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BACTERIOPHAGE–DERIVED DOUBLE-STRANDED RNA (LARIFAN) EXERTS VARIABLE EFFECTS ON HUMAN BLOOD MONOCYTES DEPENDING ON AGE AND SEX OF DONORS

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To date, great attention is paid to sex and age differences in the therapeutic effectiveness of drugs, including those that impact the immune system. Bacteriophage-derived dsRNA is the main component of the medicinal product Larifan, which exhibits interferonogenic activity. This study aimed to estimate the effect of Larifan on the activation status of human peripheral blood monocytes collected from donors of different ages and sex. Blood samples were obtained from the healthy volunteers, divided into 4 groups: young men and young women aged from 20 to 39 years, aged men and aged women from 54 to 69 years old. EDTA-anticoagulated blood samples were exposed to 200 μg/ml Larifan for 30 min, cells were washed and treated to study phagocytic index, ROS generation and expression of phenotypic markers. Only live monocytes selected by flow cytometry were included in the analysis. It was shown that monocytes from young as well as from aged females turned out to be quite inert to the treatment with Larifan. Monocytes from young males after the treatment demonstrated a minor decrease in phagocytic activity and significant down-regulation of ROS generation. Monocytes from aged adults showed clear sex-based differences in the basal cell phenotype. Thus, compared to monocytes from women, the monocytes from men over 50 after the treatment with Larifan showed decreased phagocytic activity and CD86 expression along with increased CD206 expression. Taken together, these results indicate the need for further studies of Larifan focused on developing personalized treatment depending on the age and sex of an individual.

K e y w o r d s: double-stranded RNA, Larifan, monocytes, phagocytosis, reactive oxygen species, sex and age differences.

Monocytes are circulating mononuclear phagocytes and are one of the key effector cells of innate immunity including antiviral innate immune defense. After leaving the bone marrow, they circulate in the peripheral blood for one to three days and then can migrate to various tissues where they differentiate into tissue macrophages [1]. In the steady state (homeostasis), tissues contain a mixed macrophage population: embryonically-derived tissue-resident macrophages (TRMs) and monocyte-derived TRMs at different ratios, depending on the tissue [2]. However, during tissue injury/viral infection, Danger-Associated Molecular Patterns (Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) prompt recruiting of circulating monocytes and induce their differentiation into pro-inflammatory monocyte-derived macrophages [3]. After the recognition of viral PAMPs in the course of respiratory tract infections, TRMs provide one of the main sources of type I interferons (IFN) along with epithelial cells. Type I IFN, in turn, activates the expression of hundreds of interferon-stimulated genes. The latter encode multiple effector molecules with different antiviral mechanisms of action [4].

Still, a lot of viruses, including SARS-CoV-2, can suppress IFN responses at different stages, thus facilitating viral load growth [5]. As a result, numerous PAMPs stimulate phagocytic cells, including recruited monocytes and monocyte-derived TRM, causing hyperinflammatory activation of the im-

mune system known as the cytokine storm. This pathological reaction is frequently observed in patients with COVID-19 and other infectious diseases and may be responsible for dangerous complications such as acute respiratory distress syndrome, multisystem organ failure, and death [6].

Since IFN inhibition plays such an important role in the development of cytokine storm and related complications, drugs with interferonogenic properties may be beneficial for overcoming this inhibition and ensuring proper antiviral response [7], so that the viral overaccumulation leading to cytokine storm may be prevented. One such promising drug is Larifan. This drug consists of dsRNA and is produced from *E. coli* cells infected with f2sus11 bacteriophage. It exhibits interferonogenic activity and has been shown to inhibit the replication of SARS-CoV-2 *in vitro* and *in vivo* in golden Syrian hamsters, as well as to reduce infection-induced pulmonary lesions in those animals [8]. Moreover, the ability of Larifan to reprogram macrophages has been demonstrated on glioma-associated microglia and rat peritoneal macrophages [9,10].

It is known that men and women have different immune reactivity: men demonstrate weaker immune responses, making them more prone to infectious diseases, but less vulnerable to autoimmune diseases than women [11]. This observation is also confirmed by higher COVID-19 mortality rates in men [12]. Such dichotomy is largely mediated by levels of sex hormones. Many immune cells, including monocytes and macrophages, express receptors of sex hormones, and thus can be directly regulated by hormonal influences [13]. Moreover, other factors like genetics and environment play a role in sex differences in immune responses [14]. In addition, to date, great attention has been paid to sex differences in the therapeutic effectiveness, amenability and adverse effects of chemically synthesized and biological drugs, including those that impact the immune system cells [15].

