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ORIGINAL RESEARCH

PEG-Polymeric Nanocarriers Alleviate the Immunosuppressive Effects of Free 4-Thiazolidinone-Based Chemotherapeutics on T Lymphocyte Function and Cytokine Production

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Purpose: Our study aimed to assess the effects of anticancer 4-thiazolidinone-based free water-insoluble therapeutics Les-3288 and Les-3833 and their waterborne complexes with branched PEG-containing polymeric carriers (A24-PEG550 and A24-PEG750) on immune response.

Methods: Human peripheral blood was used to study in vitro lymphocyte proliferative function, leukocyte phagocytic activity and respiratory burst, and cytokine production.

Results: The binding of the polymer to the anticancer drug Les-3288, which is intended to mitigate the immunosuppressive effects of the free drug on the proliferative activity of T lymphocytes and T-dependent B cells, demonstrated comparable efficacy for both A24-PEG750 and A24-PEG550 nanocarriers. Furthermore, it was observed that the drug-polymer complex significantly increased the reduced levels of IFN-γ and TNF-α resulting from free Les-3288. Conversely, the reduced levels of IL-6 and IL-4 remained unchanged. Administration of either form of Les-3288 had no effect on the phagocytic activity of monocytes, granulocytes or the respiratory burst of granulocytes. Due to the reduced cell viability and increased cytotoxicity associated with Les-3833, tenfold lower doses were selected for the immune assays. The effects of free Les-3833 on lymphocyte proliferative function resulted in significant stimulation of T-dependent B cells. The binding of Les-3833 to the smaller carrier, A24-PEG550 was found to maintain the stimulatory effect on B lymphocytes. While no effect of free Les-3833 on the granulocyte phagocytic activity was observed, binding of Les-3833 to both polymeric carriers resulted in a decrease in granulocyte phagocytic activity and respiratory burst, with no observable effect on monocytes. Monitoring of cytokine production showed no significant effect of either form of Les-3833 on the production of IFN-γ and IL-6. In the context of TNF-α and IL-4, the positive effect of polymer binding on restoring suppressed cytokine levels induced by the Les-3833 free drug was slightly more favorable for A24-PEG750.

Conclusion: The drug complexation with novel PEGylated carriers is a promising way for efficient therapeutic development.

Keywords: anticancer compounds, lymphocytes, phagocytic activity, respiratory burst, cytokines

Introduction

The poor water solubility of drugs has been a severe problem in the clinical application and development of efficient anticancer compounds. Many highly active and promising new substances are rejected because of their low solubility. The

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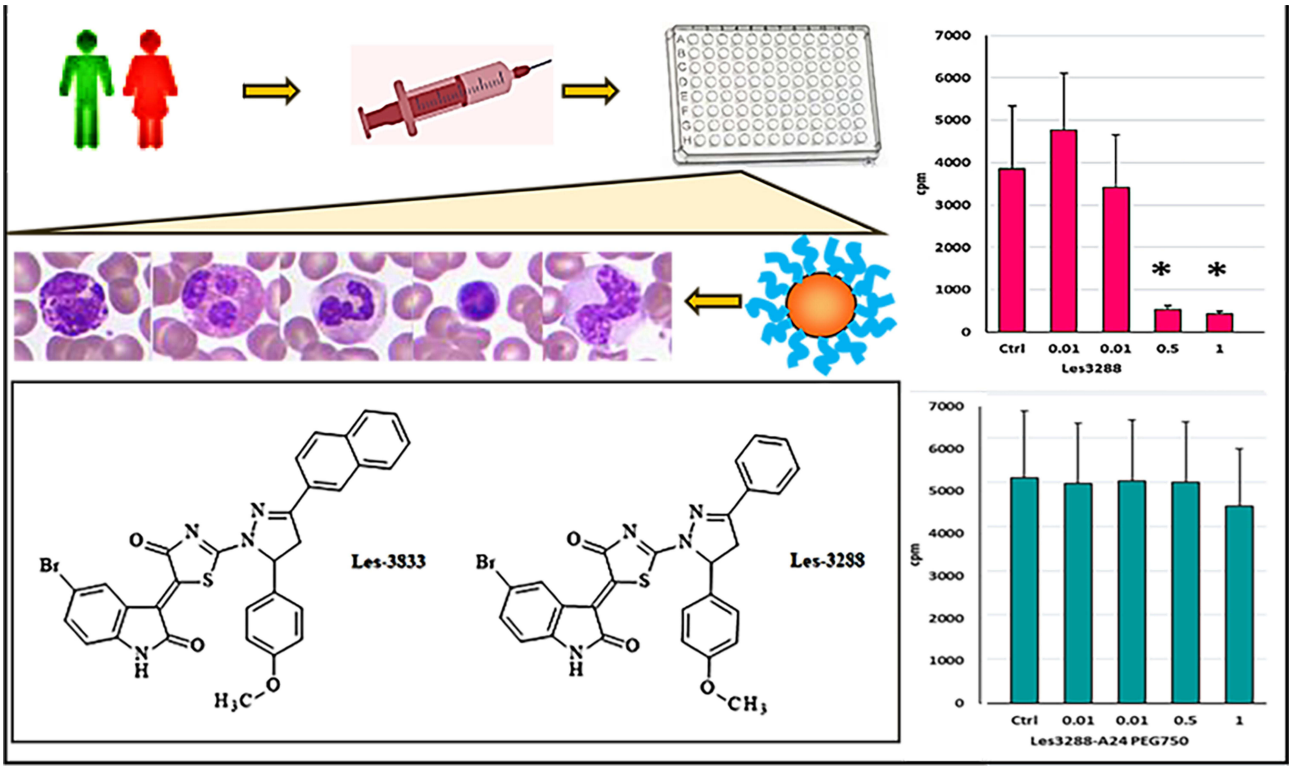
Graphical Abstract



