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COMPARATIVE ASSESSMENT OF THE IMPACT OF INTERFERONOGENIC PREPARATION LARIFAN ON MONOCYTES FROM AGED C57BL/6 AND BALB/C MICE *IN VITRO*

Background. ***Blood monocytes play a crucial role in immunity as effector cells of innate immunity. However, they can also promote hyperinflammation, as was described in COVID-19. Many viral infections trigger hyperinflammation by inhibiting type I interferon synthesis, necessitating search of interferon-based or interferonogenic treatments like Larifan – bacteriophage-derived dsRNA with interferonogenic and immunomodulatory properties. Global statistics indicate that viral infections, including SARS-CoV-2, as well as hyperinflammation occur more frequently in males, especially in the older age group, and significantly depends on genetically determined profile of immune reactivity. The aim of this study was a comparative assessment of the impact of Larifan on the metabolic profile of peripheral blood monocytes from aged male C57BL/6 and BALB/c mice in vitro.***

Methods. ***Male aged C57BL/6 and BALB/c mice were used in this study. Blood samples were collected from facial vein and treated with Larifan in vitro. Phagocytic activity, ROS production, and expression of phenotypic markers were assessed by flow cytometry. Only live monocytes were gated and included in the analysis. Data are presented as median and interquartile range (IQR). Statistical differences were calculated using Kruskal–Wallis test, with significance set at $p < 0.05$.***

Results. ***BALB/c mice showed a lower baseline phagocytic index than C57Bl/6, but phagocytosis percentages were comparable. Treatment with Larifan reduced the phagocytosis percentage in both strains, yet the phagocytic index rose in BALB/c mice after dsRNA exposure. ROS production was higher in C57Bl/6 mice, with Larifan reducing ROS levels significantly in both strains. CD80 baseline expression levels were higher in BALB/c, and dsRNA increased CD80-positive cells as well as decreased expression level of CD80 in BALB/c mice only. CD206 expression was lower in BALB/c but unaffected by Larifan, while dsRNA reduced both number of CD206-positive cells and CD206 levels in C57Bl/6 mice.***

Conclusions. ***The metabolic profile of monocytes differs between Th1-dominant C57Bl/6 and Th2-biased BALB/c mice, with higher baseline indicators in C57Bl/6 mice. Larifan treatment exerts anti-inflammatory effects by reducing ROS synthesis in both strains, with BALB/c mice also displaying increased phagocytosis and reduced antigen-presenting capability.***

Keywords: ***bacteriophage-derived dsRNA, blood monocytes, phagocytic activity, reactive oxygen species, CD80, CD206.***

Background

Blood monocytes are one of the key populations of circulating leukocytes that are recruited to the site of infectious inflammation, including that caused by viral infections. They can function as antigen-presenting cells, thus stimulating T-cell-mediated immunity, and differentiate into various immune cell types, including macrophages and dendritic cells (Austermann, Roth, & Barczyk-Kahlert, 2022). In addition, monocytes play an important role in the development of hyperinflammation in the lungs under conditions of uncontrolled viral load, as was recently demonstrated in the case of hyperinflammation accompanied by a cytokine storm associated with acute respiratory distress syndrome in patients with SARS-CoV-2 infection (Vanderbeke et al., 2021). Hyperinflammation is characteristic of many viral infections due to the ability of viruses to inhibit the synthesis of type I interferons or evade their biological effects (García-Sastre, 2017). Therefore, restoring the synthesis of type I interferons using interferonogenic drugs is considered one of the promising pathogenetic therapeutic and preventive approaches for controlling viral infections (Mesic et al., 2022). One such drug is Larifan, an immunomodulatory agent with interferonogenic activity based on bacteriophage double-stranded RNA (Vaivode et al., 2022; Hurmach et al., 2018; Pjanova et al., 2021). However, its effect on peripheral blood monocytes – important effector cells of inflammation – has not been extensively studied yet.