Another important factor influencing infectious disease mortality is aging, which causes a progressive decline of all biological systems, including immunity [16]. However, the data on the sex differences in immune reactivity and drug response of old individuals are scarce.

Considering all the above, this study aimed to investigate the effect of Larifan on the activation status of human peripheral blood monocytes collected from donors of different ages and sexes.

Materials and Methods

Study participants. Peripheral blood samples were obtained from 12 healthy volunteers, which were divided into 4 groups: young men (n = 3) and young women (n = 3) – aged from 20 to 39 years; aged men (n = 3) and aged women (n = 3) – from 54 to 69 years.

Subjects with a history of somatic diseases were excluded from the study. The research received approval from the local ethics committee, and all participants provided informed consent before its initiation.

Study design. EDTA-anticoagulated whole blood samples were exposed to 200 μg/ml Larifan at 37°C for 30 min. After this, cells were washed and treated according to protocols described below to study phagocytosis, ROS production, expression of phenotypic markers, and examined using flow cytometry. CD86 and CD206 were used as conventional phenotypic markers of pro- and anti-inflammatory polarization of phagocytes, respectively [17].

Phagocytosis assay. Blood samples were incubated at 37°C for three hours with carboxylate-modified polystyrene latex beads conjugated to fluorescent red (d = 2 μm; Sigma-Aldrich, USA) at a ratio of 50 beads per cell in RPMI-1640 medium (Sigma-Aldrich, USA) [18, 19]. Following the incubation, erythrocytes were lysed using a lysis buffer. The cells were then washed with cold PBS, fixed in 0.04% paraformaldehyde, and examined using flow cytometry. Fluorescence was measured in the ECD-A channel. The phagocytosis index was calculated using the formula: $[Gmean_{pos}/P_{pos}] – [Gmean_{neg}/P_{neg}]$, where P_{pos} – the percentage of positive cells, $Gmean_{pos}$ – the mean channel fluorescence intensity, P_{neg} – the percentage of positive cells in the negative control, and $Gmean_{neg}$ – the mean channel fluorescence intensity of the negative control.

Only live cells, selected based on scatter parameters, were included in the analysis, and monocytes were identified based on their forward and side scatter characteristics.

Intracellular ROS assay. Reactive oxygen species (ROS) generation was quantified using 2'7'-dichlorodihydro-fluorescein diacetate (carboxy-H2DCFDA, Invitrogen). Once inside cells, this compound is transformed by intracellular esterases into a non-fluorescent form (carboxy-H2DCF), which is subsequently oxidized by intracellular ROS to a fluorescent derivative, carboxy-DCF [20]. To perform

the measurement, 200 µl of whole blood treated with EDTA was mixed with 4.3 µl of PBS containing 10 µM carboxy-H2DCFDA and incubated for 30 minutes at 37°C. This allowed the esterases time to hydrolyze the ester or acetate groups, making the dye sensitive to oxidation. Red blood cells were then lysed using a lysis buffer. The cells were then washed with cold PBS, fixed in 0.04% paraformaldehyde, and examined using flow cytometry, with excitation at 488 nm and emission at 525 nm. Only live cells, selected based on scatter parameters, were included in the analysis, and monocytes were gated based on their forward and side scatter characteristics.

Staining of cell surface markers. Erythrocytes in samples were lysed using a lysis buffer. Cells were washed with PBS. 5 µl of antibodies against CD86 and CD206 (Beckman Coulter, France) were added to the cell suspension in a volume of 50 µl. The samples were incubated for 25 min in the dark at room temperature, washed twice by centrifugation in 2 ml of cold PBS supplemented with 0.02% EDTA, fixed in 0.04% paraformaldehyde, and examined using flow cytometry. Only live cells, selected based on scatter parameters, were included in the analysis, and monocytes were identified based on their forward and side scatter characteristics.

Statistical analysis. The Kruskal-Wallis nonparametric ANOVA with the post-hoc Dunn test was used for comparisons between groups. Significance was considered at P-value < 0.05.

Results and Discussion

As mentioned above, blood monocytes play an important role in host defense against infections of various localization. These cells are central for the activation of adaptive immunity since they can differentiate into dendritic cells – one of the main types of antigen-presenting cells [21]. Furthermore, monocytes themselves can present antigens to T helper cells [22]. In the context of the monocyte-macrophage axis, blood monocytes migrate into tissues upon infection or other pathologies and differentiate mainly into proinflammatory (classically activated, or M1) macrophages, while tissue-resident macrophages are naturally more anti-inflammatory (alternatively activated, or M2) – they specialize on maintaining tissue homeostasis and various tissue-specific functions [23]. Thus, overactive monocyte-derived macrophages are very likely to be the key players that promote cytokine storm. Moreover, there is a report about monocytes contributing substantially to atypical cytokine storm in severe COVID-19 cases [24].

