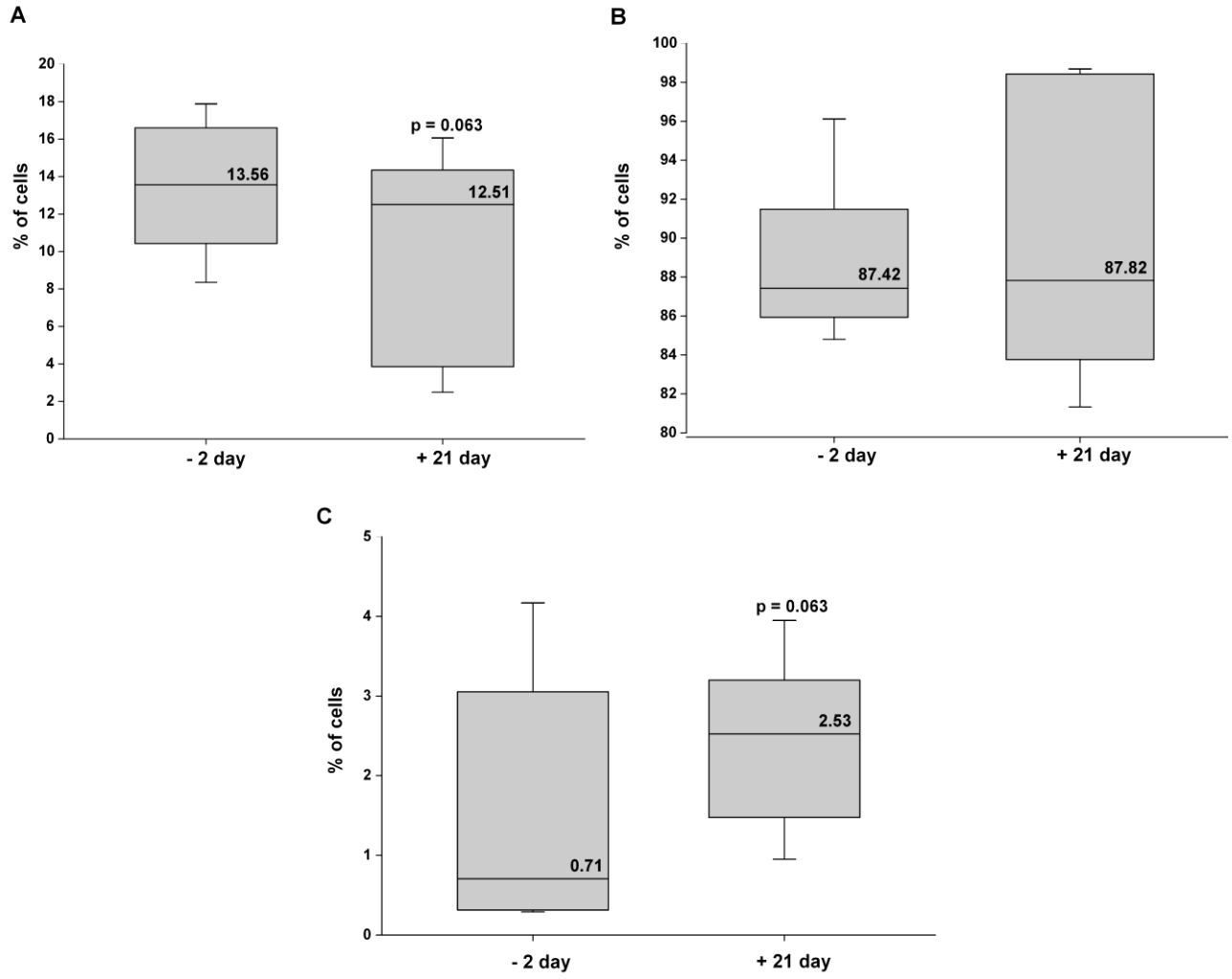

Fig. 2. The influence of 21-day bed rest on the subpopulation composition of peripheral blood granulocytes bearing on their surface clusters of differentiation responsible for binding Fc fragments of certain immunoglobulins. A – $CD64^+$ granulocyte subpopulation; B – $CD16^+$ granulocyte subpopulation; C – $CD23^+$granulocyte subpopulation

Co-receptors

An important role in the recognition of antigenic patterns is played by co-receptors, which are auxiliary molecules that act in combination with pattern-recognition receptors. CD14 is a co-receptor for a protein complex involved in the recognition of lipopolysaccharide (LPS), which is one of the most common and characteristic components of the Gram-negative bacteria cell wall.

On day 21 of bed rest, there were no statistically significant changes in the percentage of $CD14^+$ subpopulations of human peripheral blood granulocytes compared to baseline values (Fig. 3B).

