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POLARIZED ACTIVATION OF HUMAN PERIPHERAL BLOOD PHAGOCYTES BY BACTERIOPHAGE–DERIVED DOUBLE-STRANDED RNA (LARIFAN) *in vitro*

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Aim. This study aimed to examine the effect of Larifan on metabolic characteristics of human blood monocytes and granulocytes *in vitro*.

Methods. Four healthy adult men aged 21–26 years were recruited to participate in the study as blood donors. The metabolic profile of human blood monocytes and granulocytes was evaluated by phagocytic activity, reactive oxygen species production, nitric oxide generation, and arginase activity. Phagocytosis of FITC-labeled inactivated Staphylococcus aureus and reactive oxygen species generation were estimated by flow cytometry. Arginase activity was assessed in cell lysates, and nitric oxide generation in supernatants was examined using the Griess reaction.

Results. Phagocytic index and reactive oxygen species generation were found to be lower in both human blood monocytes and granulocytes treated with Larifan. The drug caused a dose-dependent increase in nitric oxide production, as well as a decrease in the arginase activity of blood monocytes.

Conclusions. Our results indicate the ability of Larifan to reinforce the antiviral properties of resting phagocytes along with containment of oxidative stress development.

Key words: monocytes; granulocytes; phagocytosis; reactive oxygen species; nitric oxide; arginase; metabolic polarization.

Type I and III interferons (IFNs) are innate cytokines that are broadly expressed across many cells, and therefore they are important in the first line of defense against viruses. However, many viruses, particularly SARS-CoV-2, are known to inhibit these IFN responses at various points, from cytokine production to receptor signaling, leading to an increase in viral load [1].

Pathogens, including viruses, are known to express some conserved motifs which are not found in host organism, known as pathogen-associated molecular patterns (PAMPs). Those molecular patterns can be recognized by the corresponding pattern recognition receptors of the phagocytic cells, leading to the proinflammatory activation of those cells [2]. Due to the SARS-COV-2 viral load increase resulting from IFN inhibition, multiple PAMPs stimulate phagocytes, leading to the overactivation of a body's immune system that is manifested as the cytokine storm. And

cytokine storm is considered to be one of the main causes of acute respiratory distress syndrome and multi-organ failure [3].

Since COVID-19 is accompanied by delayed type I IFN response [1], regulation of interferon production can be considered a promising therapeutic option [4]. One such drug candidate is Larifan, comprising a heterogeneous population of dsRNA obtained biotechnologically from *E. coli* cells infected with f2sus11 amber mutant bacteriophage. It is already approved and registered for human use at the State Agency of Medicines of the Republic of Latvia as a treatment option for herpes virus infections and secondary immunodeficiency (Reg. No.04-0230). Moreover, it was demonstrated that pre- and post-infection administration of Larifan inhibited SARS-CoV-2 replication both *in vitro* and *in vivo* in golden Syrian hamsters. Also, Larifan decreased the severity of the infection-induced pathological lesions in the lungs of those animals, and is characterized by interferonogenic activity [5].

We have previously studied the effects of Larifan on the metabolic profile of macrophages of different localization. It was found that intranasally delivered Larifan is capable of re-educating glioma-associated microglia, thus abolishing the creation of pro-tumoral microglia infiltrates [6]. Also, in another experiment, Larifan increased nitric oxide (NO) synthesis and reduced arginase activity and reactive oxygen species (ROS) generation in rat peritoneal macrophages under normoxic conditions [7].

Given the literature data about the remarkable metabolic plasticity of monocytes and macrophages [8], as well as our results showing the ability of Larifan to reprogram the metabolic profile of tissue-resident macrophages, it can be assumed that this drug may prime blood phagocytes for a fight against SARS-COV-2 infection.

The purpose of this study was to examine the effect of Larifan (bacteriophage-derived DSRNA) on metabolic characteristics of human peripheral blood monocytes and granulocytes *in vitro*.

Materials and Methods

Study participants included four healthy adult men aged 21–26 years. The exclusion criteria were a history of somatic disease. Approval was obtained from the local ethical committee, and informed consent was obtained from all subjects before the commencement of the study.