In our experiments, there were no statistically significant effects of Larifan on phagocytosis percentage and phagocytic index of blood monocytes of healthy male and female volunteers under 50 years. Only a tendency to the lowering monocyte phagocytic activity was observed after exposure to the preparation in both male and female young persons (Fig. 1, *A* and *B*). In contrast, in aged healthy persons, changes of monocyte phagocytic function after the treatment with Larifan *in vitro* differed depending on the sex. In aged females, the percentage of phagocytizing monocytes tended to rise (Fig. 1, *C*), whereas phagocytosis intensity was 2.5 times lower in treated cells as compared to untreated (Fig. 1, *D*). The opposite is true for aged men: treatment with Larifan was associated with a decrease of median values of monocyte phagocytosis percentage by 1.6 times, and with a moderate increase of phagocytic index values (Fig. 1, *C* and *D*).

Studies on humans and mice have shown that the phagocytic activity of neutrophils and macrophages is usually higher in females compared to males, thus corroborating our results concerning monocytes from aged adults [25-27]. Monocytes in humans present a heterogeneous population and consist of three subsets: CD14++CD16– classical monocytes, CD14++CD16+ intermediate monocytes, and CD14+CD16++ non-classical monocytes. Classical monocytes are the most prevalent subset (about 85-90% of the total monocyte population), which is recruited in inflamed tissue areas. Proinflammatory activation of classical monocytes is associated with an increase in their phagocytic activity, whereas anti-inflammatory metabolic shift (also known as 'tolerated' phenotype) is accompanied by a decrease in phagocytic capacity [28, 29]. Overall, Larifan had a multidirectional effect on monocyte phagocytic activity depending on age and sex. Considering the limited number of study participants, this issue warrants additional experiments.

Increased ROS generation is considered as marker of monocyte pro-inflammatory metabolic profile, and their involvement in local and systemic inflammatory processes [30,31]. In our experiments, basal values of the percentage of ROS-producing monocytes, as well as the level of ROS generation, were significantly higher in men under the age of 50 years as compared to women from the same age group (Fig. 2, *A*, *B*). Treatment with Larifan sig-

