

ROS PRODUCTION AND PHAGOCYtic ACTIVITY IN HUMAN BLOOD PHAGOCYTES TREATED WITH BACTERIOPHAGE PREPARATION PYOFAG

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The growing use of bacteriophages in the treatment of antibiotic-resistant infections highlights the need to clarify their direct effects on innate immune cells. This study investigated the effect of polyvalent phage preparation Pyofag on oxidative metabolism and phagocytic function of nonsensitized peripheral blood monocytes and granulocytes under physiological whole-blood conditions. Blood samples from healthy donors were collected using lithium heparin, incubated with Pyofag at phage-to-cell ratios of 1:2, 1:5, and 1:10, centrifuged and the cell pellet was used. ROS production was assessed with the use of DCFDA, phagocytic activity was estimated by fluorescent polystyrene latex beads-uptake intensity. The percentage of phagocytic cells and their mean fluorescence index (MFI) were measured by flow cytometry. It was shown that Pyofag induced statistically significant moderate ROS generation in both phagocyte populations only at the highest dose, remaining markedly lower than that induced by phorbol 12-myristate 13-acetate. Upon Pyofag treatment, only minor reduction in the proportion of phagocytosing monocytes and minor increased in the percentage of phagocytosing granulocytes was observed with no effect on MFI. The mild oxidative activation and stable phagocytic performance observed in Pyofag-treated blood phagocytes point to a noninflammatory, balanced immunomodulatory profile, supporting the safety of this phage preparation for potential systemic administration.

Key words: bacteriophages, Pyofag, monocytes, granulocytes, phagocytosis, reactive oxygen species, immune modulation.

The growing prevalence of antibiotic-resistant infections has renewed interest in bacteriophage therapy. In the WHO European Region, approximately 133,000 deaths per year are directly attributed to antimicrobial resistance, with more than half a million additional deaths indirectly associated [1]. As phage therapy has been extended to deep-seated and systemic infections, its routes of administration have expanded beyond topical and oral use. Advances in production technologies have enabled the isolation of therapeutic phages at near-GCP quality, supporting the increasing application of intravenous administration [2].

Once in the bloodstream, phages mainly interact with circulating monocytes and neutrophils, including the nonsensitized resting phagocyte pool.

Understanding how bacteriophages interact with resting phagocytes is central to evaluating the safety of phage therapy, helping to identify beneficial effects as well as potential risks, and thereby ensuring its safe and effective clinical use. Phagocytic cells interact with bacteriophages primarily through nonspecific binding to surface components such as sialic acid residues, mucins, and integrins, as well as through pattern-recognition receptors, including Toll-like receptors (TLRs) and scavenger receptors [3]. Bacteriophage nucleic acids can also be detected by a wide range of intracellular nucleic acid sensors [4]. Together, these interactions are capable of modulating key phagocyte functions. Specifically, engagement of pattern-recognition receptors, particularly TLR9, has been shown to modulate neutrophil res-

piratory-burst activity and NET formation, and may also influence phagocytic function [5-8]. Monocyte interactions with bacteriophages, mediated through scavenger receptors that bind negatively charged phage particles and TLRs that detect phage components, including DNA, can modulate the expression of co-stimulatory molecules, cytokine production, and other functions. These effects appear to depend on the phage used, the disease state, and the condition of the host immune system [9]. It is well established that, in addition to their direct antibacterial action, phages can modulate host immune responses and exert both pro- and anti-inflammatory effects [10]. Specifically, bacteriophage DNA recognition by intracellular DNA-sensing pathways, including cGAS–STING, may lead to NF- κ B activation and the induction of pro-inflammatory responses [11]. Although phage-driven anti-inflammatory effects were initially attributed solely to reduced bacterial burden and diminished infection-induced signaling, evidence now shows that phages can directly modulate immune cells. Their anti-inflammatory actions – both established and hypothesized – include interference with signaling pathways such as TLR–MyD88–NF- κ B, induction of anti-inflammatory mediators like IL1RN, and other yet-uncharacterized mechanisms [3].

