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PHENOTYPE REMODELING IN NEUTROPHILIC GRANULOCYTE SUBSETS CD64$^-$CD32$^+$CD16$^+$CD11B$^+$NG, CD64$^+$CD32$^+$CD16$^+$CD11B$^+$NG IN *DE NOVO* EXPERIMENTAL MODEL OF VIRAL-BACTERIAL INFECTION *IN VITRO*

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Abstract. A search for new targeted therapeutic strategies based on examining immunopathogenetic mechanisms for emerging co-infections is relevant and may further contribute not only to optimizing choice of immunotropic drugs, but also to achieving positive clinical and immunological remission for abnormal infectious processes. Previously, our studies found that recurrent viral-bacterial respiratory infections are associated with dysfunction of neutrophilic granulocytes (NG) with varying degree of intensity in altered effector properties. NG dysfunctions are often associated with diverse phenotypic profiles characterized by varying density for expression level of functionally significant trigger receptors. The aim of the study was to pinpoint phenotype transformation in CD64$^-$CD32$^+$CD16$^+$CD11b$^+$, CD64$^+$CD32$^+$CD16$^+$CD11b$^+$ neutrophilic granulocytes in experimental model of viral-bacterial infection *in vitro*. We examined 52 peripheral blood samples collected from 13 healthy adult volunteers. Viral, bacterial and virus-bacterial infection was modelled *in vitro* by incubating blood-derived cell samples with formyl-methionyl-leucyl-phenylalanine (fMLP), double-stranded RNA (dsRNA) or in combination followed by assessing changes in immunophenotyping of CD64$^-$CD32$^+$CD16$^+$CD11b$^+$NG, CD64$^+$CD32$^+$CD16$^+$CD11b$^+$NG by using using MAbs CD16-ECD, CD64-FITC, CD32-PE, CD11b-PC5 conjugates (Beckman Coulter International SA, France). It was demonstrated that NGs from healthy adult volunteers were dominated by CD64$^-$CD32$^+$CD16$^+$CD11b$^+$NG as well as minor subset CD64$^+$CD32$^+$CD16$^+$CD11b$^+$ NG varying in expression density of membrane molecules. Percentage of the minor subset CD64$^+$CD16$^+$CD32$^+$CD11b$^+$ NG was significantly increased after exposure with dsRNA, fMLP and dsRNA$^+$fMLP compared to untreated samples. Comparative analysis re vealed that various immunotropic agents differed in affecting expression of surface receptor molecules CD16, CD32 and unidirectional effects, but of varying magnitude altering CD11b marker both in major and minor subsets. Preincubation with dsRNA followed by adding fMLP allowed to find that they co-stimulated expression of surface receptors in both NG subsets. We generated an experimental model of viral-bacterial co-infection *in vitro* by using fMLP and dsRNA and observed types of phenotype transformation in CD64$^-$CD32$^+$CD16$^+$CD11b$^+$ NG and CD64$^+$CD32$^+$CD16$^+$CD11b$^+$ NG subsets. This model can be used to evaluate transformation of other NG subset phenotypes, NG functional activity, features of NET formation as well as impact of various immunotropic agents on NG.

Key words: *neutrophilic granulocytes, phenotype, subpopulation, coinfection, experimental model, fMLP, dsRNA.*

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РЕМОДЕЛЛИНГ ФЕНОТИПА СУБПОПУЛЯЦИЙ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ CD64⁻CD32⁺CD16⁺CD11B⁺НГ, CD64⁺CD32⁺CD16⁺CD11B⁺НГ В СОЗДАННОЙ *DE NOVO* ЭКСПЕРИМЕНТАЛЬНОЙ МОДЕЛИ ВИРУСНО-БАКТЕРИАЛЬНОЙ ИНФЕКЦИИ В СИСТЕМЕ *IN VITRO*