Monocyte isolation. Monocytes were isolated from the buffy coat by double-density gradient centrifugation as described by Menck et al. [9] with slight modifications. Briefly, the buffy coat was subjected to a Ficoll-Hypaque gradient centrifugation (400 g, 30 min) to harvest peripheral blood mononuclear cells (PBMCs). Isolated PBMCs were washed twice by centrifugation in PBS-EDTA (1 mM), and were then layered on a slight hyperosmolar Percoll gradient (density = 1.064 g/m) followed by centrifugation (500 g, 30 min). Cell viability was determined by the Trypan blue exclusion test. The percentage of monocytes after the Percoll gradient was higher than 90% as confirmed by morphology and FACS analysis using anti-CD14 antibodies (BD).

Study design. To estimate the effect of Larifan on ROS generation and phagocytic activity of monocytes and granulocytes whole blood samples collected with EDTA were treated with the drug at growing concentrations (50, 100, and 150 μg/ml) for 30 min and then were analyzed by flow cytometry. To estimate the effect of Larifan on arginase activity and NO generation, isolated monocytes were treated with the drug at growing concentrations (50, 100, and 150 μg/ml) for 18h. Conditioned media and cells were harvested after the treatment with Larifan. Aliquots of media were sampled immediately and analyzed for nitrites. Arginase activity was analyzed in harvested cells.

Intracellular ROS assay. ROS levels were measured using 2′7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Invitrogen), which is converted into a non–fluorescent derivative (carboxy-H2DCF) by intracellular esterases as described earlier [10]. Carboxy-H2DCF is membrane impermeable oxidized to fluorescent derivative carboxy-DCF by intracellular ROS. 200 μl of EDTA-anticoagulated whole blood was incubated with 4.3 μl of PBS containing 10 μM carboxy–H2DCFDA for 30 min at 37 °C. A short recovery time was allowed for the cellular esterases to hydrolyze the acetoxymethyl ester or acetate groups and render the dye responsive to oxidation. Erythrocytes were lysed with lysis buffer. The cells were then transferred to polystyrene tubes with cell–strainer caps (Falcon, Becton Dickinson) and analyzed with flow cytometry (excitation: 488 nm, emission: 525 nm). Only living cells, gated according to scatter parameters, were used for the analysis. Neutrophils or monocytes were gated according to forward and side scatter.

Phagocytosis assay. The flow cytometry phagocytosis assay was performed as described above [10]. *Staphylococcus aureus* Cowan I cells (collection of the Department of Microbiology and Immunology of Taras Shevchenko National University of Kyiv) were grown on beef–extract agar and subsequently were heat-inactivated and fluorescein isothiocyanate (FITC) labeled.

The stock of FITC-labeled *S. aureus* at a concentration of 1×10^7 cells/mL in a volume of 5 µL was added to 200 µl of heparinized whole blood. A tube with whole blood only served as a negative control. All samples were incubated at 37 °C for 30 min. At the end of the assay, phagocytosis was arrested by the addition of a cold stop solution (PBS with 0.02% EDTA and 0.04% paraformaldehyde). Erythrocytes were lysed with lysis buffer. The fluorescence of phagocytes with ingested bacteria was determined by flow cytometry. Neutrophils or monocytes were gated according to forward and side scatter. Phagocytosis index (PhI) was calculated with the following formula:

$$[Gmean_{pos} / P_{pos}] - [Gmean_{neg} / P_{neg}],$$

where P_{pos} — percent of positive cells, $Gmean_{pos}$ — mean channel fluorescence, P_{neg} — percent of positive cells in the negative control, $Gmean_{neg}$ — mean channel fluorescence of the negative control.

Measurement of NO production. Griess reaction was used to measure the quantity of NO in each supernatant [11]. The amount of NO per 10^6 cells was calculated by dividing the NO metabolite readings by the total number of viable cells. The mean and standard error were computed as normalized values.