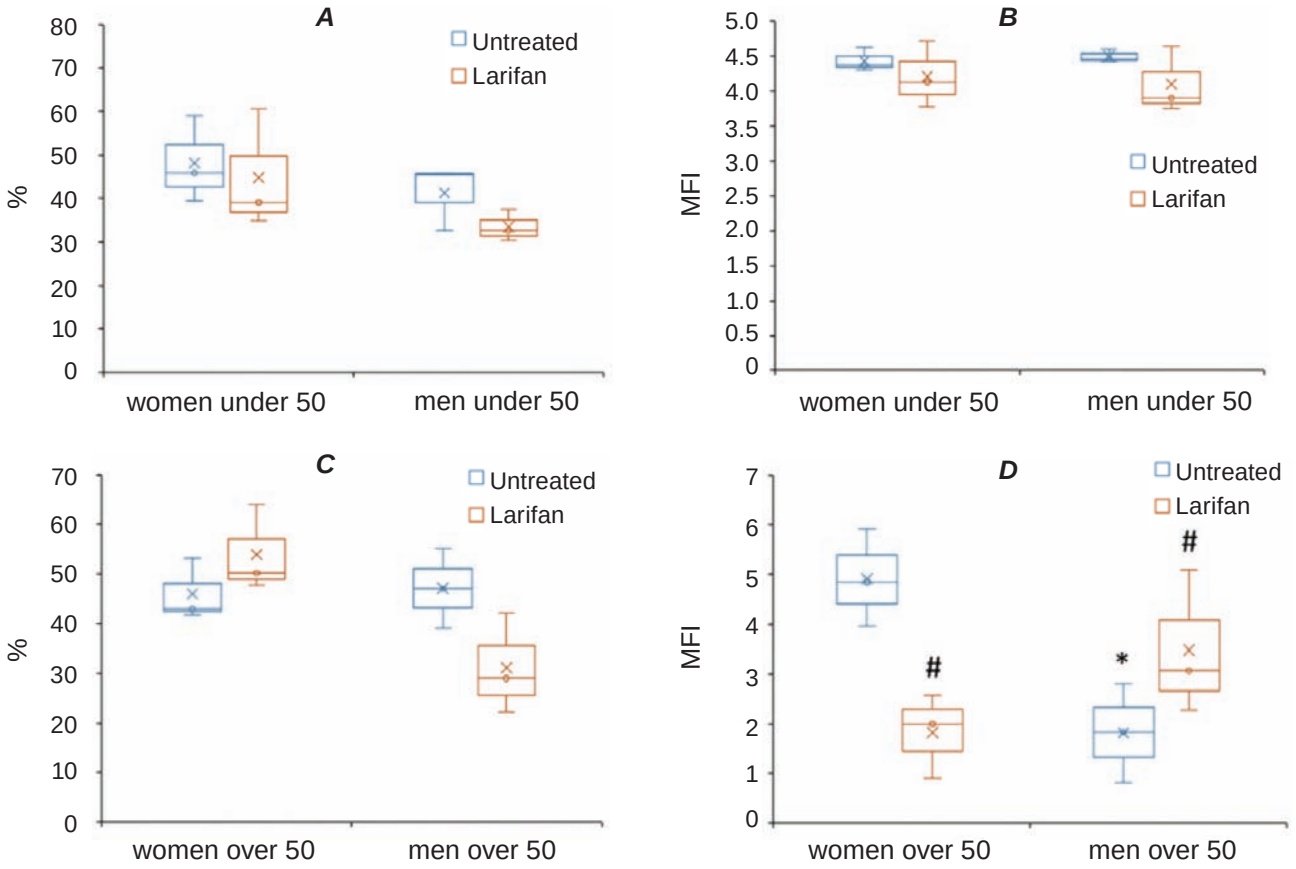

*Fig. 1. The effect of Larifan on the phagocytosis percentage (**A**, **C**) and phagocytic index (**B**, **D**) of human blood monocytes obtained from donors of different ages and sexes. MFI – mean fluorescence intensity. *P < 0.05 as compared to basal (untreated) age-matched female samples; #P < 0.05 as compared to corresponding untreated samples*

nificantly decreased both indices in males and had no meaningful effect on both parameters in age-matched females. In the group of aged persons, basal values of the aforementioned indices did not differ significantly in men and women. Treatment with Larifan did not affect the percentage of ROS-generating monocytes in both males and females (Fig. 2, *C*). Levels of ROS production markedly (2,7 times) increased in female cells upon treatment with Larifan, and there was a strong but insignificant trend towards such an increase in Larifan-treated male phagocytes (Fig. 2, *D*).

The sex-dependent difference in ROS generation by blood monocytes taken from young donors is confirmed by multiple studies demonstrating higher ROS production and lower activity of antioxidant defense mechanisms in human, as well as rodent males [32-34]. It is speculated that those differences may account for longer lifespans in females as opposed to males [35]. Moreover, it was shown that ROS are involved in the pathogenesis of COVID-19 [36], which may also be responsible for higher COVID-19 mortality in males to some extent. The inhibitory effect of Larifan on ROS production by monocytes of young men is in full concordance with our previous study on young men [37], and our experiments with Larifan on male Wistar rats [9,10]. Thus, treatment with Larifan may help fight excessive oxidative stress, which is beneficial for general health, as well as in the COVID-19 context. Importantly, Larifan did not suppress ROS generation in young women, so it can be assumed that this drug should not disturb the protective antimicrobial function of ROS in the presence of powerful antioxidant systems.

There are reports that both phagocytosis and respiratory bursts as the mechanisms of eliminating of ingested microbes decrease during aging [38, 39]. However, in addition to phagolysosomes, ROS are produced in other cellular locations and involved in diverse biological processes [40], which may explain