The incidence of viral infections and the course of the inflammatory process, including hyperinflammation, depend on genetically determined features of immune reactivity, age, and gender (Cisneros et al., 2022; Dunn, Perry, & Klein, 2024). There is a lot of reports about the possible role of different hormonal and immune factors in sex-based differences of immune response (Martínez de Toda et al., 2023). In particular, men have higher levels of testosterone, which is capable of inducing mitochondrial reactive oxygen species (ROS) generation, leading to higher oxidative stress in males. Furthermore, ROS are proinflammatory molecules that indirectly activate the NLRP3 inflammasome – important component of innate

immunity responsible for proinflammatory cytokine production (Alves et al., 2020; Ma et al., 2020). In contrast, female sex hormones, namely estrogen and progesterone, prevent NLRP3 inflammasome overactivation (Zhang, Tang, & Tao, 2021). Also, female leukocytes express higher levels of some innate immune receptors, particularly TLR4 and TLR7 (Martínez-García et al., 2020; Souyris et al., 2018). Additionally, female plasmacytoid dendritic cells produce higher quantity of IFNα in response to TLR7 ligands compared to males, leading to enhanced activation of CD8+ T cells (Meier et al., 2009). As a result, men have higher level of basal inflammation, while women produce stronger immune response to the stimulus, making the latter more resistant to infections (Martínez de Toda et al., 2023).

Another important factor determining immune response to viral infections is age. In general, aging is characterized by a decline of multiple immune parameters and weakened immunity, on the one hand, and development of chronic basal inflammation called inflammaging, on the other (Fulop et al., 2018). According to global statistics, the incidence of viral infections, including SARS-CoV-2, is higher in men, particularly those of older age (Jacobsen, & Klein, 2021). Therefore, the aim of this study was a comparative assessment of the impact of Larifan on the metabolic profile of peripheral blood monocytes from aged male C57BL/6 and BALB/c mice *in vitro*.

Methods

Study design. In this study, blood samples were taken from 5 C57BL/6 and 5 BALB/c male mice, aged 18–22 weeks. Prior to experimentation, the animals were housed under standard conditions in the vivarium of the Educational and Scientific Center "Institute of Biology and Medicine" at Taras Shevchenko National University of Kyiv, with controlled temperatures of 20 ± 2 °C, a 12-hour light/dark cycle, and ad libitum access to food and water. The University's Bioethics Committee approved the animal protocol following the Animal Protection Act (protocol No 4, 10.10.2022). All procedures with animals adhered to Ukraine's Law No. 3447-IV "On the Protection of Animals from Cruelty," the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1997), and the ethical principles from the First National Congress on Bioethics of Ukraine (September 2001), as well as other relevant national and international regulations.

Mice were manually restrained, and facial vein was pierced approximately 3 mm behind and 1 mm above the lateral tactile hair on a cheek, using a scarifier. Then, blood was immediately collected into heparin-coated Eppendorf 1.5 ml Safe-Lock tube.

Larifan, a preparation consisting of a heterogeneous mix of double-stranded RNAs (dsRNAs), was sourced from Larifan Ltd. This dsRNA mixture is derived from *E. coli* cells infected with the f2sus11 amber mutant bacteriophage and subsequently lyophilized. Isolated blood samples were pre-treated with Larifan at a concentration of 200 µg/mL for 30 minutes *in vitro*. After that, cells underwent testing to assess their phagocytic activity, oxidative metabolism, and expression of phenotypic markers.

Flow cytometry. To study the phagocytic activity of monocytes, red fluorescent carboxylate-modified polystyrene latex beads (2 µm diameter, Sigma-Aldrich, USA) were utilized as endocytic targets. Whole blood samples were incubated with the latex beads at a 1 : 50 ratio for 3 h at 37 °C and 5 % CO_2. After incubation, red blood cells were lysed using concentrated ammonium chloride-based lysis buffer for 10 min in the dark. Cells were rinsed with PBS, fixed in 0.04 % paraformaldehyde, and examined on a DxFlex flow cytometer (Beckman Coulter, Inc., USA) (Daigneault et al., 2010). Phagocytic activity was represented by the percentage of fluorescently labeled cells and the phagocytosis index (PI), calculated as the mean fluorescence intensity. For ROS assessment, cells (1×10^5) were exposed to 2′7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) for 30 min at 37 °C and 5 % CO_2, erythrocytes were lysed, sample were then washed with PBS, fixed in 0.04 % paraformaldehyde, and analyzed by DxFlex flow cytometry (Pjanova et al., 2021). Monocyte phenotypes were identified with AlexaFl488-labeled anti-CD80 (BioLegend, USA) and AlexaFL647-labeled anti-CD206 antibodies (Becton Dickinson, Pharmingen, USA). The analysis included only live cells, identified by scatter parameters, with monocytes specifically gated according to their forward and side scatter properties. Data were gathered on a DxFlex flow cytometer and analyzed with Kaluza C Analysis Software (Beckman Coulter, Inc., USA).