nanobiotechnologies proposed as an alternative to the solvent-based drug solubilization for encapsulation and delivery of the existing and new poorly water-soluble anticancer drugs might be lipid-based, polymer-based, and albumin-based.[1,2] Nanomaterial-based drug design and development are priority tasks in modern pharmaceutical research. The transition from macro- to nanosized particles is accompanied by a change in the interatomic distances and periods of the crystal lattice, which causes the appearance of new properties of the nanostructures. The nanoparticles more easily penetrate the cells of tissues and organs of the human body and are biologically more active through a large surface area per unit of mass, compared to the macro-sized particles. Particle size <100 nm in diameter is considered to be optimum for in vivo application and biodistribution.[3] The excessive reduction of particles' size is undesirable because nanoparticles sized <50 nm are more actively absorbed by the liver and spleen, which leads to increased toxicity, and the extremely small nanoparticles (<5 nm) are intensively removed by diffusion in the kidney. Despite the low quantity of conducted clinical trials and the fact that only one formulation has gotten the FDA approval, the surface-active polymeric carriers including functional polymer coated water-insoluble drug nanoparticles have a huge potential in cancer therapy, being the second ones in studies after using the conventional platforms like "liposomes".[4] The major challenges in developing safe nanoscale carriers are: a) low toxicity; b) physical stability in blood vessels; c) compatibility with body metabolites; d) controlled effect on cell damage; e) the potential to improve targeted delivery of anticancer drugs to the tumor.[5] The nanocarriers should be designed with high tumor selectivity and slow release of the active cytotoxic compound, which reduces systemic toxicity of the drug and improves its distribution and circulation time in the body. The biocompatibility of nanomaterials in vivo strongly depends on the lack of their immunogenicity. Ideally, the nanocomposites should have minimal immunogenic activity.

Polyethylene glycol (PEG) conjugation is a rapidly evolving strategy to solve hurdles in therapeutic delivery and is being used as an add-on tool to traditional drug delivery methods.[6] Successful PEGylation can amend the pharmacokinetic and pharmacodynamic outcomes of therapeutics. Specifically, the primary interest is in their ability to decrease uptake by the reticuloendothelial system, prolong blood residence, decrease degradation by metabolic enzymes and reduce protein

immunogenicity.[6] PEGylation involves modification of the therapeutics by linking one or more PEG molecules to it. The metabolism of PEG-based hydrogels is correlated with their molecular weight, where PEG-nanocarriers with lower molecular weights demonstrate higher metabolism performance and vice versa.[7] In nano-drug-delivery systems, the hydrophobic chains in amphiphilic copolymers entrap hydrophobic drugs, which form the inner core, while the hydrophilic chains form the outer shell, thus contributing to aqueous solubility.[7] During the formation of these amphiphilic copolymers, PEGs are applied as hydrophilic portions owing to their excellent aqueous solubility, biosafety, and stealthy properties.[7] The structure or components of amphiphilic copolymers can influence their particle size, surface charge, morphology, and other physico-chemical properties, thus inducing different therapeutic effects.[7,8] Hand in hand with the benefits of PEGylation as the gold standard for surface modification, the unanticipated immune reactions against PEG-nanocarriers have been reported. The production of anti-PEG antibodies at the first injection, along with the subsequent activation of accelerated blood clearance (ABC) and hypersensitivity reactions is known as complement activation-related pseudoallergy (CARPA).[9] This represents one of the key motivations for the investigation of PEGylated polymer carriers, particularly focusing on the synthesis of novel PEG-containing polymer structures, as well as the size and morphology of micelle-like complexes loaded with pharmaceuticals. To systematically compare and study the effects of the structure and components of nanocarriers, in this study, two different PEG derivatives were applied as nanocarriers to prepare nano-drug-delivery system, in which hydrophobic Les derivatives were selected as a model drug. In our study, we focused on the potential benefits of PEGylation, such as enhanced solubility and reduced immunosuppressive effects of Les-3288 and Les-3833. In addition to the effectiveness of the anticancer drug, it is important to determine not only their toxic effects at cellular and tissue levels but also the biosafety of the action of the applied drugs in the treated organism.[10] It is known that adverse reactions of different severity may accompany the effect of drugs with high biological activity. Thus, a critical requirement of the applied drugs is the optimal balance between their efficacy (therapeutic effect) and toxicity.[11] Thus, searching for new ligands or targeting moieties needed to drive the drug carrier to specific organs or tumors to achieve the site-specific delivery of the chemotherapy-containing nanocarriers to cancerous tissue is a great task in oncopharmacology. The drug carriers capable of circumventing such mechanisms are of special importance. Another innovation needed to improve the PNCs is to achieve a release of the anticancer agents by stimuli-sensitive carriers in a controlled way under the microenvironment conditions. At using anticancer drugs, many problems that are directly related to negative side effects of their action in the body can appear. The unaddressed drug delivery mechanism leads to undesirable effects on the body. If one can address the drug's action, its acting concentration could be reduced significantly, which would minimize its adverse side effects.