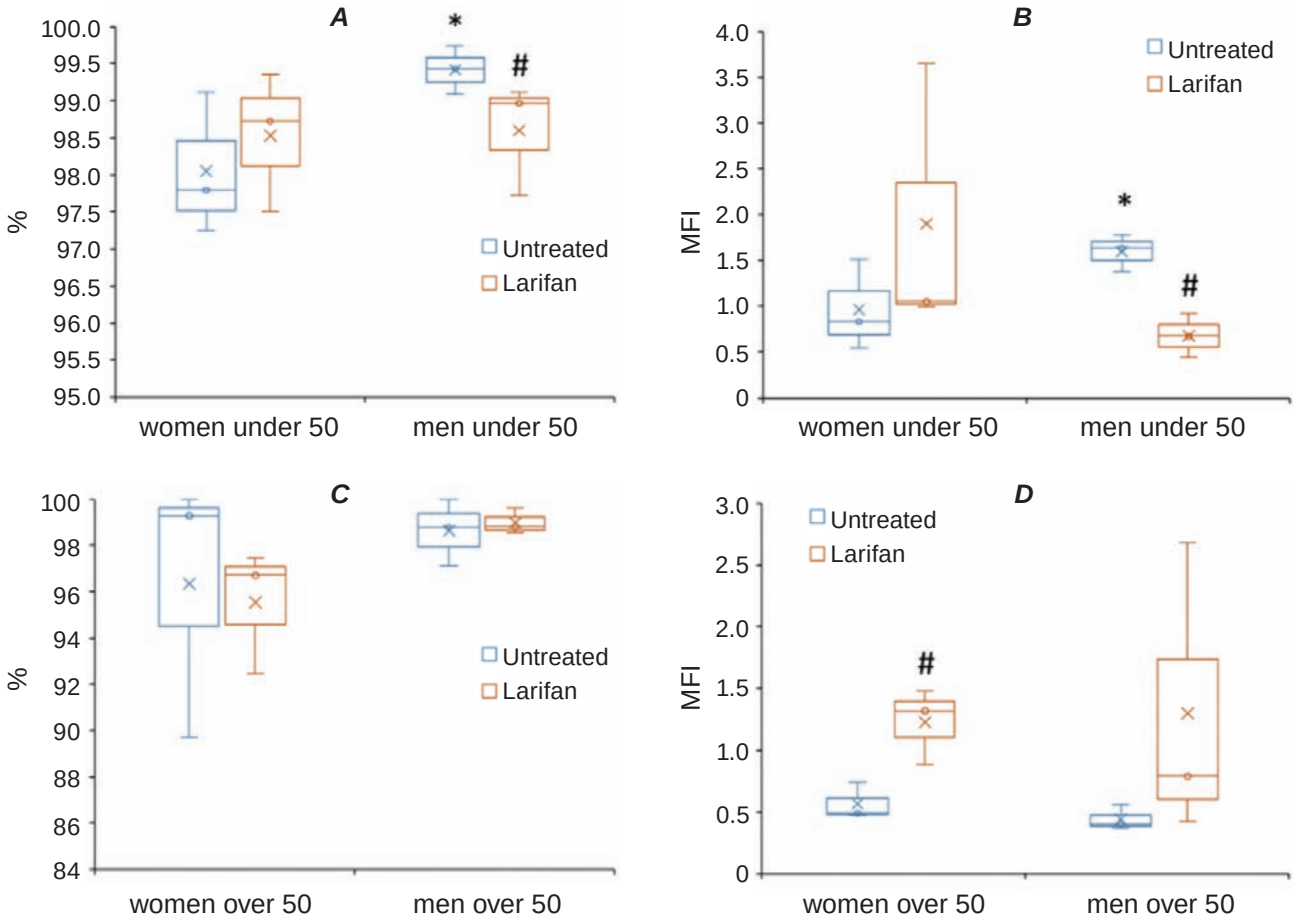

*Fig. 2. The effect of Larifan on the ROS percentage (**A**, **C**) and level of ROS production (**B**, **D**) of human blood monocytes obtained from donors of different ages and sexes. MFI – mean fluorescence intensity. *P < 0.05 as compared to basal (untreated) age-matched female samples; #P < 0.05 as compared to corresponding untreated samples*

evidence of oxidative damage in the elderly despite suppression of respiratory burst [41]. Studies show that there is a significant increase in oxidative stress in women after menopause compared to young women, leading to less pronounced sex differences in oxidative stress markers in elderly populations [42]. This explains the absence of a significant difference in ROS production between men and women over 50 in our study. It is difficult to interpret unequivocally the stimulatory effect of Larifan on ROS production by cells from aged donors of both sexes. Oxidative stress is usually already present in aged adults, and it can play an adverse role in the course of COVID-19 [36], so ROS overproduction may be harmful. Contrary to that, ROS generation is an important defensive mechanism against infections of different etiology, including viral diseases, and the cellular source of ROS causing oxidative stress in old adults should also be considered [43]. More studies are needed to address this question.

There were no sex-dependent differences in the basal percentage of CD86-expressing cells, as well as in the level of expression of this marker by monocytes in study participants under the age of 50 years (Fig. 3, *A* and *B*). Treatment with Larifan did not influence those indices in cells collected from both males and females of the younger group. By contrast, in the aged group, modest sex differences were observed in CD86 expression by monocytes. The percentage of CD86-expressing monocytes was significantly lower in untreated samples collected from males over 50 compared to females of this age group (Fig. 3, *C*). Treatment with Larifan did not affect CD86+ cell percentage. At the same time, the level of CD86 expression by monocytes was significantly higher in untreated cells taken from males