Granulocyte clearance receptor

CD36 is a thrombospondin receptor. CD36 has been found to be required by apoptotic neutrophilic granulocytes to induce phagocytosis by macrophages. In this case, thrombospondin acts as a common bridge molecule that binds macrophages to CD36 on neutrophil apoptotic bodies (DeLeon-Pennell *et al*., 2016).

When analyzing the effect of bed rest on the granulocyte $CD36^+$ subpopulation percentage, no statistically significant changes were found compared to baseline values (Fig. 3C). These results are consistent with the previously stated assumption that in the subjects who are conditionally healthy people exposure to bed rest did

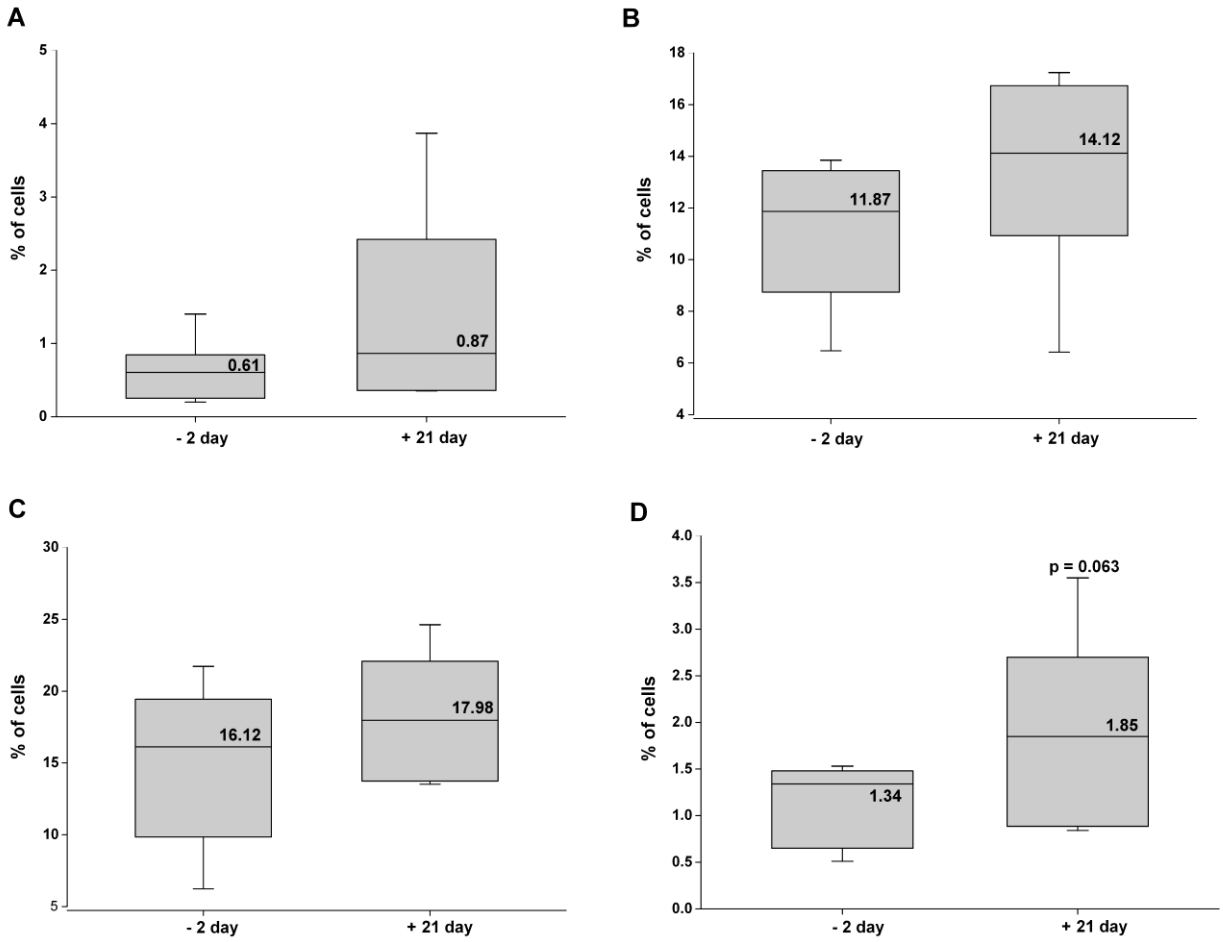

Fig. 3. The impact of 21-day bed rest on the subpopulation composition of peripheral blood granulocytes bearing on their surface clusters of differentiation involved in antigen recognition (A, B), granulocyte clearance (C), and regulatory IL-2 binding (D). A – $CD286^+$ granulocyte subpopulation; B – $CD14^+$ granulocyte subpopulation; C – $CD36^+$ granulocyte subpopulation; D – $CD25^+$ granulocyte subpopulation

not lead to the activation of apoptotic processes, including in relation to the granulocyte $CD36^+$ subpopulation.