In our previous studies, we reported the design and synthesis of heterocyclic hybrid molecules containing 4-thiazolidinone, 2,3-dihydro-1H-indol-2-one, and pyrazoline moieties.[12] Among this series of pyrazoline-thiazolidinone-isatin hybrids, we determined that Les-3833 was the most active compound towards in the non-small-cell lung cancer cell line HOP-92, colon cancer line HCT-116, CNS cancer cell line SNB-75, ovarian cancer cell line NCI/ADR-RES, and renal cancer cell line RXF 393.[13] In biological studies, we compared Les-3833 with a less active compound from the same group – Les-3288 (Figure 1).

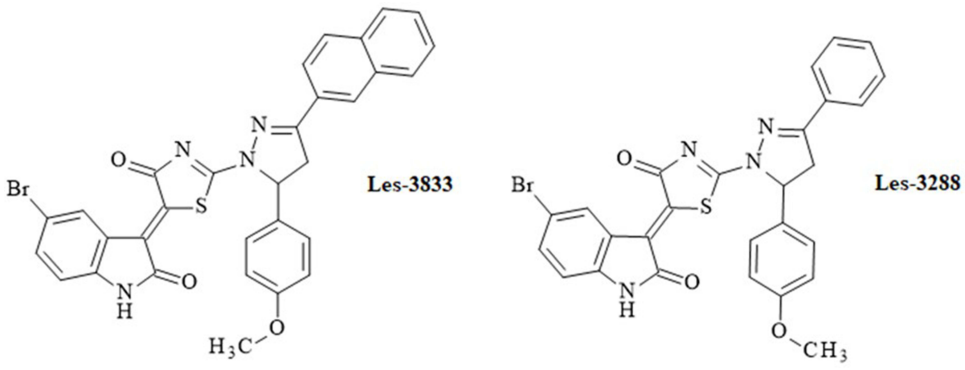


Figure 1 Structure of the Les-3288 and Les-3833 molecules.

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The thiazolidinone-based compounds were successfully tested as promising anticancer therapeutics in vitro and in vivo in free form in DMSO solution and in the waterborne delivery system after complexing with novel branched copolymers containing side PEG chains of different lengths. Previous studies of the complexes of these carriers with water-soluble (Doxorubicin) and water-insoluble (KP1019), thiazolidinone derivatives,[14–18] thiazole,[19–21] as well as kinase inhibitor based on chlorine benzene derivative of maleimide[13] revealed their non-toxicity, stability and enhanced therapeutic efficiency. We demonstrated that 4-thiazolidinone derivatives (Les-3288, Les-3833, and Les-3882) showed less general toxicity compared with Dox in the experimental animals, as demonstrated by the measured biochemical parameters.[12,22] Thus, the binding of an antitumor drug with a PNCs and drug application in the form of a stable water delivery system can reduce the toxic effects in the animals, compared with the action of these substances in free form.[23,24]

PEGylated nanocarriers (PNCs) loaded with 4-thiazolidinone derivatives for anticancer therapy administered via injection will inevitably encounter the human immune system. Aware of all the pros and cons associated with PNCs, we aimed to investigate some other aspects of nanoparticle-induced modulation of immune responses. We have had a strong motivation to explore the response of human peripheral blood cells to the first interaction with the free antitumor drugs or their complexes with PNCs and to provide new insights into the immunosafety of systems under investigation to address new challenges in our research. The aim of our study was to assess the effect of 4-thiazolidinone derivatives (compounds Les-3288 and Les-3833) and their complexes with PNCs on the function of human lymphocytes, phagocytic activity and respiratory burst of leukocytes and in vitro production of cytokines.

Materials and Methods

Anticancer Drugs

The 4-thiazolidinones derivatives (compounds Les-3288 and Les-3833, Figure 1) were synthesized at the Department of Pharmaceutical, Organic and Bioorganic Chemistry of Danylo Halytsky Lviv National Medical University, Ukraine, as previously described.[12]

Before use in cell culture, these compounds were dissolved in dimethyl sulfoxide (DMSO, Arterium, Lviv, Ukraine). The solution was additionally kept for 5 min in a boiling water bath and diluted in distilled water in order to reach the working concentrations. The final concentration of the DMSO in culture medium was below 0.1%. Doxorubicin (Dox) was bought in a local pharmacy from a Pfizer (Italy) representative in Ukraine.