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Резюме. Поиск новых таргетных терапевтических стратегий, базирующихся на изучении иммунопатогенетических механизмов возникновения коинфекций, является актуальным и может в дальнейшем способствовать не только оптимизации выбора иммунотропных лекарственных средств, но и достижению позитивной клинико-иммунологической ремиссии нетипично протекающих инфекционных процессов. Ранее нашими исследованиями было установлено, что возвратные вирусно-бактериальные респираторные инфекции ассоциированы дисфункциями нейтрофильных гранулоцитов (НГ) с разной степенью выраженности нарушений их эффекторных свойств. Зачастую дисфункции НГ сопряжены с различными фенотипическими профилями, характеризующимися разными уровнями и плотностью функционально значимых триггерных рецепторов. Целью исследования было уточнение вариантов трансформации фенотипа субпопуляций CD64⁻CD32⁺CD16⁺CD11b⁺НГ, CD64⁺CD32⁺CD16⁺CD11b⁺НГ в созданной экспериментальной модели вирусно-бактериальной коинфекции *in vitro*. Исследовано 52 образца периферической крови 13 здоровых взрослых добровольцев в возрасте от 21 до 32 лет. Для воспроизведения условий вирусной, бактериальной и вирусно-бактериальной инфекции образцы инкубировали с формил-метионил-лейцил-фенилаланин (fMLP), двухцепочечной РНК (дцРНК) и совместно, затем определяли фенотипические характеристики субпопуляций CD64⁻CD32⁺CD16⁺CD11b⁺НГ, CD64⁺CD32⁺CD16⁺CD11b⁺НГ с использованием конъюгатов МКАТ CD16-ECD, CD64-FITC, CD32-PE, CD11b-PC5 (Beckman Coulter International S.A., Франция). Анализ полученных данных продемонстрировал, что НГ здоровых взрослых лиц представлены мажорной субпопуляцией CD64⁻CD16⁺CD32⁺CD11b⁺ НГ и минорной субпопуляцией CD64⁺CD16⁺CD32⁺CD11b⁺ НГ с разной плотностью мембранных молекул. Минорная субпопуляция CD64⁺CD16⁺CD32⁺CD11b⁺ НГ значительно увеличилась под влиянием дцРНК, fMLP и дцРНК + fMLP по сравнению с интактными образцами. Сравнительный анализ моновлияния иммунотропных субстанций позволил выявить их разные эффекты в отношении действия на поверхностные рецепторные молекулы CD16, CD32 и однонаправленные, но разной интенсивности на CD11b, как в мажорной, так и в минорной субпопуляциях. Преинкубация с дцРНК с последующим добавлением fMLP в группе исследования позволила выявить эффекты совместного стимулирующего влияния субстанций на уровни поверхностных рецепторов обеих субпопуляции НГ. Нами была создана экспериментальная модель вирусно-бактериальной коинфекции в системе *in vitro* с использованием fMLP и дцРНК и установлены варианты трансформации фенотипа субпопуляций CD64⁻CD32⁺CD16⁺CD11b⁺НГ и CD64⁺CD32⁺CD16⁺CD11b⁺НГ. Данная модель может быть использована для оценки вариантов трансформации фенотипа других субпопуляций НГ, изучения функциональной активности НГ, особенностей формирования NET, влияния на НГ различных иммунотропных субстанций.

Ключевые слова: *нейтрофильные гранулоциты, фенотип, субпопуляция, коинфекция, экспериментальная модель, fMLP, дцРНК.*

Introduction

An urgent problem of modern clinical immunology is the creation of new technologies to restore the normal functioning of the immune system in various infectious diseases of bacterial and viral etiology, including mixed infections. The problem of mixed infection in the modern world is recognized as one of the most urgent, since so far this sixth part of the world's set has been affected by this combined pathology [3, 22]. In recent years, in Russia there has been an absolute and relative increase in the incidence of children and adults with various infectious diseases [6, 7]. It has been shown that many viral infections cause a worsening of the course of the chronic inflammatory processes of the respiratory tract of bacterial etiology due to *S. aureus*, *Str. pneumoniae* [25, 26, 35, 37]. Viral-bacterial mixed infections can significantly change the clinical picture of each infection separately and lead to a more severe course of diseases that are not amenable to standard therapy methods [2, 18].

Neutrophilic granulocytes (NG) are multifunctional cells that have a powerful protective anti-infective effector potential and, in addition, carry out, as a positive, and if necessary, negative regulation of the innate and adaptive immune system [10, 30, 36]. On the other hand, under the influence of certain viruses and/or bacteria, NGs can change their properties. In particular, NG hyperactivation that occurs under the influence of pathogens leads to damage to tissues and organs. At the same time, some viruses

and bacteria can cause damage to NGs themselves, which leads to the appearance of defects in their functioning. In some cases, an acute viral infection can cause neutropenia, functional, regulatory, and other dysfunctions of NG, while cell damage can be either mono or combined. The occurrence of defects in the functioning of NG causes various complications, and, first of all addition of bacterial infections [12]. Grunwell J.R. и et al. (2019) described neutrophil dysfunctions in the respiratory tract in children with acute respiratory failure arising from viral and bacterial co-infections of the lower respiratory tract [23].

Previously, our studies found that immunocompromised children with recurrent viral and bacterial respiratory respiratory infections associated with latent and/or recurrent herpesvirus infections revealed NG dysfunctions with varying degrees of severity of their effector properties. These NG dysfunctions were associated with various phenotypic NG profiles characterized by different levels and expression densities of functionally significant trigger receptors.