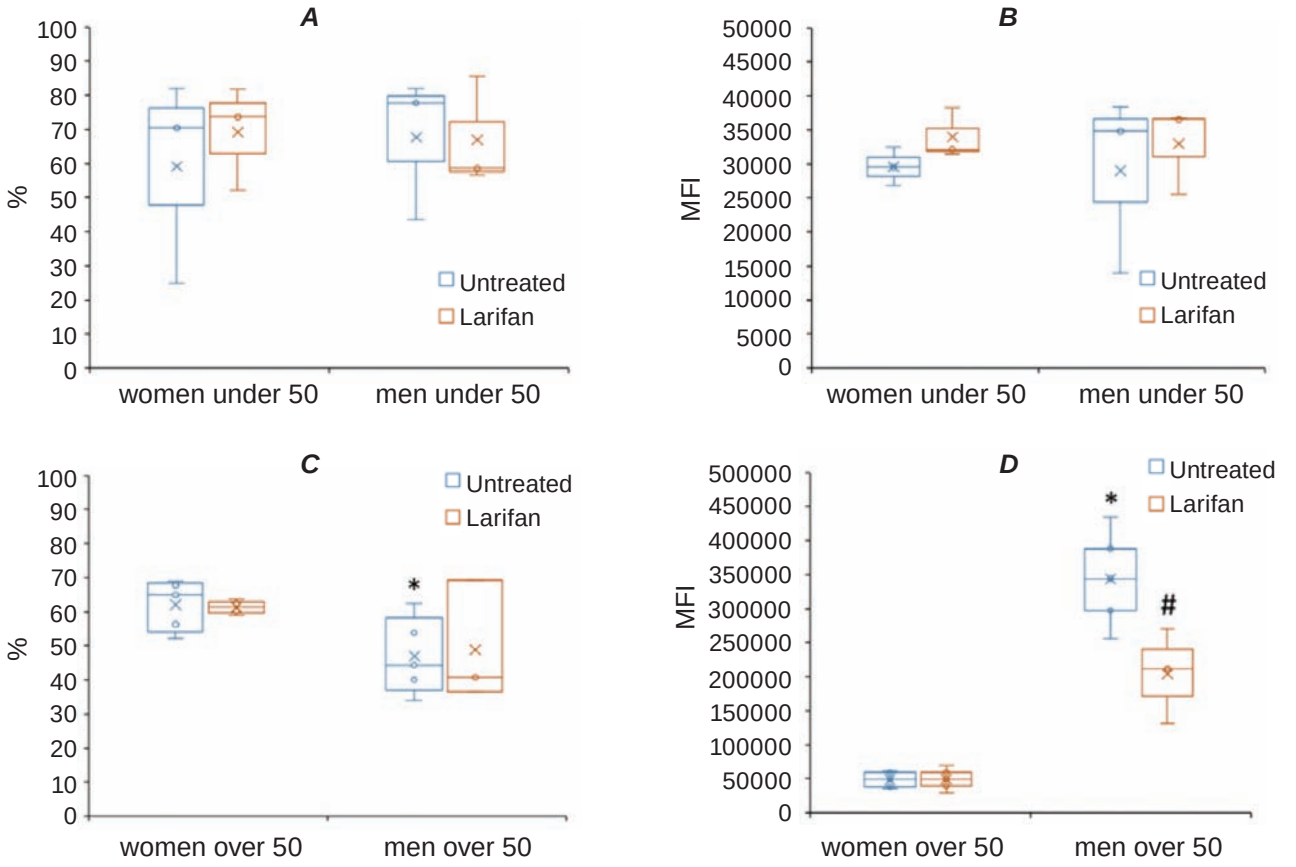

*Fig. 3. The effect of Larifan on the percentage of CD86-expressing phagocytes (**A**, **C**) and CD86 expression level (**B**, **D**) of human blood monocytes obtained from donors of different ages and sexes. MFI – mean fluorescence intensity. *P < 0.05 as compared to basal (untreated) age-matched female samples; #P < 0.05 as compared to corresponding untreated samples*

over 50 compared to age-matched females (Fig. 3, *D*). Larifan down-regulated CD86 expression level in monocytes collected from males over the age of 50 years, while not affecting CD86 expression by cells of age-matched females.

The meaning and significance of CD86 for assessing and characterizing monocyte polarized activation have not yet been fully explored. Literature data concerning this issue are quite controversial. Increasing the expression of the CD86 is most often mentioned in connection with monocyte differentiating into macrophages, which is most characteristic for dominate classical subset [44]. Moreover, Borst et al., 2018 revealed the protective and immunomodulating role of stimulating type I IFN signaling in peripheral blood monocytes. They have shown that IFN-I receptor (IFNAR)-triggering in monocytes by viral nucleic acids delays the development of monocyte-derived tissue-resident proinflammatory macrophages and promotes their M2-polarization, in such a way preventing hyperinflammation [45]. Therefore, we tend to assume that a decrease in CD86 expression in monocytes from aged male participants after the treatment with Larifan can be a sign of acquiring immunomodulatory anti-inflammatory profile.