Polymeric Nanocarrier (PNC)

The PNCs for drug delivery were synthesized at the Department of Organic Chemistry of Lviv Polytechnic National University (Ukraine), using a methodology described earlier.[25] PEGylated branched polymeric carriers were synthesized via two approaches, namely: 1) interaction of monomethyl substituted PEG (mPEG) with side epoxide groups of the backbone copolymers of glycidyl methacrylate (PNC: A24-PEG550, A24-PEG750) and 2) copolymerization of PEG-methacrylate (PEGMA) with dimethyl maleate (DMM) (PNC: PEGMA-DMM). The structures and some characteristics of Branched PEG-containing carriers are presented on Figure 2 and were previously described.[25–28] The polymer structures were confirmed by functional analysis[29] and NMR spectroscopy.[30] All NMR spectra were recorded in the DMSO-d[6] on a Bruker AV300 NMR spectrometer operating at a frequency of 300 MHz (Billerica, MA). Molecular weights of the polymers were measured using

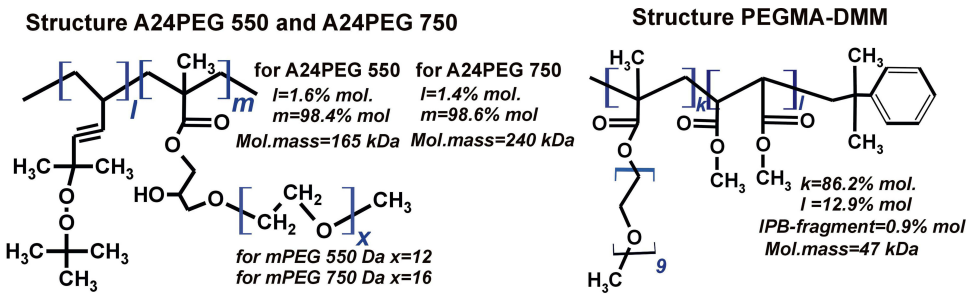

Figure 2 Structure of the polymeric nanocarriers (PNCs) A24-PEG550, A24-PEG750 and PEGMA-DMM.

size exclusion chromatography (SEC) with Waters 150°C chromatograph with a built-in RI detector (Waters Corporation, Milford, MA), a Shodex 602 column (Kawasaki, Japan), the flow rate varied (0.5 and 2.5 $cm^3 \cdot min^{-1}$).

To prepare the water PNC solutions, 0.09 g of PNC was dissolved in 0.9 mL DMSO. This PNC solution was added to 8.0 mL of 0.9% NaCl solution. Then, the solution was stirred for 0.5 h and sonicated for 10 s. A high-frequency UZDN-1A (Ukrrospribor Ltd, Sumy, Ukraine) unit was used for ultrasonic treatment with transducer for 22 kHz frequency, the applied power was 40 W. In aqueous solutions, the amphiphilic PNC forms micelle-like structures that can solubilize water-insoluble compounds (eg, drugs) or these compounds can be adsorbed onto the surface of the nanoparticles, thereby increasing their water dispersibility and biocompatibility.

To prepare water drug delivery systems the mixture of water-insoluble derivatives of 4-thiozolidinone and PNCs in DMSO solution was dropped into saline water solution as follows: 0.045 g of PNC was dissolved in 0.15 mL of DMSO, and 0.0015 g of Les-3288 (or Les-3833) was dissolved in 0.10 mL of DMSO. The solutions of PNCs and 4-thiazolidinones were mixed and added to 4.25 mL of 0.9% NaCl solution and sonicated for 10 s as described above.

The dispersions of complexes of the PNC with 4-thiazolidinone derivatives are highly stable and protected from aggregation and sedimentation by the adsorbed PNC shell on the thiazolidinone nanoparticle surface. Changes in sizes of the nanoparticles dispersed in the water system are negligible at multiple dilutions with water, as well as after 6 months of aging of the water systems of complexes of PNC with 4-thiazolidinones.[17] To confirm the stability of the dispersions, the sizes of the colloidal structures were measured every day for 2 weeks and then every 5 days for 6 months. Figure 3A shows the results of measuring the hydrodynamic parameters of micellar structures formed by PNC, as well as PNC-drug colloidal structures. Figure 3B shows the results of studying the stability of aqueous PNS-drug colloidal structures.

Table 1 presents some colloidal chemical characteristics of polymer carriers and their complexes with drugs. The size of polymer micelles and polymer complexes with drugs were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments GmbH, Stuttgart, Germany) and DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA, USA).

The size and morphology of PNC micelles as well as of PNC + drug nanoparticles were studied using a transmission electron microscope JEM-200A (JEOL, Japan) at an accelerating voltage of 200 kV. A Zeiss Supra 40/40VP scanning electron microscope (Carl Zeiss Group, Germany) was also used in this study. Zeta potential of the PNC micelles as well as of PNC + drug nanoparticles were measured by electrophoretic light scattering using the Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK).