The full realization of the effector and regulatory activity of NG is closely related to a certain group of surface membrane proteins CD64, CD32, CD16, CD11b, which form a certain NG phenotype. FcγRs activates endocytosis, phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), the formation of reactive oxygen species (ROS), an oxygen explosion, the secretion of granular enzymatic and non-enzymatic proteins, cytokines, and the formation of neutrophilic extracellular nets (NET) [10, 11, 12, 16, 31].

CD64 (FcγRI) — a high affinity receptor capable of binding human IgG1, IgG3 and IgG4 in monomeric form, is practically not expressed on the membrane surface of unactivated NG blood in healthy individuals [27]. CD64 is a marker for inflammatory processes and infection [20, 21, 29, 38]. CD64 on activated NGs binds to IgG, activates NADPH oxidase, the formation of reactive oxygen species, which leads to increased phagocytosis and the launch of ADCC via an oxygen-mediated mechanism [15, 17].

The expression of the low affinity CD32 (FcγRIIa) receptor for IgG on the NG membrane surface in response to pathogens or cytokines triggers the Ca^{2+}-transit and signaling of tyrosine phosphorylation, which leads to activation of NADPH oxidase complex. In addition, CD32 mediates endocytosis, stimulation of secretory activity, cytotoxic mechanisms and immunomodulatory functions of NG [34, 39].

CD16 (FcγRIIIb) is a low-affinity IgG receptor responsible for the cytotoxic function of NG. CD16 is not a phagocytosis receptor, but the binding of FcγRIIa to FcγRIIIb and the combination of CD16 with IgG initiates signaling cascades for ADCC, degranulation phagocytosis, oxygen burst and proliferation [1, 24]. High expression of CD16 molecules indicates an increased functional activity of NG. A decrease or absence of CD16 on the membrane surface of NG can indicate immaturity of NG and/or "reverse differentiation" of NG, leading to a decrease in the total number of leukocytes, up to leukopenia, which leads to tissue necrosis or bacterial infection [1]. In addition, it was shown that CD16 molecules are shedding on the cytoplasmic membrane of apoptotic NG [41]. At the same time, it was shown that the CD16 receptor is able to function together with the CD11b/CD18 receptor and enhance FcγRII-mediated internalization.

CD11b (Mac-1) is a neutrophilic surface antigen, which is the α-subunit of the β2-integrin adhesion molecule, a transmembrane heterodimeric NG receptor for the CR3b component of complement [19]. Neutrophilic CD11b is a marker of phagocytosis of activated NG. CD11b is stored in intracellular granules and is additionally expressed on the surface of NG after activation, which promotes adhesion of NG to the endothelium and transendothelial migration into tissues — to the loci of inflammation. An increase in the expression level of CD11b NG molecules is observed in various infections [33]. In addition, Mac-1 is a signaling partner for other receptors, such as the fMLP receptor, LPS receptor (CD14), and Fc receptors. Mac-1 associated with the actin cytoskeleton of NG and signaling proteins is able to regulate chemotaxis, migration, adhesion, phagocytosis, respiratory burst and NG degranulation. Impaired expression of CD11b on NG violates the regulatory mechanisms of the immune system. Blocking CD11b leads to a defect in the activation of Fcγ receptors and impaired phagocytic function of NG [28, 32].

There is no doubt that the cooperation of various receptors plays an important role in the implementation of the effector functions and regulatory mechanisms of NG, the interaction of which can both enhance and weaken the effect of each other [8].

The search for new targeted therapeutic strategies based on the study of immunopathogenetic mechanisms of co-infections is relevant. The study of these mechanisms can help optimize the choice of immunotropic drugs and achieve clinical and immunological remission of atypically occurring infectious processes. In this regard, the development of experimental model *in vitro* of viral-bacterial infections, in which it would be possible to assess the reorganization of the above functionally significant NG receptors, is of particular relevance. Such an experimental model, on the one hand, would allow revealing the features of the transformation of the receptor apparatus; on the other hand, its development is necessary to search for new immunotropic substances for optimal methods for the correction of NG dysfunctions arising from viral-bacterial infections.

Aim: to evaluate the transformation of the phenotype of the subsets $CD64^{-}CD32^{+}CD16^{+}CD11b^{+}$, $CD64^{+}CD32^{+}CD16^{+}CD11b^{+}$ neutrophilic granulocytes in created *de novo* experimental model of viral-bacterial infection *in vitro*.

Materials and methods

We examined 52 samples of peripheral blood of 13 healthy adult volunteers (7 women, 6 men) aged 21 to 38 years old. To develop a new experimental model of viral-bacterial infection *in vitro*, 4 groups were formed:

1. Comparison group 1 — intact NG;
2. Comparison group 2 — model of bacterial infection (incubation of NG with fMLP — an analogue of a bacterial pathogen);
3. Comparison group 3 — model of viral infection (incubation of NG with dsRNA — an analogue of a viral pathogen);
4. Study group — created *de novo* model of viral-bacterial infection (incubation of NG with dsRNA, and then with fMLP).