The major obstacle to successful phage therapy is the rapid emergence of phage resistance, which can lead to treatment failure [12]. Resistance to phages may arise through modifications of bacterial surface receptors involved in adsorption or through antiviral systems such as CRISPR–Cas and DNA restriction–modification mechanisms [13]. This has led to increasing use of phage cocktails, in which phages with different receptor specificities and genomic characteristics act together, reducing the likelihood of resistance development and enhancing therapeutic stability [14-16].

Despite growing interest in both phage therapy for multidrug-resistant infections and bacteriophage immunomodulatory potential, data on the influence of phages on the metabolic activity of unsensitized circulating phagocytes remain limited, particularly when comparing single-phage agents and phage mixtures. Therapeutic formulations often include phage cocktails, where multiple phages with different receptor specificities may act synergistically, potentially producing immune effects that differ from those of individual phages.

Given these considerations, the aim of this study was to evaluate the effects of the polyvalent bacteriophage preparation Pyofag on the metabolic activity of peripheral blood phagocytes obtained from healthy, nonsensitized donors.

Materials and Methods

Study participants. Peripheral blood was obtained from healthy female individuals aged 20–25 years ($n = 10$) at a university medical facility by qualified medical personnel after written informed consent. Subjects were excluded if they were currently taking medications that affect immune reactivity or had a history of acute or chronic immune-mediated or inflammatory disease. All participants provided written informed consent prior to sample collection and analysis, and the study was approved by the local ethics committee (Protocol No. 2, February 27, 2025).

Phage cocktail. The agent used was Pyofag® polyvalent bacteriophage (Pharmex Group LLC, Ukraine for NeoProBioCare Inc.), which is active against pyogenic and enteric pathogens. Each milliliter contains at least 1×10^5 bacteriophage particles specific to *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Proteus mirabilis*.

Study design. Whole blood, collected in lithium heparin-coated VACUETTE® blood collection tubes (Greiner Bio-One, Austria), was aliquoted 50 μ l/sample and incubated for 30 minutes at 37°C with Pyofag at doses corresponding to 2, 5, or 10 viral particles (VP) per phagocyte. Saline was added to the unstimulated control samples, while phorbol 12-myristate 13-acetate (PMA; Thermo Fisher Scientific, USA) was used as a positive control at 10 ng/ml to assess phagocytic activity and at 20 ng/ml to measure reactive oxygen species (ROS) production. PMA was used as a positive control as protein kinase C activator, which drives NADPH oxidase assembly and cytoskeletal remodeling, resulting in a strong increase in both ROS production [17, 18] and phagocytic activity [19]. After incubation, the samples were centrifuged at $300 \times g$ for 7 min, the supernatant was discarded, and the samples were processed as described below (Fig. 1).

ROS-production assay. ROS generation was assessed as previously described [20], with minor modifications. To the cell pellet, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Invitrogen,