CD25 as a potential marker of granulocyte activation

CD25 is a low-affinity α-chain of the IL-2 receptor and is found in activated T- and B-lymphocytes, monocytes/macrophages (Mahmoodpoor *et al*., 2018). There is very little data on the presence of CD25 on the granulocyte membrane, and it is believed that CD25 is not characteristic of granulocytes. However, a few animal studies suggest that CD25 can be used as a marker of granulocyte activation in acute infectious diseases (Zoldan *et al*., 2014). We decided to consider CD25 as a potential marker of human peripheral blood granulocyte activation and see how simulated microgravity would affect the percentage of $CD25^+$ granulocyte subpopulation.

As a result of this study, we found that there was a tendency ($p = 0.063$) to an increase in the percentage of $CD25^+$ subpopulation of peripheral blood granulocytes in subjects after 21 days of bed rest compared with the values before the start of the experiment (Fig. 3D). This may indicate that during bed rest there was an increase in the concentration of IL-2 in the blood and/or some stimulation in order to increase the sensitivity of granulocytes to IL-2 by increasing CD25 positive granulocytes.

Discussion

It is known that granulocytes migrate from the bone marrow to the blood when they are mature, and either are in the free circulation or move along the endothelium of blood vessels in the process of rolling. Adhesion is the first step in the activation of granulocytes in response to a chemoattractant formed in the focus of inflammation. The CD11b/CD18 complex is directly involved in the attachment of granulocytes to endothelial cells (Liew & Kubes, 2019). In our study, we did not observe changes in the percentage of $CD11b^+$, $CD18^+$, and $CD11b^+CD18^+$ granulocyte subpopulations after simulated microgravity in bed rest, however, the analyzed percentage of positive granulocyte subpopulations for these adhesive molecules was about 99% in all cases before and after the experiment. This suggests that there was no shift in the ratio of mature and immature granulocytes in the peripheral blood of the subjects. The high percentage of terminally differentiated CD11b/CD18 granulocytes did not change significantly.

A lower percentage of the $CD64^+$ granulocyte subpopulation may indicate a lower level of IgG-opsonized infectious agents in subjects during bed rest and/or inhibition of IgG-dependent granulocyte functions. Obviously, in this case, the involvement of CD16 as an auxiliary molecule for CD64 should not occur, which we observe, since the percentage of $CD16^+$ subpopulation of granulocytes does not modify on day 21 of the experiment compared to the values before bed rest. At the same time, there is a trend towards an increase in the $CD23^+$ subpopulation of granulocytes responsible for binding and responding to IgE.

The study of such pattern-recognizing receptors as CD14 receptor for LPS of gram-negative bacteria and TLR6 for lipoproteins of gram-positive bacteria and saccharides of the fungi cell wall demonstrated that the percentages of $CD14^+$ and $TLR6^+$ granulocyte subpopulation after the experiment did not statistically significantly differ from the corresponding indicators before the experiment. These results are consistent with the findings obtained in the research of $CD64^+$ and $CD16^+$ subpopulations of granulocytes and indicate the absence of infectious agents of a bacterial or fungal nature and/or a weakening of the response to them when exposed to bed rest.

The study of the CD14 co-receptor to LPS of gram-negative bacteria and TLR6 to lipoproteins of gram-positive bacteria and saccharides of the fungi cell wall showed that the percentages of CD14- and TLR6-positive granulocytes after the experiment did not statistically significantly differ from the corresponding indicators before the experiment. These results are consistent with the findings obtained in the study of $CD64^+$ and $CD16^+$ subpopulations of granulocytes and may indicate a potential absence of infectious agents of a bacterial or fungal nature and/or a weakening of the response to them under bed rest conditions, however, this assumption requires additional studies.

When assessing the percentage of CD36-positive granulocytes involved in the apoptosis of these cells and necessary for the clearance of granulocytes from the inflammatory focus after they have performed their protective functions, no influence bed rest on the percentage of CD36+ granulocyte subpopulation was revealed. Probably, no processes triggering a pathologically high level of granulocyte apoptosis occurred during bed rest, as a result of which, on the 21st day of the experiment, the pool of $CD36^+$ granulocytes remained at the baseline indicators level.

In our study, a 21-day microgravity model resulted in an increase in the percentage of $CD25^+$ subpopulations of peripheral blood granulocytes in subjects. Since there was also a trend towards an increase in the CD23+ subpopulation of granulocytes, it is probably advisable in the future to test the possibility of a relationship between the functional activity of CD23 and CD25 on the surface of granulocytes during simulated microgravity.

Thus, the influence of simulated microgravity on the phenotypic features of human peripheral blood granulocytes was evaluated for the first time. Tendencies to a decrease in the percentage of $CD64^+$ granulocyte subpopulation and an increase in the percentage of $CD23^+$, as well as $CD25^+$ granulocyte subpopulations of

the subjects after 21 days of anti-orthostatic hypokinesia were revealed. The data obtained can be utilized for further studies of the microgravity impact on the human granulocyte receptor profile, as well as on the functional features of these cells.

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