To create experimental conditions for viral infection *in vitro*, we incubated samples with double-stranded RNA (dsRNA). DsRNA is a key activator of innate immunity in viral infections and is a powerful inducer of interferons.

In order to reproduce the conditions of a bacterial infection *in vitro*, we selected N-formyl-methionyl-leucyl-phenylalanine (fMLP), which is a bacterial pathogen and, according to various researchers, has activating effects on NG. fMLP may be of exogenous origin (bacteria tripeptide) or endogenous origin (localized in the mitochondria of human cells). fMLP is one of the powerful chemotactic factors of NG, which is able to bind to heterodimeric G-proteins — cell surface receptors. fMLP activates signaling pathways mediated by phosphatidinositol-specific phospholipase C (PLC), phospholipase D (PLD), phosphotidylinositol-3 kinase (PI3K), and mitogen-activated protein kinases (MAPK) which leads to the activation of phagocytosis, chemotaxis, reactive oxygen species generation and release of microbicidal molecules from NG granules.

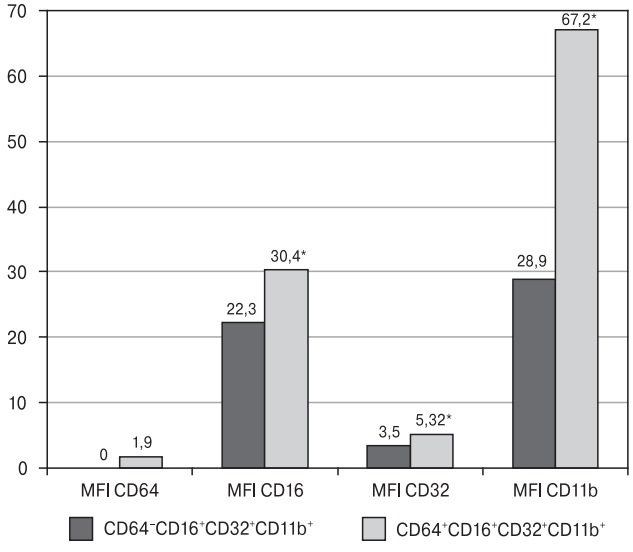

Figure 1. Characteristic of receptors of $CD64^-CD16^+CD32^+CD11b^+$ NG and $CD64^+CD16^+CD32^+CD11b^+$ NG subsets in healthy adult subjects

Note. * — statistically significant differences, $p < 0.05$.

The experimental study was carried out in several stages:

Stage I — investigation the phenotype of subsets $CD64^-CD32^+CD16^+CD11b^+$NG, $CD64^+CD32^+CD16^+CD11b^+$NG in comparison group 1 (intact NG);

Stage II — investigation phenotype of subsets $CD32^+CD16^+CD11b^+$NG, $CD64^+CD32^+CD16^+CD11b^+$NG in comparison group 2 after incubation samples with fMLP (10^{-7} M) for 15 minutes at T 37°C;

Stage III — investigation the phenotype of subsets $CD32^+CD16^+CD11b^+$NG, $CD64^+CD32^+CD16^+CD11b^+$NG in comparison group 3 after incubation samples with dsRNA (10^{-6} g/l) for 30 minutes at T 37°C;

Stage IV — investigation the phenotype of subsets $CD32^+CD16^+CD11b^+$NG, $CD64^+CD32^+CD16^+CD11b^+$NG in study group in created *de novo* model of viral-bacterial infection (after incubation samples with dsRNA (10^{-6} g/l) for 30 minutes at T 37°C, and then with fMLP (10^{-7} M) for 15 minutes at T 37°C).

The transformation of the phenotype of NG subsets under the influence of substances fMLP, dsRNA and dsRNA + fMLP was evaluated by flow cytometry (FC 500, Beckman Coulter, USA) using MAbs conjugates CD16-ECD, CD64-FITC, CD32-PE, CD11b-PC5 (Beckman Coulter International S.A., France). We determined the number (in %) of subsets of $CD32^+CD16^+CD11b^+$NG, $CD64^+CD32^+CD16^+CD11b^+$NG and the intensity of expression of each receptor (CD64, CD32, CD16, CD11b) according to MFI.

Statistical processing of the obtained data was carried out using computer programs Microsoft Exel 2016 and StatPlus 2010 using nonparametric Wilcoxon and Mann–Whitney tests. The results were presented as the median (upper and lower quartile) Me (Q1; Q3). Significance of differences was determined at $p < 0.05$.