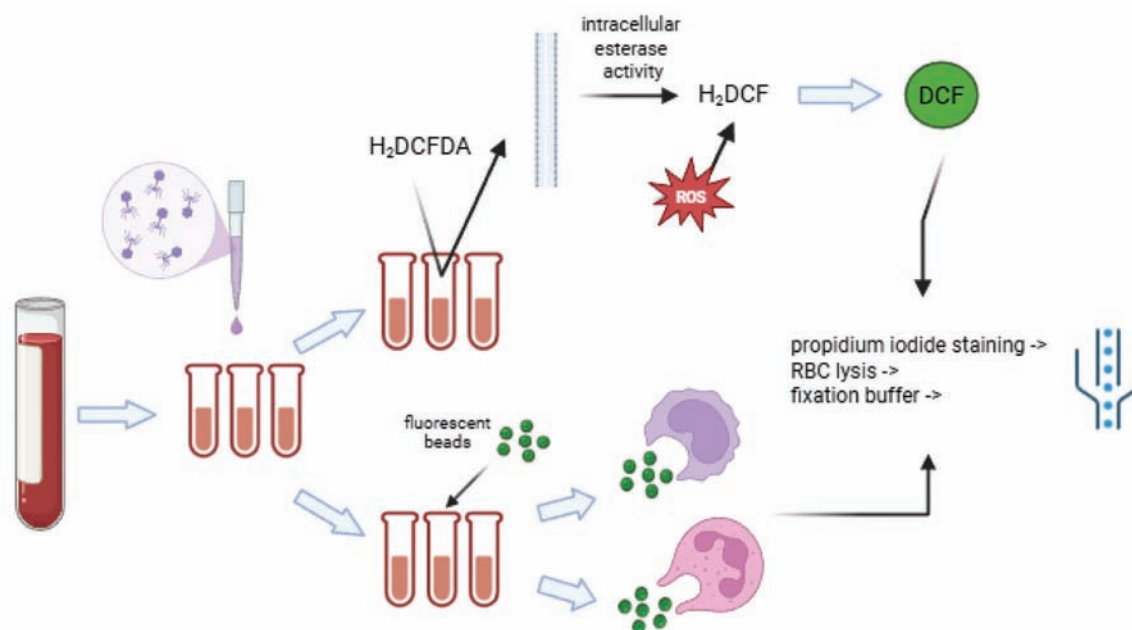


Fig. 1. Study design. Following incubation of whole-blood samples with bacteriophages, parallel workflows for oxidative burst measurement using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and for phagocytosis assessment using fluorescent beads were performed prior to flow cytometric analysis

USA) was added to all samples except the blank control, resulting in a final concentration of 20 μM . The samples were incubated for 30 min at 37°C. Subsequently, cold ammonium chloride-based lysis buffer (0.154 M NH_4Cl , 7.2 mM K_2CO_3 , and 1 mM EDTA, prepared in dH_2O and adjusted to pH 7.2; all chemical reagents from Thermo Fisher Scientific, USA) was added, and the samples were incubated in the dark for 10 min, followed by centrifugation at $300 \times g$ for 7 min. The resulting pellets were washed with phosphate-buffered saline (PBS: 0.12 M NaCl, 2.2 mM KH_2PO_4 , and 5.6 mM Na_2HPO_4 , dissolved in dH_2O and adjusted to pH 7.4; all chemical reagents from Thermo Fisher Scientific, USA), stained with propidium iodide (PI; Invitrogen, USA) at a final concentration of 1 $\mu g/ml$ for 5 min at room temperature to assess cell viability, washed again with PBS, and fixed using a fixation buffer (PBS supplemented with 0.02% EDTA, 0.4% paraformaldehyde (PFA); all chemical reagents from Thermo Fisher Scientific, USA), while avoiding exposure to direct light throughout the procedure. Fluorescence was analyzed using a DxFLEX flow cytometer (Beckman Coulter, USA), with oxidized H_2DCFDA (DCF) detected at 488 nm excitation / 518 nm emission and PI fluorescence collected in the ECD channel (610/20 nm).

Phagocytosis assay. To the cell pellet in all samples except the blank control, 100 μl of RPMI-1640 (Sigma-Aldrich, USA) and carboxylate-modified yellow-green fluorescent polystyrene latex beads (1 μm in diameter; Sigma-Aldrich, USA) were added at a ratio of 50 beads per phagocyte, followed by incubation for 1 hour at 37°C. After incubation, erythrocytes were lysed and the cells were stained with PI as described above. The samples were then fixed and analyzed by flow cytometry (excitation/emission parameters same as above). The percentage of phagocytosing cells and their mean fluorescence index (MFI) were measured.

To combine the phagocytic parameters, we used the phagocytic score formula (percentage of phagocytosing cells $\times$ MFI / 100).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Data were normalized to the untreated control for each donor prior to statistical analysis. Since each donor contributed data to all experimental conditions, a repeated-measures design was applied. Normalized data were $\log_2$ -transformed to approximate a normal distribution. When the assumption of normality (Shapiro-Wilk test) was met, repeated-measures ANOVA with post hoc Tukey's test was applied; otherwise,

the nonparametric Friedman test followed by Dunn's multiple comparisons test was used. Statistical significance was defined as $P < 0.05$.