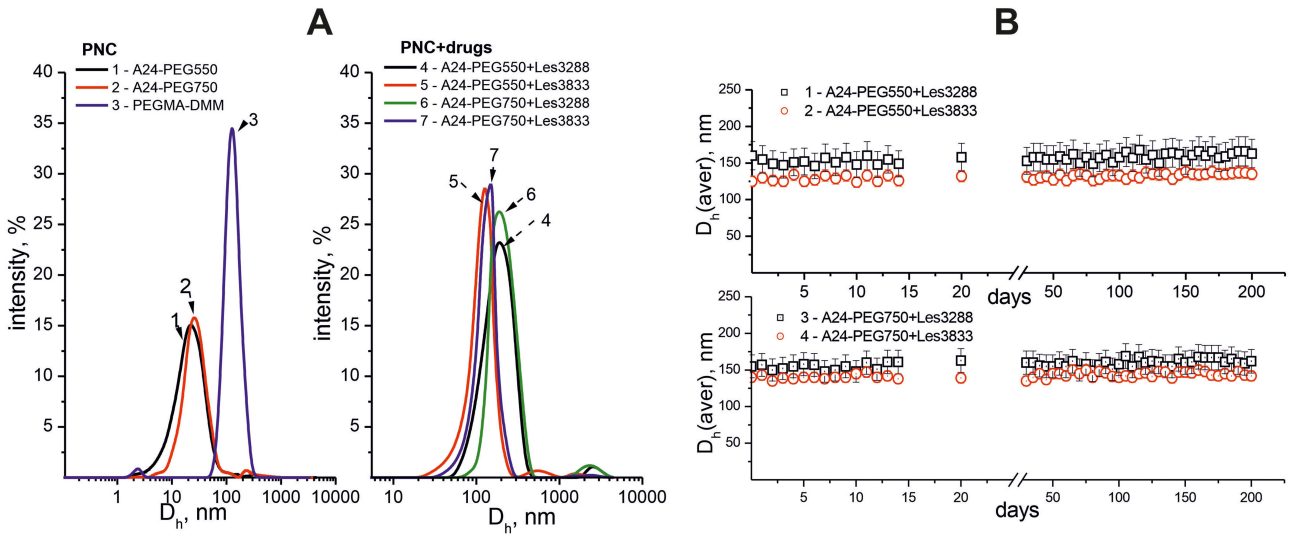

Figure 3 (**A**) Hydrodynamic diameters of colloidal structures formed by polymeric nanocarriers (PNCs) (10 mg/mL) (1,2,3) and PNC+drug (10 mg/mL + 0.3 mg/mL) (4–7): 1- A24-PEG550; 2- A24-PEG750; 3- PEGMA-DMM; 4- A24-PEG550 + Les-3288; 5 - A24-PEG550 + Les3833; 6- A24-PEG750 + Les-3288; 7 - A24-PEG750 + Les-3833; Dh, nm - hydrodynamic diameter (nm). (**B**) Dependence of the particle size of colloidal structures on the storage time of aqueous dispersions; Dh(aver), nm - hydrodynamic diameter (nm).

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Table 1 Colloidal Chemical Characteristics of Polymeric Nanocarriers (PNCs) and Complexes Les-3288+PNC and Les-3833+PNC in Waterborne Systems

Nature of the Polymer Carrier and Drug	Z-Average Hydrodynamic Diameter (nm) DLS (± SD)	Average Diameter (nm) TEM (± SD)	Zeta Potential (mV)
A24-PEG550	42 ± 28	32 ± 7	+0.08
Les-3288+A24-PEG550	155 ± 38		−0.22
Les-3833+A24-PEG550	130 ± 22		−0.25
A24-PEG750	50 ± 25	35 ± 6.5	+0.11
Les-3288+A24-PEG750	155 ± 30	130 ± 18	−0.56
Les-3833+A24-PEG750	140 ± 25	120 ± 17	−0.35
PEGMA-DMM	150 ± 40	90 ± 16	−0.15

Abbreviations: DLS, dynamic light scattering; TEM, transmission electron microscopy; SD, standard deviation.

The dispersions of complexes of the PNC with 4-thiazolidinone derivatives are highly stable and protected from aggregation and sedimentation by the adsorbed PNC shell on the thiazolidinone nanoparticle surface. The changes in sizes of the nanoparticles dispersed in the water system are negligible at multiple dilutions with water and after 6 months of aging of the water systems of complexes of PNCs with 4-thiazolidinones.

Preparation of Test Substances for in vitro Studies

All test substances for in vitro studies were prepared by the Department of Organic Chemistry of Lviv Polytechnic National University (Ukraine). Les-3288 and Les-3833 free drugs, solutions in DMSO were delivered at a concentration of 0.3 mg/mL. Stock solutions of Les-3288 drug complexes were prepared in the following concentrations: A24-PEG550 (polymer 10 mg/mL) + Les-3288 (0.3 mg/mL) and A24PEG750 (polymer 10 mg/mL) + Les-3288 (0.3 mg/mL). Stock solutions of Les-3833 drug complexes with polymers were prepared analogously: A24-PEG550 (polymer 10 mg/mL) + Les-3833 (0.3 mg/mL) and A24-PEG750 (polymer 10 mg/mL) + Les-3833 (0.3 mg/mL). Pure PNCs were delivered as follows: A24-PEG550 – drug-free control – 10 mg/mL, A24-PEG750 – 10 mg/mL and PEGMA-DMM – 10 mg/mL. After a few minutes of vortex-shaking, all samples were diluted 300 times in RPMI 1640 complete medium (without serum) to obtain concentration of 1 μg/mL (drug)/33 μg/mL (polymer). Consequently, a series dilution was performed to obtain final concentrations (1 μg/mL – 0.005 μg/mL, drug) and (33 μg/mL – 0.165 μg/mL, polymer), respectively. Before each experiment, fresh solutions/dispersions were prepared. Before mixing them with cell culture, solutions/dispersions were vortexed (BioTech, CZ) and immediately added to the heparin blood.

Collection of Blood

A licensed staff member collected peripheral venous blood from fasting adult volunteers into vacuum collection tubes containing heparin. The volunteers were in good health and screened by a doctor for any known immune disorders or acute infections. Each participant signed a written consent form and received information about the blood donation for research purposes.