Statistical analysis. The Statistica 12.0 software package was used to perform statistical analysis. Given the small sample sizes, the Shapiro-Wilk test was applied to assess if the data followed a normal distribution (Mishra et al., 2019). Results are reported as median values along with the interquartile range (IQR). Statistical comparisons were made using the Kruskal-Wallis test (Chan, & Walmsley, 1997), with significance defined at $p < 0.05$.

Results

Phagocytic index was significantly lower in BALB/c mice compared to C57Bl/6 (Fig. 1B), while phagocytosis percentage did not differ significantly between strains (Fig. 1A). Phagocytosis percentage tended to decrease in C57Bl/6 mice, and was significantly lower in BALB/c mice after the treatment with Larifan (Fig. 1A). In contrast, phagocytic index significantly increased in BALB/c mice, and tended to rise in C57Bl/6 mice after the exposure to dsRNA (Fig. 1B).

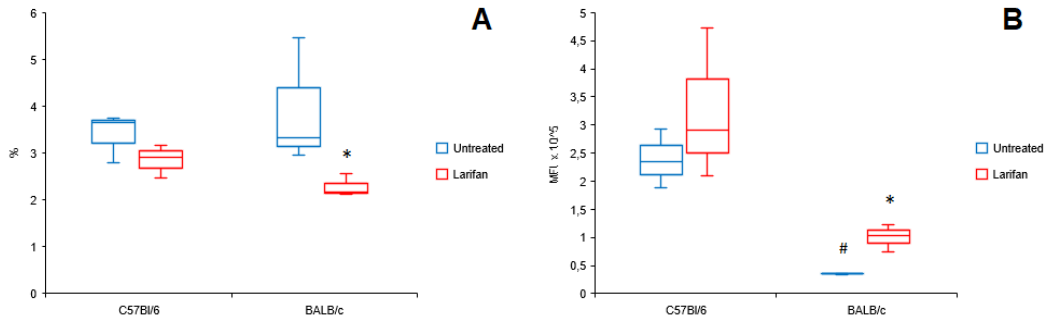

Fig. 1. Effect of Larifan on phagocytosis percentage (A) and phagocytic index (B) of murine blood monocytes obtained from animals of different strains. MFI – mean fluorescence intensity

(*$p < 0.05$ compared to corresponding untreated samples; #$p < 0.05$ compared to basal (untreated) samples from C57BL/6 mice)

ROS production was markedly lower in BALB/c mice compared to C57Bl/6. Treatment with Larifan led to statistically significant decrease of ROS generation in both studied strains of animals (Fig. 2).

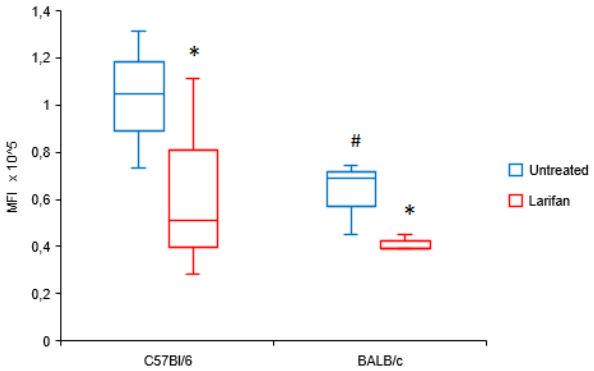

Fig. 2. Effect of Larifan on ROS production by murine blood monocytes obtained from animals of different strains (MFI – mean fluorescence intensity)
(*p < 0.05 compared to corresponding untreated samples; #p < 0.05 compared to basal (untreated) samples from C57BL/6 mice)

The percentage of CD80-expressing monocytes was significantly lower in BALB/c mice compared to C57Bl/6. dsRNA exposure markedly increased the number of CD80-positive phagocytes in BALB/c mice, and had no effect on C57Bl/6 mice (Fig. 3A). At the same time, basal expression level of this marker was considerably higher in BALB/c mice than in C57Bl/6. After the treatment with Larifan, CD80 expression level did not change in C57Bl/6 mice, and decreased significantly in BALB/c mice (Fig. 3B).