Results and Discussion

The relevance of this study lies in the fact that most existing research on the immunomodulatory properties of bacteriophages has been performed on cultured or isolated cells. By contrast, conducting experiments using whole blood enhances the translational value of the findings, as it better reflects the complexity of physiological immune responses. Whole blood preserves the physiological microenvironment, including plasma proteins, complement components, and granulocyte-to-monocyte ratios, which are substantially altered during cell isolation, thereby enabling more accurate assessment of phage-immune cell interactions.

Pyofag is highly effective against methicillin-resistant *S. aureus* [21, 22], increases sensitivity of multidrug-resistant *E. coli* to various classes of antibiotics, including beta-lactams, fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides [23].

Pyofag is approved for local and oral administration, with intraluminal and intracavitary application permitted. Despite the high level of purification, intravenous administration is not recommended, and systematic studies evaluating its interaction with blood phagocytes in nonsensitized healthy donors have not been conducted.

Pyofag-induced changes in oxidative metabolism of phagocytes. Stimulation with PMA induced a pronounced increase in ROS production in monocytes compared with the untreated control (median $\log_2$ -change = 2.52; $P = 0.0024$; Fig. 2, A). A similar trend was observed in granulocytes (median $\log_2$ -change = 4.71; $P < 0.0001$; Fig. 2, B). Exposure to the polyvalent bacteriophage preparation resulted in moderately elevated ROS levels relative to untreated cells. This effect was most pronounced in samples treated with Pyofag at a 1:10 ratio (median $\log_2$ -change = 0.81; $P = 0.03$). The magnitude of ROS production in phage-treated monocytes was substantially lower than in the PMA-stimulated condition ($P < 0.05$ for the 1:2 and 1:5 phage-to-cell ratios vs. PMA; Fig. 2, A). Although an enhanced oxidative burst is often viewed as a hallmark of pro-inflammatory phagocyte activation, increased ROS production is not inherently pro-inflammatory. Its effects depend on the intensity, duration, and cel-

lular context of ROS generation. While high ROS levels drive inflammatory responses, moderate and tightly regulated ROS can promote anti-inflammatory processes - such as apoptotic cell clearance [18, 24]. In granulocytes, median ROS levels in phage-treated samples tended to be lower than those observed in the PMA-treated control; however, this difference was statistically significant only at the highest phage-to-cell ratio (1:10; $P < 0.01$; Fig. 2, B). Although ROS levels showed a tendency to increase relative to the untreated control, no statistically significant differences were detected among the tested phage-to-cell ratios (1:2, 1:5, 1:10) in either monocytes or granulocytes. The modest elevation in ROS production detected in our study aligns with previously reported responses of granulocytes to the T4 bacteriophage [25].

Although median ROS levels in phage-treated granulocytes and monocytes were generally higher than in untreated controls, substantial inter-individual variability was observed. In some donors, phage exposure resulted in lower ROS production, and this effect did not correspond to the magnitude of the PMA-induced oxidative burst. These findings indicate that individual donor differences, independent of overall oxidative capacity, influence the cellular response to bacteriophage stimulation.

The absence of a major increase in ROS production relative to unstimulated controls suggests that Pyofag does not induce excessive pro-inflammatory activation of phagocytes *in vitro*, but rather maintains their patrolling function. These findings are consistent with a potentially noninflammatory safety profile and support further evaluation of the preparation for systemic administration, including intravenous use.

Pyofag-induced changes in phagocytic function. In monocytes, the percentage of phagocytosing cells showed numerically lower median values in phage-treated groups compared with the untreated control; however, no statistically significant differences were observed (Fig. 3, A). In contrast, in granulocytes, a statistically significant increase in the percentage of phagocytosing cells was observed at the two higher phage concentrations, with median $\log_2$ -change values of 0.45 (1:5) and 0.31 (1:10) relative to the untreated condition ($P < 0.05$; Fig. 3, B).