Determination of arginase activity. Arginase activity in cell lysates was determined using a conventional procedure [11]. In summary, cells were treated for 15 minutes at room temperature in a shaker with 100 µl/well of 0.1% Triton X-100 lysis solution. Subsequently, 10 µl of 10 mM $MnCl_2$ and 100 µl of 50 mM Tris-HCl, pH 7.5, were added to each lysate. The plates were heated to 56 °C for 7 minutes to activate the enzyme. The lysates were incubated for two hours with 100 µl of 0.5 M L-arginine (pH 9.7) at 37 °C to facilitate substrate hydrolysis. Using 800 µl of H_3PO_4 (85%), H_2SO (96%), and H_2O (1/3/7, v/v/v), the reaction was stopped. α-isonitrosopropiophenone (40 µl, 9% solution in ethanol) was added and the mixture was incubated for 30 minutes at 95 °C and then for 30 minutes at 4 °C to determine urea using colorimetric analysis. Spectrophotometric analysis was used to quantify the urea content. Each measurement was expressed as the urea level/h per 10^6 cells by dividing it by the total number of viable cells. The means and standard errors were normalized.

Statistical analysis. All experimental results are reported as mean ± standard error. Data statistical significance was determined by Student's t-test. The values of $P < 0.05$ were considered as significant.

Results and Discussion

A slight decrease of the phagocytic index was detected in human blood monocytes and granulocytes treated with all studied concentrations of Larifan compared to untreated cells (Fig. 1). Phagocytosis,

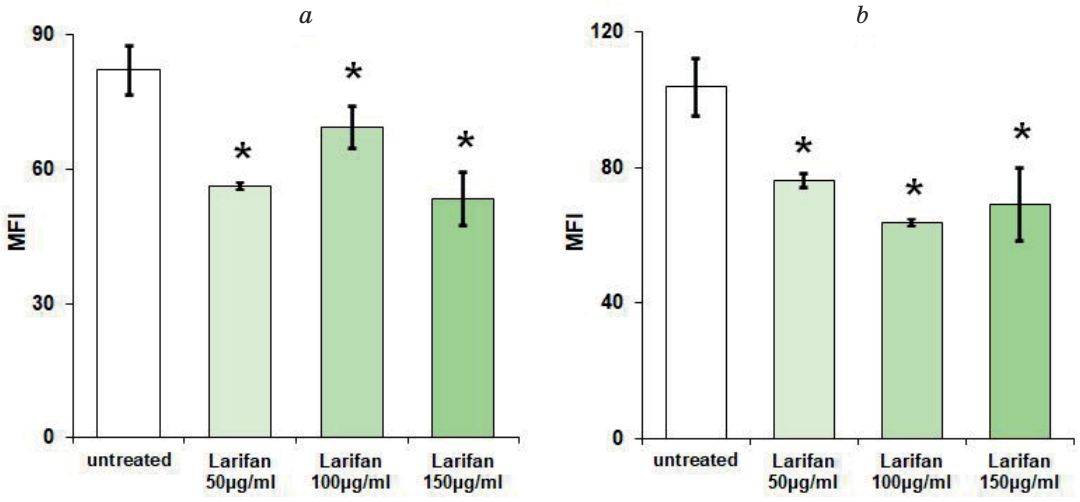

Fig. 1. **Phagocytic index in human blood monocytes:**
a — and granulocytes, *b* — treated with Larifan. MFI — mean fluorescence intensity.
The data are presented as mean ± standard error of the mean. *$P < 0.05$ as compared to untreated cells

specifically antibody-dependent, can be a double-edged sword for antiviral defense: there are reports demonstrating its protective [12], as well as deleterious role, due to phenomenon known as antibody-dependent enhancement (ADE) occurring through enhanced antibody-mediated virus uptake by Fc gamma receptor-expressing phagocytic cells [13]. There is no compelling evidence for ADE in COVID-19 patients so far [14]. Nonetheless, this phagocytosis-inhibiting effect of Larifan may be speculated to prevent ADE to some extent.

Similarly, ROS generation declined significantly ($P < 0.05$) in human blood monocytes after applying Larifan in 3 different concentrations (Fig. 2, *a*). Two higher doses of the drug also significantly lowered ROS production ($P < 0.05$) by human blood granulocytes as compared to untreated cells (Fig. 2, *b*). High ROS production is generally associated with pro-inflammatory metabolic profile of phagocytic cells, with ROS being responsible for direct antimicrobial effects mediated through interaction with bacterial DNA, RNA and proteins [15]. Similar properties of ROS were also described in the context of parasitic infections. However, it is important to note that in contrast to bacteria and parasites, viruses often benefit from increased ROS production [15–17]. To et al. (2017) have shown that Nox2-derived ROS, generated in response to infection with single-stranded RNA and DNA viruses, suppressed antiviral response by modifying highly conserved cysteine residue (Cys98) on Toll-like receptor-7, and targeted inhibition of those endosomal ROS molecules abrogated influenza A virus pathogenicity [17]. Also, according to the studies, ROS play an important role in the pathogenesis of COVID-19 [18]. We have also shown the ability of Larifan to attenuate ROS production in tissue-resident macrophages of different origins, such as rat peritoneal macrophages and microglial cells of C6 glioma-bearing rats [6, 7]. Therefore, the ROS suppressive effect of Larifan may be beneficial in the context of viral diseases, including COVID-19.