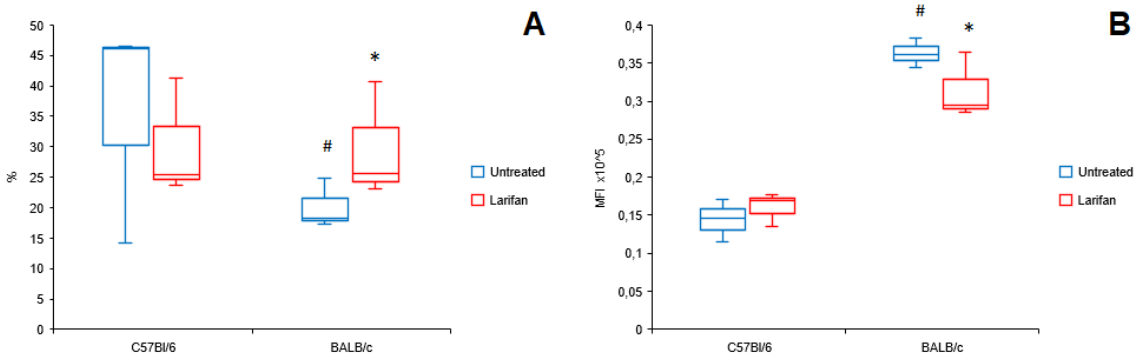

Fig. 3. Effect of Larifan on the percentage of CD80-expressing phagocytes (A) and CD80 expression level (B) of murine blood monocytes obtained from animals of different strains (MFI – mean fluorescence intensity)
(*p < 0.05 compared to corresponding untreated samples; #p < 0.05 compared to basal (untreated) samples from C57BL/6 mice)

The percentage of CD206-expressing monocytes did not differ significantly between strains (Fig. 4A), although expression level of this marker was considerably lower in BALB/c mice compared to C57Bl/6 (Fig. 4B). After the treatment with dsRNA, both the number of CD206 positive phagocytes and CD206 expression level markedly decreased in C57Bl/6 mice. Contrary to this, Larifan did not alter both parameters in BALB/c mice (Fig. 4, A, B).

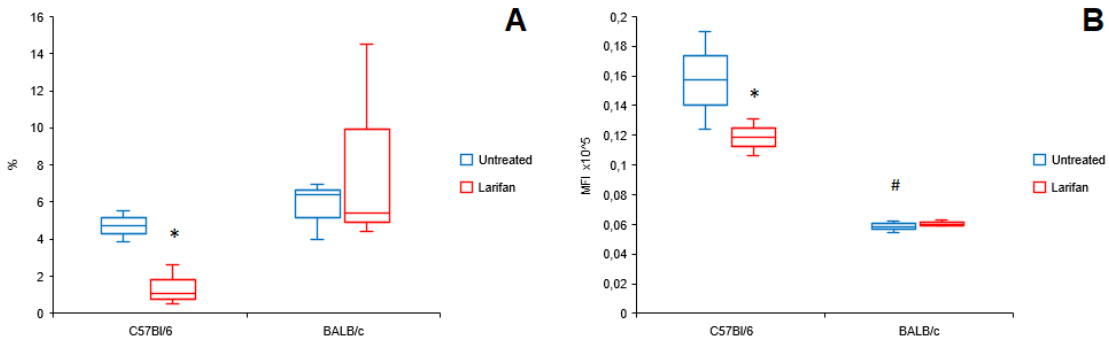

Fig. 4. Effect of Larifan on the percentage of CD206-expressing phagocytes (A) and CD206 expression level (B) of murine blood monocytes obtained from animals of different strains (MFI – mean fluorescence intensity)
(*p < 0.05 compared to corresponding untreated samples; #p < 0.05 compared to basal (untreated) samples from C57Bl/6 mice)

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Discussion and conclusions

Genetic factors play important role in shaping antiviral immunity. In order to account for genetic variability of the human population which is not present in inbred animals, we used mice of two different strains: C57BL/6 and BALB/c. C57BL/6 and BALB/c mice develop Th1- and Th2-dominant immune responses, tailored for fighting the intracellular and extracellular pathogens, respectively (Bleul et al., 2021). Therefore, C57BL/6 mice exhibit a more robust cellular immune response, while BALB/c – humoral response. Also, these strains have different gene sequences at the H2 site of the major histocompatibility class I gene locus (Resende et al., 2008). Moreover, C57BL/6 mice exhibit stronger macrophage polarization, higher resistance to intracellular parasites, and antigen processing deficiency compared to BALB/c (Breda et al., 2022; Hume, 2015).