There were no sex differences in the percentage of CD206-expressing blood monocytes in participants of both age groups (Fig. 4, *A* and *C*). Treatment with Larifan did not affect significantly the numbers of CD206-positive cells in all participants. Basal values of CD206 expression level were slightly lower in monocytes from young men as compared to those in young women (Fig. 4, *B*). Treatment with Larifan did not cause any significant changes in the expression level of this molecule in monocytes from young men, and tended to upregulate this marker in monocytes from women under the age of 50 years. In aged women, CD206 expression levels in monocytes were substantially lower than that in aged men

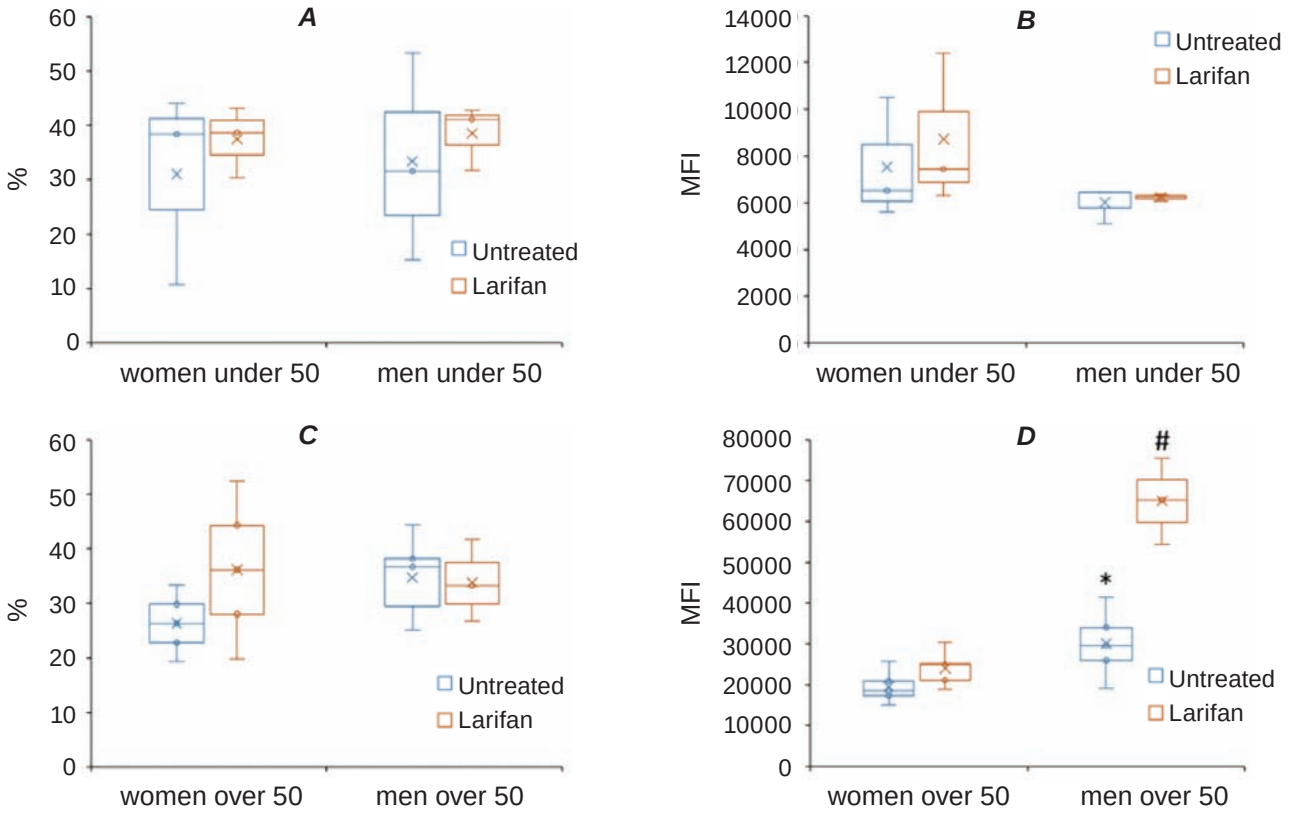

*Fig. 4. The ffect of Larifan on the percentage of CD206-expressing phagocytes (**A**, **C**) and CD206 expression level (**B**, **D**) of human blood monocytes obtained from donors of different ages and sexes. MFI – mean fluorescence intensity. *P < 0.05 as compared to basal (untreated) age-matched female samples; #P<0.05 as compared to corresponding untreated samples*

(Fig. 4, *D*). Treatment with Larifan did not affect CD206 expression level in aged female participants. By contrast, in aged male persons, we observed an increase of CD206 expression level in circulating monocytes after the treatment with Larifan *in vitro.*

An increase in CD206 expression is characteristic for anti-inflammatory monocyte shift [46, 47]. The most well-known CD206 functions include binding and endocytosis of carbohydrate moieties and the silent clearance of inflammatory molecules. This activates an anti-inflammatory and tolerogenic program in dendritic cells and macrophages, emphasizing the role of CD206 in maintaining homeostasis [48, 49].