Stimulation with PMA induced a pronounced increase in the number of phagocytosed beads in both monocytes and granulocytes compared with the untreated condition, as expected (Fig. 4). In contrast,

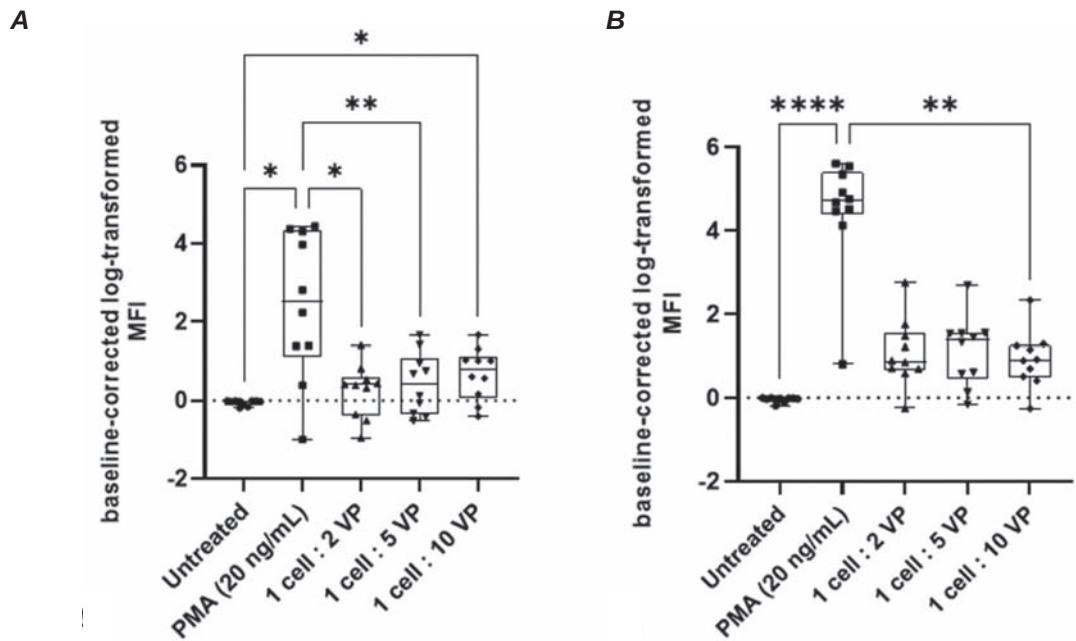


Fig. 2. Pyofag effect on ROS production in human blood monocytes (A) and granulocytes (B). Log₂-transformed, baseline-corrected mean fluorescence intensity (MFI) of oxidized H₂DCFDA (DCF) is shown. Data are presented as box-and-whisker plots: the center line indicates the median, the box represents the interquartile range (Q1–Q3), and whiskers indicate the minimum and maximum values (n = 10). *P < 0.05, **P < 0.01, ****P < 0.0001