Results

Data showed that NG in healthy adults is represented by a subset of CD64-$CD16^+CD32^+CD11b^+$ NG in 96.1 (93.7; 97.2)% — major subset and a subset $CD64^+CD16^+CD32^+CD11b^+$ NG in 0.2 (0.1; 1.9)% — minor subset; expression density of membrane molecules of these subset is different. The subset $CD64^+CD16^+CD32^+CD11b^+$ NG with CD64 receptor has a higher level of MFI CD16 — 30.4 (24.6; 36.0), CD32 — 5.32 (4.9; 6.3) and CD11b — 67.2 (54.8; 71.1) ($p_{1,2,3} < 0,05$) (Fig. 1.).

Incubation of samples with fMLP (comparison group 2) led to an 18-fold increase in $CD64^+CD16^+CD32^+CD11b^+$ NG ($p < 0.05$) and a decrease in CD64-$CD16^+CD32^+CD11b^+$ NG ($p > 0.05$) (Fig. 2).

The level of MFI CD16 increased 1.4 times ($p < 0.05$) and CD11b increased 3.3 times ($p < 0.05$) in the major subset after incubation of samples with fMLP. This indicates the readiness of NG healthy adults to develop an adequate response to exogenous and endogenous pathogens and various injuries. There was no significant effect of fMLP on the MFIs of NG receptors in the minor subset (Table).

Minor subset of $CD64^+CD16^+CD32^+CD11b^+$ NG significantly increased by 20 times ($p < 0.05$) under the influence of dsRNA in comparison group 3, the levels of membrane receptors CD32 and CD11b were also higher than in comparison group 1 ($p_1 < 0.05$, $p_2 < 0.05$). Significant effects of dsRNA influence on the CD64-$CD16^+CD32^+CD11b^+$NG subset and levels of CD16 and CD32 molecules were absent; the level of MFI CD11b was 2 times higher compared with comparison group 1 ($p < 0.05$) (Table).

Upon incubation with fMLP, the level of CD16 receptor increased statistically significantly in the subset of CD64-$CD16^+CD32^+CD11b^+$ NG ($p < 0.05$), while the effect of dsRNA showed a slight decrease ($p > 0.05$). Incubation with dsRNA led to an increase in the expression of the CD32 receptor by 1.5 times on the NG subset of $CD64^+CD16^+CD32^+CD11b^+$ ($p < 0.05$) and 1.34 times on the NG subset of CD64-$CD16^+CD32^+CD11b^+$NG ($p > 0.05$), while significant effects of fMLP were absent.

Preincubation with dsRNA followed by the addition of fMLP in the study group revealed the effects of a joint stimulating effect of substances on the MFI levels of surface receptors of both subsets of NG. The content of $CD64^+CD16^+CD32^+CD11b^+$ subsets of NG ($p < 0.05$) significantly increased, the MFI of all receptors also increased: CD64 by 1.1 times, CD16 — 1.4 times, CD32 — 1.56 times, CD11b by 1.78 times ($p_1 < 0.05$; $p_2 < 0.05$; $p_3 < 0.05$; $p_4 < 0.05$) (Table, Fig. 2). The combined effect of both substances did not change the content of CD64-$CD16^+CD32^+CD11b^+$ subsets of NG and led to an increase in the expression density of CD16 by 1.15 times, CD32 — 1.5 times, CD11b — 1.53 times ($p_1 < 0.05$; $p_2 < 0.05$; $p_3 < 0.05$) (Table).

A comparative analysis of the effect of immunotropic substances revealed multidirectional effects on the receptor molecules CD16, CD32 and the uni-

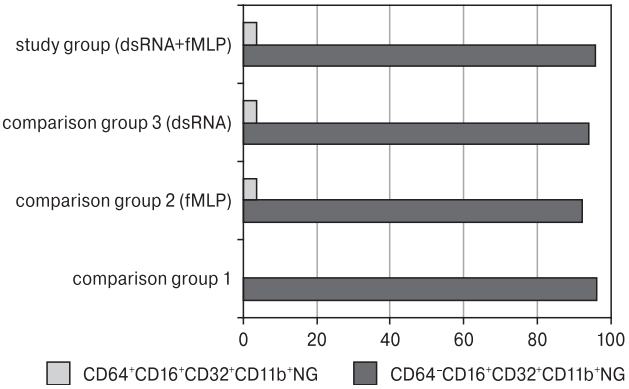

Figure 2. Transformation of $CD64^-CD16^+CD32^+CD11b^+$ and $CD64^+CD16^+CD32^+CD11b^+$ neutrophilic granulocytes subsets under the influence of fMLP and dsRNA

Note. * — differences are significant compared with comparison group 1, $p < 0.05$.