Thus, resting peripheral blood monocytes from young healthy females turned out to be most inert to the treatment with Larifan *in vitro* and exhibited no significant changes in studied characteristics. Only a tendency to the mixed metabolic shift (slight lowering phagocytic activity, insignificant increase of oxidative metabolism and CD86 expression level) was observed. Circulating resting monocytes from young healthy males after the treatment with Larifan *in vitro* demonstrated signs of anti-inflammatory shift with minor decrease phagocytic activity and significant down-regulating ROS generation. However, M1/M2 monocyte/macrophage classification is currently considered an oversimplified, and activation states of phagocytes are viewed more as a spectrum nowadays [50, 51]. Therefore, modulating effects of Larifan on monocytes from young healthy women may lie outside the scope of this simplified M1/M2 classification scheme. As opposed to younger individuals, blood monocytes from aged adults are characterized by clear sex-based differences in the basal cell phenotype. Resting monocytes from men over 50 were characterized by lowered phagocytic activity along with upregulated levels of both CD86 and CD206 compared to women from the same age group. Larifan caused an anti-inflammatory shift in the phenotype of cells taken from aged males, manifested by a decrease in the phagocytic

activity and expression of CD86 along with an increase in the expression of CD206. Such effect may be beneficial for attenuation of the negative impact of inflammaging – the state of chronic inflammation that is sustained mainly by innate immune cells in aged male adults [39]. Resting monocytes from aged healthy females were quite inert to the treatment with Larifan *in vitro* similarly to cells from young females, and demonstrated mixed phenotype, in which decreased phagocytic activity was coupled with increased ROS generation.

In conclusion, Larifan exerts a multidirectional effect on resting human peripheral blood monocytes depending on age and sex. Modulatory effects of the preparation are more pronounced in resting monocytes from aged healthy individuals. Larifan exhibited an anti-inflammatory effect on resting monocytes taken from men of both age groups, while not affecting the cells of young women and exerting a slight proinflammatory effect on the monocytes of aged female donors. Taken together, these results warrant further studies of Larifan focused on developing personalized treatment plans depending on the age and sex of an individual.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ДВОЛАНЦЮГОВА РНК ФАГОВОГО ПОХОДЖЕННЯ (ЛАРИФАН) ПО РІЗНОМУ ВПЛИВАЄ НА МОНОЦИТИ КРОВІ ЛЮДИНИ ЗАЛЕЖНО ВІД ВІКУ ТА СТАТІ ДОНОРІВ

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На сьогоднішній день значна увага приділяється статевим і віковим відмінностям у терапевтичній ефективності лікарських засобів, включаючи ті, що впливають на імунну систему. Дволанцюгова РНК, отримана з бактеріофагів, є основним компонентом лікарського препарату Ларифан, який має інтерфероногенну активність. Метою даної роботи була оцінка впливу Ларифану на активаційний стан моноцитів периферичної крові людини, отриманих у донорів різного віку та статі. Зразки крові здорових добровольців були розподілені на 4 групи: молоді чоловіки та жінки віком від 20 до 39 років, літні чоловіки та жінки віком від 54 до 69 років. Зразки крові з антикоагулянтом ЕДТА обробляли Ларифаном (200 мкг/мл) протягом 30 хв, клітини відмивали та вимірювали фагоцитарний індекс, продукцію реактивних форм кисню та експресію фенотипових маркерів. Аналізували лише живі моноцити, ідентифіковані за допомогою проточної цитометрії. Моноцити як молодих, так і літніх жінок слабо реагували на обробку Ларифаном. Моноцити молодих чоловіків після обробки продемонстрували незначне зниження фагоцитарної активності та значне зменшення продукції реактивних форм кисню. У моноцитів літніх людей виявлялися чіткі статеві відмінності фенотипу нестимульованих клітин. Порівняно з моноцитами жінок, у моноцитів чоловіків старше 50 років після обробки Ларифаном спостерігалося зниження фагоцитарної активності та експресії CD86, а також підвищення експресії CD206. Ці результати вказують на необхідність подальших досліджень Ларифану, спрямованих на розробку персоналізованого лікування в залежності від віку та статі пацієнта.

Ключові слова: дволанцюгова РНК, Ларифан, моноцити, фагоцитоз, реактивні форми кисню, статеві та вікові відмінності.

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