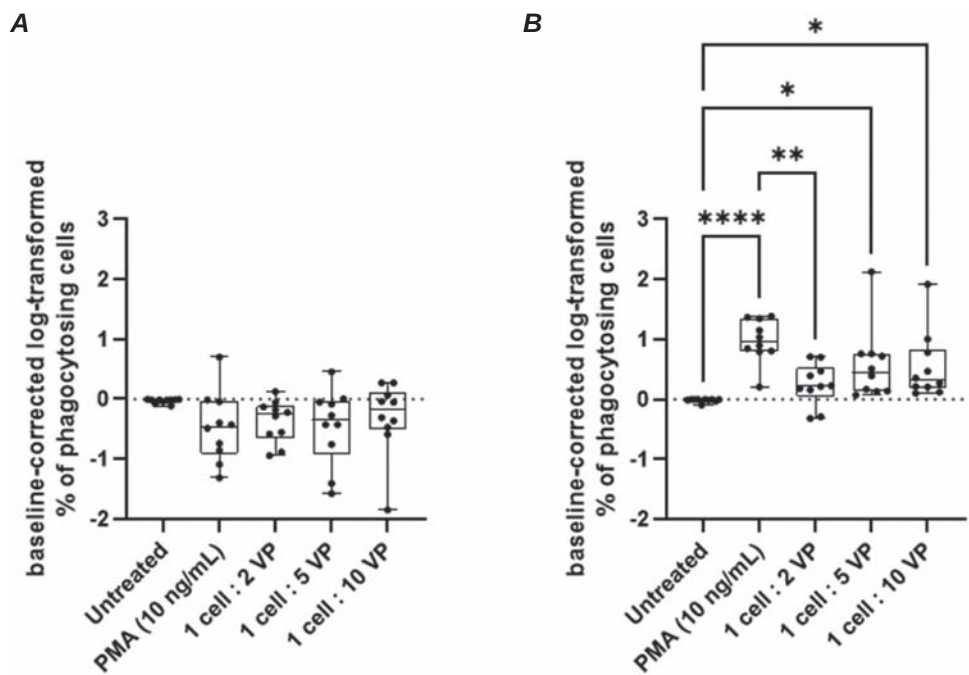


Fig. 3. Pyofag effect on % of phagocytosing human blood monocytes (A) and granulocytes (B). Log₂-transformed, baseline-corrected percentage of phagocytosing cells is shown. Data are presented as box-and-whisker plots: the center line indicates the median, the box represents the interquartile range (Q1–Q3), and whiskers indicate the minimum and maximum values (n = 10). *P < 0.05, **P < 0.01, ****P < 0.0001

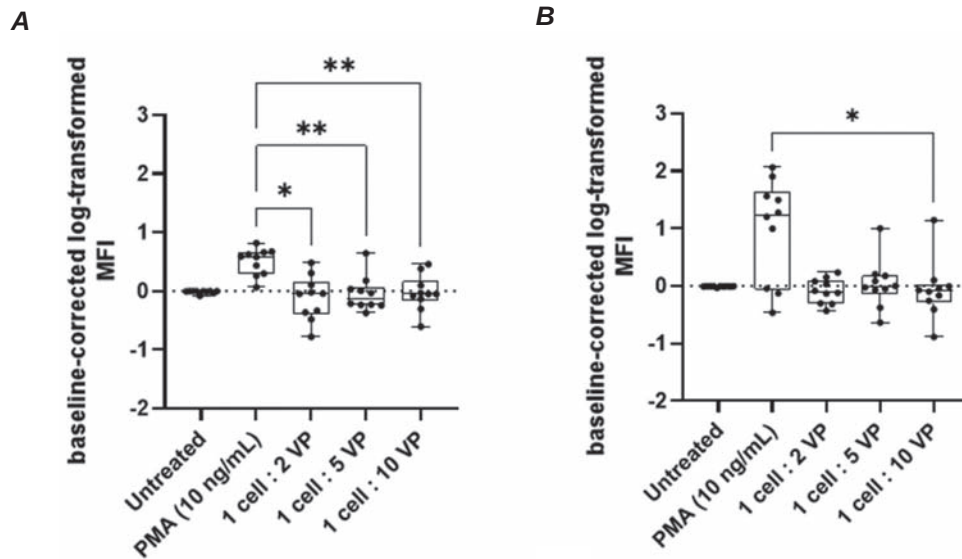


Fig. 4. Pyofag effect on phagocytic activity of human blood monocytes (A) and granulocytes (B). Log₂-transformed, baseline-corrected mean fluorescence intensity (MFI) of phagocytosed fluorescent beads is shown. Data are presented as box-and-whisker plots: the center line indicates the median, the box represents the interquartile range (Q1–Q3), and whiskers indicate the minimum and maximum values ($n = 10$). * $P < 0.05$, ** $P < 0.01$

treatment with the bacteriophage preparation did not significantly alter phagocytic activity relative to the untreated control in either cell type, with all phage-to-cell ratios remaining close to baseline levels.

In monocytes, phagocytic activity in all phage-treated groups was significantly lower than in the PMA-stimulated condition ($P < 0.05$ for 1:2, 1:5, and 1:10 vs. PMA; Fig. 4, A), indicating that phage exposure did not substantially enhance this parameter. A similar pattern was observed in granulocytes; however, a statistically significant reduction relative to PMA was detected only at the highest phage-to-cell ratio (1:10; $P = 0.01$; Fig. 4, B).