Adding 100 μg/ml and 150 μg/ml of Larifan caused a dose-dependent increase in NO production ($P < 0.05$ and $P < 0.01$, respectively) by human blood monocytes (Fig. 3, *a*). Apparently, it happens as a result of dsRNA stimulating RNA-dependent protein kinase, which in its turn activates inducible nitric oxide synthase (iNOS) through the NF-κB pathway [19]. An increase in NO production is usually regarded as a sign of proinflammatory activation of classically activated M1 macrophages [20], with NO acting as a broad-spectrum antimicrobial and antiviral agent effective, in particular, against SARS-COV-2. There are a multitude of specific and non-specific anti-viral mechanisms exerted by NO [21]. Moreover, in contrast to the widely recognized concept of NO being a pro-inflammatory molecule, there are recent reports demonstrating the ability of NO to downregulate leukocyte migration in the course of an acute inflammatory reaction, as well as to inhibit the production of pro-inflammatory cytokines and chemokines [22, 23].

The same higher doses of Larifan (100 μg/ml and 150 μg/ml) reduced the

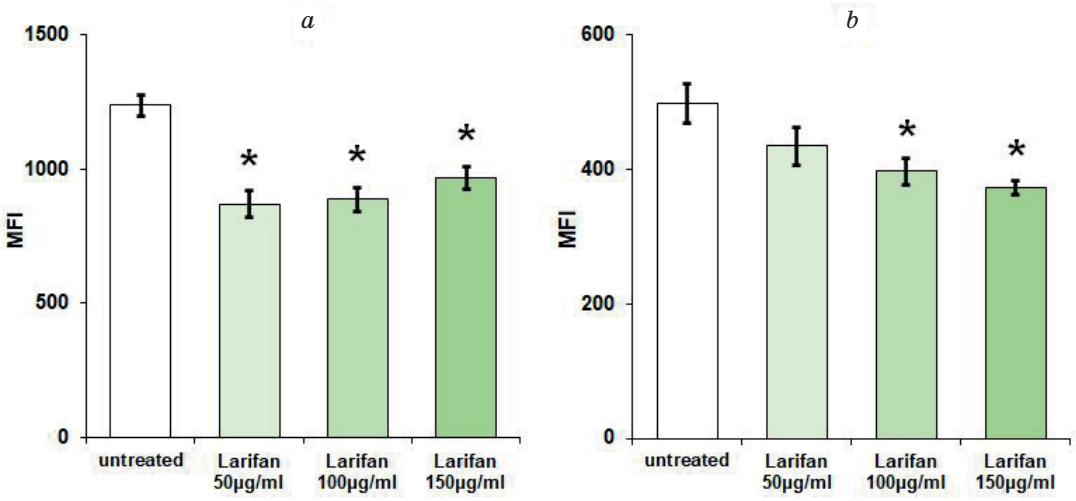

Fig. 2. **Reactive oxygen species generation in human blood monocytes:**
a — and granulocytes, ***b*** — treated with Larifan. MFI — mean fluorescence intensity.
The data are presented as mean ± standard error of the mean. * $P < 0.05$ as compared to untreated cells.