Table. The effect of fMLP and dsRNA on phenotype of $CD64^-CD16^+CD32^+CD11b^+$ and $CD64^+CD16^+CD32^+CD11b^+$ neutrophilic granulocytes subsets in healthy adults *in vitro*, Me (Q1; Q3)

Группы Groups	$CD64^-CD16^+CD32^+CD11b^+$				
	NG, %		MFI CD16	MFI CD32	MFI CD11b
Comparison group 1	96,1 (93,7; 97,2)		22,3 (18,3; 24,9)	3,5 (3,09; 4,28)	28,9 (19,5; 37,9)
Comparison group 2 (fMLP)	91,5 (90,0; 95,7)		30,5* (27,5; 36,9)	3,8 (3,21; 5,1)	**71,4*** (69,15; 98,3)
Comparison group 3 (dsRNA)	93,4 (91,7; 96,2)		21,6^ (19,1; 24,5)	4,7 (2,97; 6,3)	**57,9*^** (46,0; 65,3)
Study group (dsRNA + fMLP)	94,7 (94,7; 96,2)		25,5*• (25,5; 34,8)	5,2*# (5,12; 6,9)	**73,2*•** (73,2; 98,25)
Группы Groups	$CD64^+CD16^+CD32^+CD11b^+$				
	NG, %	MFI CD64	MFI CD16	MFI CD32	MFI CD11b
Comparison group 1	0,2 (0,1; 1,9)	1,9 (1,45; 2,09)	30,4 (24,6; 36)	5,32 (4,9; 6,3)	67,2 (54,8; 71,1)
Comparison group 2 (fMLP)	3,7* (2,9; 5,7)	2,26 (1,8, 2,4)	31,3 (27,2; 35,5)	6,1 (5,1; 7,0)	79,8 (62,1;81,7)
Comparison group 3 (dsRNA)	4,2* (2,9; 5,7)	2,15 (1,8; 2,2)	22,6 (19,5; 34,2)	**8,2*^** (7,34; 11,3)	**87,7*^** (85,3; 108)
Study group (dsRNA + fMLP)	3,85* (3,8; 5,66)	2,1* (2,13; 2,43)	40,7*#• (40,65; 74,2)	**8,3*#•** (8,25; 10,3)	**119,9*#•** (119,0;166)

Notes. * — the differences are significant compared with the comparison group 1, $p < 0.05$; ^ — differences are significant between comparison group 2 and comparison group 3, $p < 0.05$; # — differences are significant between the study group and the comparison group 2, $p < 0.05$; • — differences are significant between the study group and the comparison group 3, $p < 0.05$.

directional effect on CD11b, both in the major and minor subsets (Fig. 3).

We identified a tendency to increase MFI CD32 in the study group in the major subset; the level of MFI CD11b increasing under the mono influence of dsRNA or fMLP ($p_1 < 0.05$; $p_2 < 0.05$) remains at the level of the effect induced by fMLP. In contrast, the MFI CD16 receptor is reduced in equipment density, which is similar to the fMLP effect.

Sequential incubation with dsRNA and fMLP caused statistically significant effects of an increase in the number of CD16 and CD64, which was not established with mono-influence of substances in a subset of $CD64^+CD16^+CD32^+CD11b^+NG$. Noteworthy are the higher levels of MFI CD32 and CD11b detected by the combined effect of dsRNA and fMLP in the study group compared with the effects identified in comparison group 3.

Discussion

NGs fight viral and bacterial infections through the implementation of phagocytic activity, the ability to extracellularly degranulate enzymatic and non-enzymatic proteins, exosomes, initiating the development of a basic inflammatory response, forming NET, producing reactive oxygen species, synthesizing and secreting pro- and anti-inflammatory cytokines and, thus, regulate the work of both the innate and adaptive immune systems. It has been shown that there are subsets of NG with both pro-inflammatory and anti-inflammatory properties. At the same time, NGs are plastic and, depending on the conditions in which they are (contact with various antigens, change of cytokine background, hormonal effects, etc.), can change the phenotype, acquiring new or losing existing useful properties. In turn, various viruses and bacteria lead their struggle for survival and can cause both quantitative and functional disorders of NG: neutropenia, defects in phagocytic activity, which leads to violations of their killing properties and microbicidal activity, suppression of the production of reactive oxygen species, depression of antiviral and antibacterial activity, inability to form NET, negative transformation of functionally significant subsets, and etc. Defective functioning of NG leads to the appearance of atypically occurring, not responding to the standard therapy of viral and bacterial infections, as well as viral-bacterial mixed infections, which negatively affects their prognosis and outcomes of diseases [4, 5, 10, 11, 14].