Thus, across both phagocyte populations, bacteriophage treatment maintained phagocytic activity at levels comparable to the untreated baseline and did not induce the heightened phagocytic response observed under PMA stimulation.

Whether internalization of phage particles influences the ability of phagocytes to subsequently engulf other targets remains unresolved. Although a competitive limitation of phagocytic capacity is theoretically possible at high phage loads, current studies provide no clear consensus [26].

When considering both measured phagocytic parameters (percentage of phagocytosing cells and MFI), distinct tendencies were observed between monocytes and granulocytes. In monocytes, expo-

sure to the Pyofag was associated with a slight reduction in overall phagocytic performance relative to untreated cells (Fig. 5, A). In contrast, granulocytes showed an opposite trend, with a modest increase in phagocytic capacity following phage treatment, most evident at the higher phage-to-cell ratios (median log₂-change values of 0.41 for 1:5 and 0.30 for 1:10; Fig. 5, B).

Though, these trends did not reach statistical significance when compared with the untreated control, indicating that bacteriophage exposure did not meaningfully enhance or suppress phagocytic function in either cell type under the tested conditions.

Taken together, these results suggest that bacteriophages do not act as strong activators or inhibitors of basal phagocytic activity, and that monocytes and granulocytes may differentially adjust their functional responses to phage contact. This difference may reflect intrinsic variability in receptor expression or engagement of intracellular signaling pathways between two phagocyte populations.

In the literature, reduced bacterial phagocytosis has been observed *in vitro* following preincubation of phagocytes with bacteriophages; however, *in vivo* studies have not demonstrated a corresponding decrease in phagocytic activity [25, 27].

However, the present results should not be directly equated with bacterial phagocytosis. The

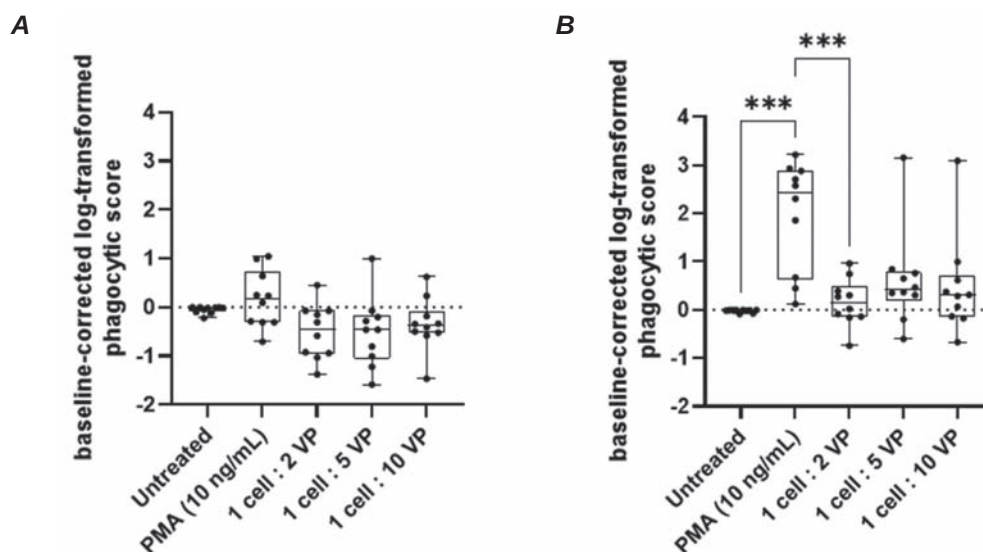


Fig. 5. Pyofag effect on the phagocytic score of human blood monocytes (A) and granulocytes (B). Log₂-transformed, baseline-corrected phagocytic score values (% of phagocytosing cells×MFI/100) are shown. Data are presented as box-and-whisker plots: the center line indicates the median, the box represents the interquartile range (Q1–Q3), and whiskers indicate the minimum and maximum values (n = 10). ****p* < 0.001