Assessment of Endotoxin Contamination

Endotoxin contamination in free drugs dissolved in DMSO, complexes with PNCs, and pure PNCs was evaluated using a commercial chromogenic Limulus Amoebocyte Lysate (LAL) assay (BIOENDO), following the manufacturer's instructions. Preliminary controls were conducted to determine any potential interference from the test substances with the assay results. A calibration curve was created based on the absorbance values of the standards measured at a wavelength of 545 nm. No endotoxin was detected in any of the samples, and the assay sensitivity was 0.01 EU/mL.

Separation of Peripheral Blood Mononuclear Cells

Lymphocyte Separation Medium (LSM) LymphoSep™ (Biosera) was used for in vitro isolation of peripheral blood mononuclear cells (PBMC) from peripheral blood donated by adult volunteers. Procedure was provided according to the user manual. Briefly, heparinized blood was diluted with Phosphate Buffered Saline (PBS, Oxoid) at a 1:1 ratio, carefully layered over the separation medium and centrifuged at 800 × g for 30 min. After the formation of the PBMC layer, it was aspirated, transferred and resuspended in PBS and centrifuged for 10 min at room temperature at 160 × g. PBMCs obtained by repeated centrifugation were resuspended in the culture medium and used for the assessment of the cell viability.

Cell Viability

The RealTime-Glo™ MT Cell Viability Assay (Promega) was utilized to assess the cell viability of 4-thiazolidinone compounds. Luminescence was measured after a 72-hour exposure of isolated human peripheral blood mononuclear cells (PBMC) to the selected 4-thiazolidinone compounds, luminescence was measured. The results were presented as a percentage of cell viability compared to control untreated cells, which served as a baseline for comparison.

Proliferative Activity of Lymphocytes

The proliferative activity of lymphocytes was evaluated using liquid scintillation. Human peripheral blood was stimulated in vitro with the T cell mitogen phytohemagglutinin (PHA, Merck) and the T-dependent B cell mitogen pokeweed (PWM, Merck). Radioactive 3H-methyl-thymidine (Hartmann Analytics) was incorporated into the DNA of newly synthesizing cells. The radioactivity of these proliferating cells was quantified as counts per minute (cpm).

Specifically: Heparinized human blood (n = 6) was diluted 1:15 in complete RPMI 1640 medium (Merck), supplemented with 10% fetal calf serum (FCS, Merck), L-glutamine (Merck), and gentamycin (Sandoz). The diluted blood was dispensed in a volume of 150 µL in three technical replicates into the wells of a 96-well microtiter culture plate. PHA (25 µg/mL) and PWM (2.5 µg/mL) were each added to the different wells (25 µL per well) to stimulate lymphocyte proliferation. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

After an initial 24-hour incubation period, test compounds including Les-3288, Les-3833 (free drug in DMSO) and their complexes with A24-PEG550 and A24-PEG750, as well as polymer nanocapsules (PNCs) of A24-PEG550, A24-PEG750 and PEGMA-DMM were added to the cultures (25 µL per well). Controls to which the treated cultures were compared were supplemented with an appropriate volume (25 µL per well) of RPMI 1640 medium. In addition, Dox (0.1 mg/mL, Medac) was used as a cytotoxic control to assess the suppression of lymphocyte proliferation. The cultures were then incubated for an additional 48 hours, increasing the total incubation time to 72 hours.

Twenty-four hours before the end of the incubation, cultures were pulsed with 1 µCi of 3H-methyl-thymidine (Perkin Elmer) in 20 µL of medium to measure DNA synthesis. After the full 72-hour incubation, the cells were harvested onto glass fiber filter paper (Perkin Elmer), which was subsequently placed in scintillation fluid (Perkin Elmer) to measure radioactivity. Results were reported as counts per minute (cpm) in triplicate for each condition. Additionally, potential interference from the test substances was assessed by measuring cpm in cultures with the corresponding concentrations added to the wells 5 minutes before harvesting the cells.

Phagocytic Activity and Respiratory Burst of Leukocytes

The phagocytic activity of monocytes and granulocytes, as well as the respiratory burst of granulocytes, was assessed using FITC-labelled opsonized *Staphylococcus aureus* bacteria (SPA-FITC) and hydroxyethidium bromide (substrate for the assessment of respiratory burst), following the previously described flow cytometry protocol.[31] Peripheral blood from adult voluntary donors was used for this purpose. Heparinized peripheral blood (n = 8) diluted in a 1:1 ratio in complete RPMI 1640 medium (Merck) containing 10% FCS (Merck), L-glutamine (Merck), and gentamycin (Sandoz) were allocated in three technical replicates into the 96-well microtiter culture plate wells (175 µL per well) under a sterile condition. The cultures were exposed to 25 µL of test substances (free drugs and complexes as stated in Proliferative activity of lymphocytes) in the well and incubated at 37°C in a 5% CO2 atmosphere for 24 hours. Culture medium in volume of 25 µL was introduced as