For the first time, we performed a comparative analysis of the phenotype reorganization of two functionally significant subsets of blood $CD64^-CD32^+$

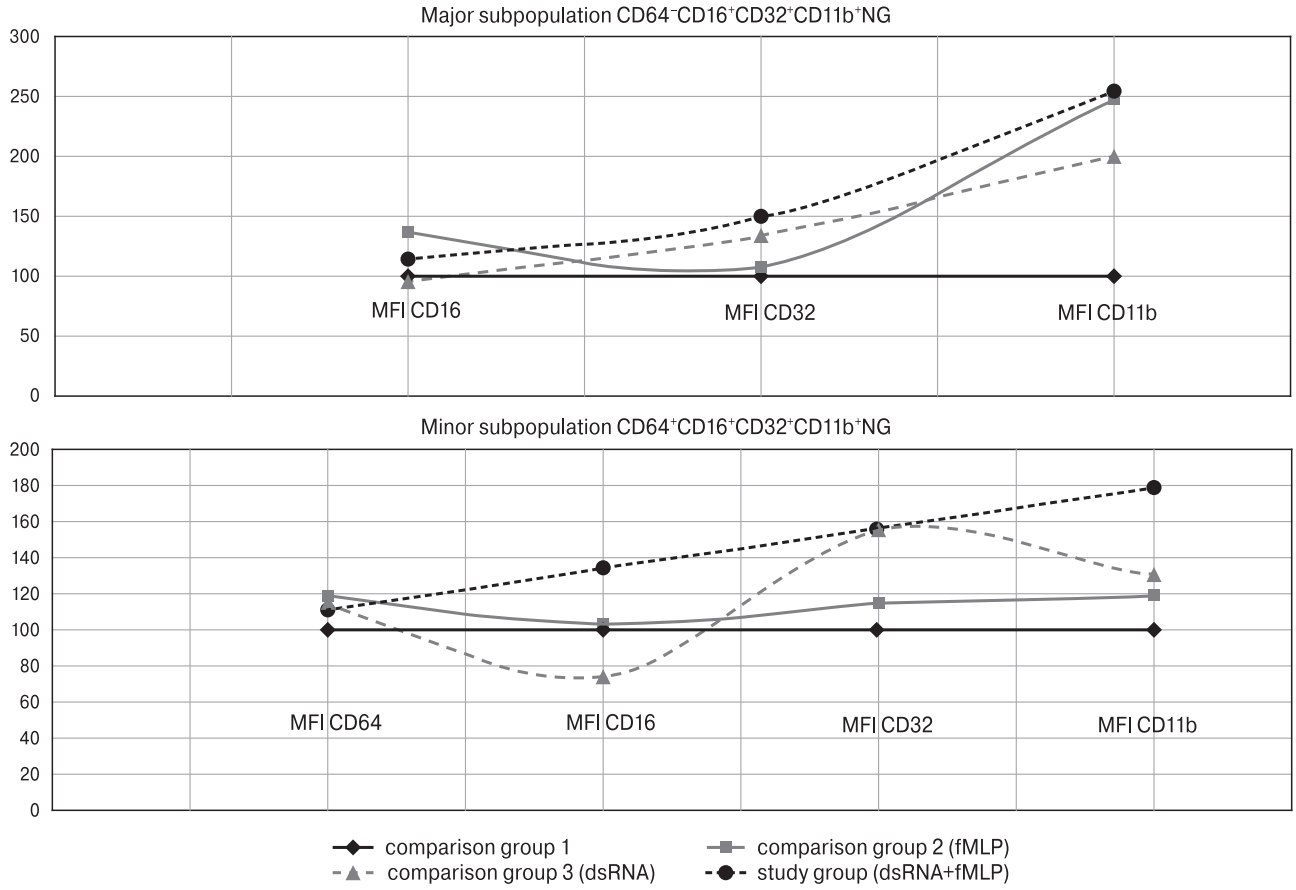

Figure 3. Subset phenotype transformation under the influence of immunotropic substances *in vitro*

$CD16^+CD11b^+NG$ and $CD64^+CD32^+CD16^+CD11b^+$ NG in models of viral infection, bacterial infection and viral-bacterial co-infection.

The results demonstrate that in the peripheral blood under physiological conditions the major subset of $CD64^-CD32^+CD16^+CD11b^+NG$ predominates. This subset is 96.1% and is characterized by moderate expression of CD16, CD32, CD11b molecules and very low level or complete absence of 64 molecules. The minor subset is only 0.2%, has a CD64 receptor, and higher expression levels of CD16, CD32, CD11b than in the major subset. The data obtained suggest that the major subsets are inherent in the properties of the "watchful guard", ready, if necessary, to rebuild and activate their potential. A minor subset, circulating in a very small amount, is more active and able to provide immediate protection.

The contact of a major subset of NG with a bacterial pathogen leads to an increase in the expression of molecules of CD16 and CD11b on the surface membrane. It is known that an increase in the expression of CD16 molecules indicates an increased functional activity of NG [1, 24]. Therefore, an increase in the expression density of CD16 on the NG membrane promotes the launch of ADCC, an increase in phagocytic activity, transmembrane degranulation, production of reactive oxygen species, etc. It is also known that a simultaneous increase in the expression of CD11b and CD16 molecules can enhance CD32-mediated internalization.