uptake of bacteria is mediated by specific pattern-recognition and opsonin-dependent mechanisms, including recognition through TLRs, complement receptors, and Fcγ receptors (FcγR) that bind antibody-opsonized pathogens. Engagement of these receptors activates intracellular signaling cascades that promote actin remodeling, phagosome maturation, and the activation of antimicrobial effector functions [28]. In contrast, the uptake of inert latex beads is primarily driven by nonspecific mechanisms, such as macropinocytosis or receptor-independent membrane engulfment, and does not involve pathogen-associated molecular pattern (PAMP) recognition. Consequently, bead internalization does not fully reproduce the receptor signaling, phagosomal processing, or oxidative and enzymatic activation that accompany bacterial ingestion. Therefore, while the bead-based assay provides a standardized measure of the phagocytic potential of monocytes and granulocytes, it reflects general engulfment capacity rather than immune receptor-driven phagocytosis of microbial targets.

Several limitations of this study should be acknowledged. The study was based on short-term *in vitro* functional assays of human blood phagocytes from a limited number of healthy donors and focused on early functional readouts. Moreover, phagocytic

capacity was assessed using inert particles, which do not fully replicate receptor-driven immune responses to microbial targets. These factors should be considered when interpreting the findings.

Conclusion. Taken together, the mild oxidative activation and stable phagocytic performance observed in Pyofag-treated peripheral blood phagocytes suggest a noninflammatory, balanced immunomodulatory profile, providing a favorable prerequisite for further evaluation of the safety of this phage preparation for potential systemic administration. However, to confirm and extend these findings, future investigations should assess additional indicators of immune modulation, including cytokine and chemokine release (TNF-α, IL-1β, IL-6, IL-10), surface activation and polarization markers (CD11b, CD14, CD16, HLA-DR, CD80/86, CD206), and metabolic mediators such as nitric oxide and arginase activity. Integrating these parameters with time-resolved analyses of intracellular signaling pathways will help clarify whether Pyofag exerts subtle pro- or anti-inflammatory effects and establish its full immunological safety profile.

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

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ПРОДУКЦІЯ АКТИВНИХ ФОРМ КИСНЮ ТА ФАГОЦИТАРНА АКТИВНІСТЬ ФАГОЦИТІВ КРОВІ ЛЮДИНИ ПІД ВПЛИВОМ БАКТЕРІОФАГОВОГО ПРЕПАРАТУ ПІОФАГ

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Зростає застосування бактеріофагів у лікуванні, спричинених антибіотикорезистентними збудниками інфекцій, зумовлює необхідність з'ясування їх безпосереднього впливу на клітини вродженого імунітету. У цьому дослідженні вивчали вплив полівалентного бактеріофагового препарату Піофаг на оксидативний метаболізм і фагоцитарну функцію несенсибілізованих моноцитів і гранулоцитів периферичної крові за фізіологічних умов у цільній крові. Зразки крові здорових донорів відбирали з використанням літій-гепарину та інкубували з Піофагом у співвідношеннях фагів до клітин 1:2, 1:5 та 1:10; після чого центрифугували та використовували клітинний осад. Продукцію активних форм кисню (АФК) оцінювали з використанням DCFDA, а фагоцитарну активність – за інтенсивністю поглинання флуоресцентних полістирольних латексних частинок. Відсоток фагоцитуючих клітин і середній індекс флуоресценції (MFI) визначали методом проточної цитометрії. Показано, що Піофаг індукував статистично значуще помірне утворення АФК в обох популяціях фагоцитів лише за найвищої дози, при цьому рівень активації залишався значно нижчим порівняно з індукованим форбол-12-міристат-13-ацетатом. За дії Піофага спостерігали лише незначне зменшення частки фагоцитуючих моноцитів та незначне підвищення відсотка фаго-

цитуючих гранулоцитів без змін значень MFI. Помірна оксидативна активація та стабільні показники фагоцитарної активності фагоцитів цільної крові за дії Піофага свідчать про його незапальний, збалансований імуномодуючий профіль, що підтверджує безпечність цього бактеріофагового препарату для потенційного системного застосування.

Ключові слова: бактеріофаги, Піофаг, моноцити, гранулоцити, фагоцитоз, активні форми кисню, імуномодуляція.

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