a negative control, Doxorubicin (0.1 mg/mL, Medac) in the same volume served as a cytotoxic immunosuppressive control. After 24-hour incubation, 30 µL of blood exposed was transferred to 3 tubes (1 tube labelled control and 2 tubes labelled test – technical duplicates) and 10 µL of hydroxyethidium bromide (diluted in a 1:100 ratio in complete RPMI 1640 medium without serum; Polysciences) was added. Vortex-mixed (Bio Tech) tubes were incubated (Jouan) at 37°C for 15 min in a water bath. Following the 15-min incubation, a standardized amount of SPA-FITC (3 µL, 1.4×10^6 per test; Molecular Probes) was pipetted only into tubes referred to as test. All vortex-mixed tubes were incubated in the water bath for an additional 15 min at 37°C, then quickly transferred onto ice, and 800 µL of ice-cold lysis solution was added to each tube to stop phagocytosis. Moreover, 3 µL of SPA-FITC was pipetted only into tubes labelled control. After vortex-mixing and 10-min lysis of erythrocytes, the phagocytic activity as well as the respiratory burst were measured using a Cytomics FC 500 flow cytometer (Beckman Coulter). Three thousand cells were analyzed. The results are presented as the percentage of phagocytic cells and the percentage of granulocytes exhibiting a respiratory burst.

In vitro Production of Cytokines

Human heparinized blood (n = 8–9) was diluted 1:15 in complete RPMI 1640 medium (containing 10% FCS), and PHA at a concentration of 25 µg/mL was added. The mixture was then incubated at 37°C for 72 hours. Nanoparticle dispersion was introduced 24 hours into the 72-hour incubation period. After the full incubation period, supernatants from three technical replicates of cell cultures were collected and stored at −70°C. The concentrations of IFN-γ, TNF-α, IL-6, and IL-4 in the cell supernatants were measured in two technical replicates using ELISA kits, following the manufacturer's instructions (Biolegend).

Statistical Analysis

Statistical analysis was conducted using SPSS software. Results are presented as the mean with the standard error of the mean (SEM). Measurements from each individual were averaged and used as a single value for analysis. The Shapiro–Wilk test was utilized to assess the normality of the data distribution. The Paired *t*-test was employed for normally distributed datasets, while the Wilcoxon test was used for non-normally distributed datasets to evaluate differences between the exposed and control groups. Differences were considered statistically significant at $p < 0.05$. Statistical significance is denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Results

Cell Viability

Cell viability was measured using luminescence method in human PBMC in vitro exposed to PNCs and 4-thiazolidinone compounds for 72h. Results were expressed as percentage of control untreated cells.

The effects of Les-3288 either in free-form or conjugated with PNCs are depicted in Figure 4A. Cell viability of Les-3288 free compound – solution in DMSO is comparable to that bound to the PNCs A24-PEG550 and A24-PEG750. One

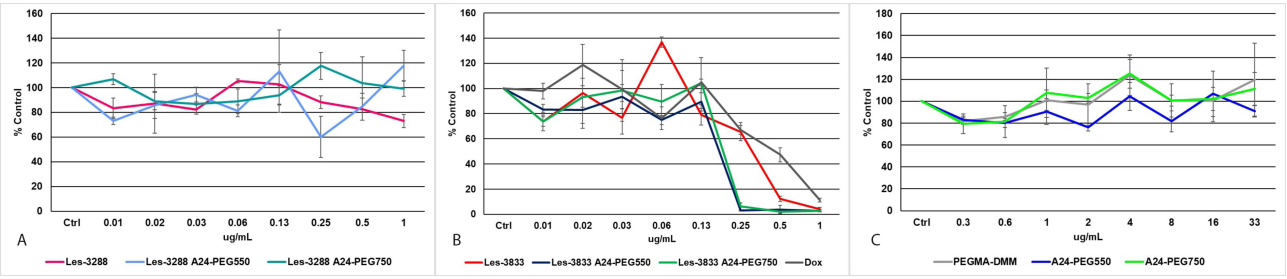

Figure 4 The effect of binding Les-3288 and Les-3833 to the polymeric nanocarriers, and polymeric nanocarriers themselves on cell viability. Viability of cells was measured using luminescence method. Human peripheral blood mononuclear cells were exposed for 72h to (**A**) Les-3288 – free drug in DMSO, complex Les-3288 A24-PEG550 and complex Les-3288 A24-PEG750; (**B**) Les-3833 – free drug in DMSO, complex Les-3833 A24-PEG550 and complex Les-3833 A24 PEG750; (**C**) polymeric nanocarriers PEGMA-DMM, A24-PEG550 and A24-PEG750; Ctrl – control. Data are depicted as % of Control using line graphs. Points indicate mean cell viability ± SD; SD - standard deviation.

low value of cell viability (60%) occurred in cell cultures exposed to Les-3288 bound to the PEG nanocarrier A24-PEG550. As the low value was measured in a single concentration 0.25 µg/mL only and the viability of cells exposed to the lower (113%) and higher concentrations (85%, 118%) was high, value was considered as the outlier value.

Cell viability of compound Les-3833 is shown in Figure 4B. Les-3833 either in free-form or conjugated with PNCs displayed more significant cytotoxicity when applied to the cells in comparison with other 4-thiazolidinone derivative Les-3288. Cytotoxicity was highly pronounced in concentrations 0.25 µg/mL - 1 µg/mL and similar to that found in cultures administered with Dox (Figure 4B).