Human and murine blood monocytes are classified in three main subpopulations: classical, intermediate and non-classical. Classical monocytes are characterized by strong phagocytic and bactericidal activity, production of ROS, nitric oxide, myeloperoxidase, lysozyme, and the chemokines IL-8, CCL2, and CCL3. They also express the CCR2 receptor, which is critical for monocyte emigration from the bone marrow (Stansfield, & Ingram, 2015). These cells possess essential markers for migration to inflammation sites, where they carry out phagocytic and cytolytic activities, and are thus considered precursors of pro-inflammatory (M1) macrophages. Increased levels of non-classical monocytes are observed in pathological conditions such as sepsis, acute and chronic viral and bacterial diseases. They are considered precursors of anti-inflammatory (M2) macrophages. These cells express high levels of major histocompatibility complex class II molecules and costimulatory molecules, and show relatively weak phagocytic activity but effectively present antigens to T-lymphocytes and secrete IFN-alpha (Tak et al., 2017). Nevertheless, non-classical monocytes are also capable to produce pro-inflammatory cytokines, such as TNF-alpha. Their numbers increase in various inflammatory diseases, including atherosclerosis (Schlitt et al., 2004). Intermediate monocytes remain insufficiently studied. This monocyte subpopulation has an immunoregulatory function, exerting a marked stimulatory effect on Th cells (Zawada et al., 2011). Alongside non-classical monocytes, they are major producers of pro-inflammatory cytokines, such as TNF-alpha, IL-1beta, and IL-12. Additionally, they exhibit moderate phagocytic activity, limited respiratory burst capacity, and synthesize the chemokines IL-8, MCP-1, and CCL3 (Belge et al., 2002).

Phagocytosis is a process important for both acute inflammatory response (i. e. engulfing pathogens or foreign particles with their subsequent degradation and antigen-presentation) and resolution of inflammation (e.g. clearance of apoptotic and necrotic cells, as well as cell debris). Therefore, it is important to interpret modulation of phagocytic cell function in complex with other parameters. Decreased basal phagocytic activity and ROS production in monocytes from BALB/c mice compared to C57Bl/6 mice coincides with Th2- and Th1-polarized immunity of those strains (Bleul et al., 2021).

CD80 is an inducible co-stimulatory molecule providing a signal to the T cell CD28 receptor, thus amplifying T-cell antigen receptor signaling in a process of antigen presentation (Smith-Garvin, Koretzky, & Jordan, 2009). Therefore, its expression serves as the marker of pro-inflammatory activation of phagocytes. Conversely, activation of CD206 on phagocytes initiates an anti-inflammatory and tolerogenic response, leading to the release of anti-inflammatory cytokines, reduction in pro-inflammatory cytokines, and suppression of ROS production (van der Zande et al., 2021). Interestingly, BALB/c mice had higher level of CD80 and lower level of CD206 expression compared to C57Bl/6. This seeming conflict with the data on Th1- and Th2-biased immune responses of C57Bl/6 and BALB/c mice may be due to the use of older animals in our study, and difference in aging trajectories between studied animal strains. Study of Costantini et al. (2018) on human monocytes revealed that the ratio of proinflammatory monocytes increases with aging in older patients with acute myocardial infarction.

In general, treatment with Larifan caused anti-inflammatory effect on monocytes from C57Bl/6 mice. It manifested in significant decrease of ROS production. However, both percentage and expression level of anti-inflammatory marker CD206 decreased in this strain of mice after exposure to dsRNA. Kapellos et al. (2019) have shown that human monocytes can respond to different environmental factors, including dietary components and lifestyle changes, as well as development of various pathologies. Also, Constantini et al. (2018) used multiple phenotypic markers to study blood monocytes of healthy individuals, and found that each of the 3 main populations of monocytes (classical, non-classical and intermediate) were composed of 4 phenotypic subpopulations (CD80+CD163-, CD80+CD163+, CD80-CD163- and CD80-CD163+) in different proportions. Moreover, they found correlation of particular subpopulations of monocytes with healthy aging and acute myocardial infarction (Costantini et al., 2018). Therefore, it can be assumed that after the treatment with Larifan, blood monocytes of aged C57Bl/6 mice may have acquired some specific functional profile combining low ROS production and low CD206 expression.

In BALB/c mice, treatment with Larifan also lowered ROS production. Moreover, dsRNA exposure elevated phagocytic index simultaneously with inhibition of CD80 expression. Since CD80 expression is required for efficient antigen presentation, such type of phagocytic response may be relevant during the resolution of inflammation. Taken together, these results show anti-inflammatory polarization of monocytes from BALB/c mice after the exposure to Larifan.