The number of NG minor subsets of $CD64^+CD32^+CD16^+CD11b^+$ NG significantly increased from 0.2% to 3.7% — by 18.5 times, due to the fact that about 4% of NG major subset, being in closed experimental system, transformed into an activated minor subset under the influence of the bacterial pathogen.

The high affinity CD64 receptor is practically not expressed on the membrane surface of non-activated NG in the blood in healthy individuals [27]. The appearance of the CD64 molecule on the NG membrane has a direct connection with the activation of cells by the bacterial antigen, followed by translocation of this molecule from the granular apparatus of the cell to the surface membrane of NG. It should be noted that the level of membrane molecules CD11b, CD16, CD32 in the minor subset did not change under the influence of the bacterial pathogen and remained at the level of comparison group 1.

After dsRNA exposure, the minor subset of $CD64^+CD32^+CD16^+CD11b^+$ NG significantly increased from 0.2% to 4.2% due to the fact that the NG major subset reoriented into the activated minor subset due to the translocation of the CD64 molecule from the cytoplasmic space to the surface membrane. In addition, there was a significant increase in the expression density of CD32 and CD11b receptors, and a decrease in CD16 molecules, which, apparently, is associated with shedding of CD16 under the influence of dsRNA.

In the created model of viral-bacterial co-infection obtained by sequential exposure to dsRNA and then fMLP, we revealed completely different profiles of phenotypic changes, both major $CD64^-CD32^+CD16^+CD11b^+NG$ and minor $CD64^+CD32^+CD16^+CD11b^+NG$ subsets. Taking into account the specific features of the phenotype of the $CD64^-CD32^+CD16^+CD11b^+NG$ subset, it can be concluded that this subset is more active in the model of viral-bacterial infection than in other experimental models.

In the minor subset of $CD64^+CD32^+CD16^+CD11b^+NG$ under the influence of the bacterial pathogen and dsRNA, we found a significant increase in the number of NG expressing CD64 from 0.2% to 3.85% — 19.25 times. The expression density of CD32, CD16, CD11b molecules was the highest in the model of viral-bacterial infection, not only in comparison with healthy individuals, but also in comparison with the data obtained in the models of viral and bacterial infection. The data obtained suggest that the experimental model of viral-bacterial infection has a superactivated phenotype of a minor subset with possible defective phagocytic and oxidase microbicidal activity. Similar changes were previously discovered by J.R. Grunwell and colleagues (2019) [23], who revealed the presence of NG dysfunctions in acute distress syndrome in children with viral and bacterial co-infections of the lower respiratory tract. The NG of these patients had an increased level of activation markers on the cytoplasmic membrane, but paradoxical facts were established: a decrease in the ability of NG to kill bacteria in the reaction of phagocytosis, a defective respiratory burst, and impaired transmigration activity of NG.

In other words, a significant remodeling of the phenotype of the $CD64^-CD32^+CD16^+CD11b^+NG$ and $CD64^+CD32^+CD16^+CD11b^+$ NG subsets in *de novo* experimental model of viral-bacterial infection *in vitro* was performed. Remodeling of the phenotype of both the major and minor subsets was significantly different from the changes obtained in both the viral model and the bacterial infection model.

Conclusion

Thus, we reproduced models of bacterial infection using fMLP, viral infection using dsRNA and created a new model of viral-bacterial co-infection in a comparative experimental study *in vitro*. It was shown that functionally significant subsets of NGs $CD64^-CD32^+CD16^+CD11b^+$ NG and $CD64^+CD32^+CD16^+CD11b^+NG$ respond differently to the mono- and combined effects of fMLP, dsRNA substances. It was established that each of the models is characterized by its own options for reorienting the phenotype of subsets $CD64^-CD32^+CD16^+CD11b^+NG$ and $CD64^+CD32^+CD16^+CD11b^+NG$. The most significant transforma-

tion of the NG phenotype was obtained in a new model of viral-bacterial co-infection: there was an intense activation of the major subset $CD64^-$ $CD32^+CD16^+CD11b^+$NG and overactivation of the minor subset of $CD64^+CD32^+CD16^+CD11b^+$NG, which may be associated with acquired defects in the functioning of NG.

Created *in vitro* experimental model of viral-bacterial co-infection can be used to evaluate the transformation of other subsets NG with different phenotype, to study the features of the functional activity of NG, to assess changes in their effector, secretory and regulatory functions, and the ability to form NET.

The model of viral-bacterial co-infection that we developed can be used in immunopharmacology to study the characteristics of the effect of immunotropic substances on the NG phenotype. The data obtained are promising for the development of new targeted immunotherapeutic strategies for the correction of the negatively transformed phenotype of defectively functioning subsets of NG in viral-bacterial and other co-infections.

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