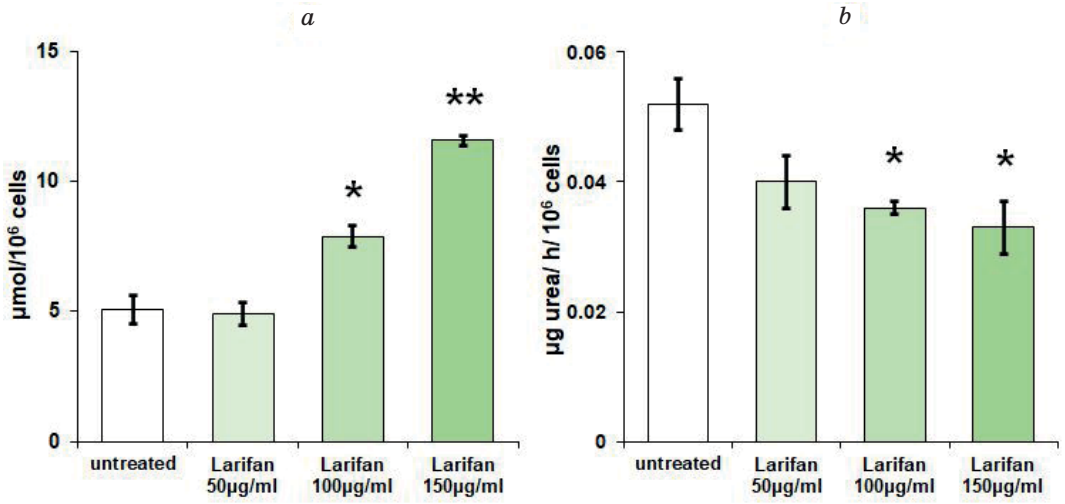

Fig. 3. **Nitrite production (*a*) and arginase activity (*b*) in human blood monocytes treated with Larifan**
The data are presented as mean ± standard error of the mean. * $P < 0.05$; ** $P < 0.01$ as compared to untreated cells.

arginase activity of human blood monocytes ($P < 0.05$) in comparison to untreated cells (Fig. 3, *b*). This decrease may be explained by the reduction of the amount of available L-arginine, which is a common substrate of iNOS and arginase, due to aforementioned significant increase in activity of iNOS (Fig. 3, *a*). Elevated arginase activity is associated with alternatively activated M2 repairing response of macrophages, which is appropriate when a viral pathogen has been eradicated and there is a need to restore tissues damaged by inflammation and viral replication [20]. Therefore, it is not surprising that arginase was inhibited by Larifan since the purpose of the latter is to enhance antiviral response when the infection is in full swing.

Conclusions

Treatment with Larifan causes a slight decrease in phagocytic activity, potent inhibition of reactive oxygen species generation along with an increase of NO release, and a moderate decrease of arginase activity. Taken together, our results indicate the ability of Larifan to reinforce the antiviral properties of resting phagocytes along with containment of oxidative stress development.

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Conflicts of Interest
Authors declare no conflict of interest.

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ПОЛЯРИЗОВАНА АКТИВАЦІЯ ФАГОЦИТІВ ПЕРИФЕРИЧНОЇ КРОВІ ЛЮДИНИ ДВОЛАНЦЮГОВОЮ РНК (ЛАРИФАН) ФАГОВОГО ПОХОДЖЕННЯ *in vitro*

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Метою роботи було вивчити вплив Ларифану на метаболічні характеристики моноцитів і гранулоцитів крові людини *in vitro*.

Методи. Донорами крові для цього дослідження були четверо здорових дорослих чоловіків віком 21–26 років. Метаболічний профіль моноцитів і гранулоцитів крові людини оцінювали за фагоцитарною активністю, утворенням реактивних форм кисню, продукуванням оксиду азоту та активністю аргінази. Фагоцитоз інактивованого *Staphylococcus aureus*, міченого FITC, і утворення реактивних форм кисню оцінювали за допомогою проточної цитометрії. Активність аргінази вимірювали в клітинних лізатах, а утворення оксиду азоту в супернатантах досліджували за допомогою реакції Грісса.

Результати. Фагоцитарний індекс і утворення активних форм кисню були нижчими як у моноцитів, так і у гранулоцитів крові людини, які отримували Ларифан. Препарат викликав дозозалежне підвищення продукції оксиду азоту, а також зниження аргіназної активності у моноцитів крові.

Висновки. Наші результати вказують на здатність Ларифану посилювати противірусні властивості фагоцитів та попереджати розвиток оксидативного стресу.

Ключові слова: моноцити; гранулоцити; фагоцитоз; реактивні форми кисню; оксид азоту; аргіназа; метаболічна поляризація.