In summary, the metabolic profile of monocytes in mice with different types of immune reactivity is different: in Th1-dominant C57Bl/6 mice nearly all studied monocyte indicators were higher compared to those in Th2-biased BALB/c mice. However, the monocyte response to the treatment was statistically more pronounced in BALB/c mice for most indicators. Interestingly, Larifan treatment inhibits ROS synthesis regardless of genotype, indicating its anti-inflammatory properties, as ROS is one of the primary markers of inflammation. Moreover, in BALB/c mice, the anti-inflammatory nature of the effect is further confirmed by simultaneous enhancement of phagocytic activity and a reduction in antigen-presenting capacity, demonstrated by a decrease in CD80 expression levels.

Authors' contribution: Roman Dovhyi – investigation, writing – original draft – Mariia Rudyk – formal analysis, writing – review & editing; Anastasiia Dvukhriadkina: investigation – Karina Ostrovska: investigation; Dace Pjanova – conceptualization.

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ПОРІВНЯЛЬНЕ ОЦІНЮВАННЯ ВПЛИВУ ІНТЕРФЕРОНОГЕННОГО ПРЕПАРАТУ ЛАРИФАН НА МОНОЦИТИ СТАРИХ МИШЕЙ ЛІНІЙ C57BL/6 ТА BALB/C *IN VITRO*

В с т у п . ***Моноцити крові відіграють ключову роль в імунному захисті організму як ефекторні клітини вродженої імунної системи. Однак вони також можуть сприяти розвитку гіперзапалення, як було описано на прикладі COVID-19. Чимало вірусних інфекцій викликають гіперзапалення, пригнічуючи синтез інтерферону I типу, що актуалізує пошук інтерфероновмісних препаратів та інтерфероногенів, таких як Ларифан – бактеріофагальна дволанцюгова РНК, які мають інтерфероногенні та імуномодуляторні властивості. Згідно з даними світової статистики вірусні інфекції, у тому числі SARS-CoV-2, а також гіперзапалення, частіше трапляються у чоловіків, особливо старшого віку, і значною мірою залежать від генетичного профілю імунної реактивності. Метою цього дослідження було порівняння впливу Ларифану на метаболічний профіль моноцитів периферичної крові, отриманої від старих самців мишей ліній C57BL/6 та BALB/c in vitro.***

М е т о д и . ***У дослідженнях використовувалися старі самці мишей ліній C57BL/6 та BALB/c. Кров отримували з лицьової вени й обробляли Ларифаном in vitro. Фагоцитарну активність, продукцію РФК та експресію фенотипових маркерів вивчали за допомогою проточної цитометрії. Гейтували та аналізували лише живі моноцити. Дані представлені як медіана та міжквартильний діапазон (IQR). Статистичні відмінності розраховували, використовуючи критерій Краскела–Уолліса, статистично значущими вважали відмінності за*** $p < 0,05$.

Р е з у л ь т а т и . ***У мишей лінії BALB/c спостерігалася менша базальна фагоцитарна активність, ніж у C57BL/6, однак відсоток фагоцитуючих клітин достовірно не відрізнявся. Обробка Ларифаном знижувала відсоток фагоцитуючих клітин в обох лініях, при цьому фагоцитарна активність зростала під впливом дволанцюгової РНК у мишей лінії BALB/c. Продукція РФК була вищою у C57BL/6, при цьому Ларифан значно знижував рівень РФК у мишей обох ліній. Базальний рівень експресії CD80 був вищим у BALB/c, водночас препарат збільшував кількість CD80-позитивних клітин і знижував рівень експресії CD80 лише у мишей цієї лінії. Експресія CD206 була нижчою у BALB/c, але не змінювалась під впливом Ларифану, у той час як препарат знижував як кількість CD206-позитивних клітин, так і рівень експресії CD206 у мишей лінії C57BL/6.***

В и с н о в к и . ***Метаболічний профіль моноцитів відрізняється у тварин лінії C57Bl/6 з Tx1-домінантною імунною відповіддю, і BALB/c з переважанням імунної відповіді типу Tx2, що проявлялося вищими базальними показниками у C57BL/6 мишей. Ларифан здійснює протизапальний ефект, знижуючи синтез РФК у тварин обох ліній, при цьому у BALB/c мишей також спостерігалось зростання фагоцитарної активності на фоні зниження здатності до презентації антигенів.***

К л ю ч о в і с л о в а : ***бактеріофагальна дволанцюгова РНК, моноцити крові, фагоцитарна активність, реактивні форми кисню, CD80, CD206.***

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