At the end of the cell viability assays, we determined the range of concentrations that will be applied in further tests with a view to use non-cytotoxic concentrations of administered substances in functional immune assays. Concentrations were determined as follows: 0.01–1 µg/mL for 4-thiazolidinone derivative Les-3288 free substance and compounds with polymers (PNC 0.33–33 µg/mL); range 0.005–0.1 µg/mL for Les-3833 and its polymeric compounds (PNC 0.165–3.3 µg/mL) and 0.33–33 µg/mL for pure PNC.

Cell viability of PNCs PEGMA-DMM, A24-PEG550 and A24-PEG750 is given in Figure 4C. Synthesized PNCs showed high cell viability within the selected range of concentrations 0.33–33 3g/mL. All three forms are not cytotoxic up to the highest tested concentration 1 µg/mL (threshold 70%).

Proliferative Activity of Lymphocytes

The proliferative activity of T lymphocytes and T-dependent B cell response were assessed in human peripheral blood. The incorporation of radioactive ^{3}H-methyl-thymidine into the DNA of proliferating cells was measured as counts per minute.

The Effect of Binding Les-3288 to the PNCs

Administration of Les-3288 free drug to the peripheral blood cultures significantly suppressed T lymphocyte and T-dependent B cell response (Figure 5) in middle and high dose applied (0.5–1µg/mL). Binding of Les-3288 drug to both pegylated nanocarriers (A24-PEG550 and A24-PEG750) significantly improved the ability of lymphocytes to respond to mitogens. Dramatic reduction in toxicity was manifested as an increase in activity of T lymphocytes from 53%, 24% (Les-3288) up to 95%, 85% of control (Les-3288 A24-PEG550) and 95%, 85% of control (Les-3288 A24-PEG750), respectively (Figure 5). Similar stimulating effect on T-dependent B cell response was observed. Les-3288 drug-suppressed immune response (14%, 11%) increased to control level due to the binding of both polymer nanocarriers (96%, 91% and 98%, 87%), respectively (Figure 5).

The Effect of Binding Les-3833 Drug to the PNCs

Due to the high cytotoxicity of Les-3833 free substance and compound complexes with nanocarriers, 10 times lower concentrations were administered to the peripheral blood cell cultures for immune assays.

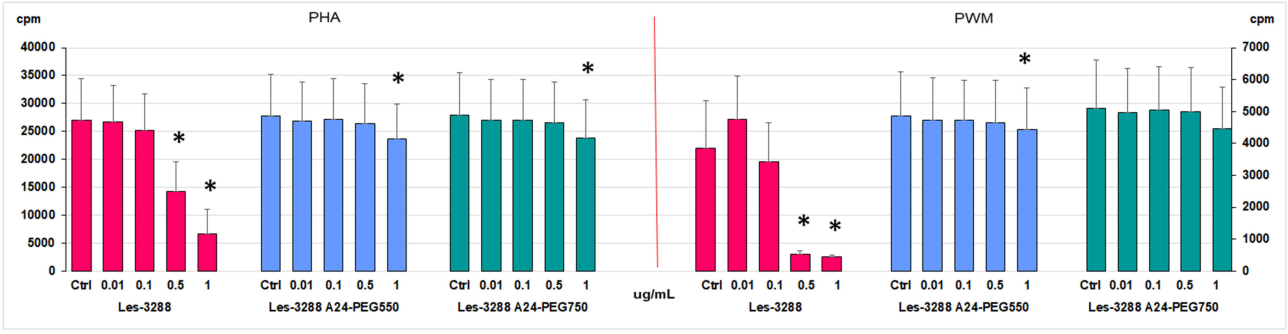

Figure 5 The effect of binding Les-3288 to the polymeric nanocarriers on proliferative activity of blood lymphocytes. The proliferative activity of lymphocytes in response to mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM) in the human peripheral blood in vitro exposed to Les-3288 – free drug in DMSO, complex Les-3288 A24-PEG550 and complex Les-3288 A24-PEG750 for 48h was measured as incorporation of [3H]-thymidine into replicating cells. Ctrl – control group (n = 6). Blood was exposed to 4 different concentrations: 0.01–1 µg/mL. Results are expressed as cpm (counts per minute)/ per culture. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean. Significance: *p < 0.05.

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T lymphocytes exposed to the middle concentration (0.05 μg/mL) of Les-3833 free drug in DMSO exhibited significant stimulation of the proliferation, while high concentration (0.1 μg/mL) significantly decreased activity (Figure 6). Differences were significant; however, we can consider them as a result of biological variability rather than biological relevance (102%, 96% of control). Surprisingly, a significant stimulating effect of Les-3833 free compound (125–134%) on T-dependent B cells was shown in peripheral blood cultures in vitro stimulated to pokeweed mitogen (Figure 6). Binding of Les-3833 to the smaller polymer carrier A24-PEG550 maintained incentive effect of compound on B lymphocytes.

The Effect of PNCs

Proliferative activity of T lymphocytes administered with PEGMA-DMM polymeric nanoparticles did not differ from unexposed controls. High concentration of both PEG-nanocarriers significantly suppressed T lymphocyte response (Figure 7). Regardless of the dose applied, no alterations in T-dependent B cell response were found in cultures exposed to PEG polymer coated nanoparticles. In case of PEGMA-DMM, low dose of PNC significantly decreased proliferative activity of T-dependent B cells (Figure 7).

Phagocytic Activity and Respiratory Burst of Leukocytes

Phagocytic activity of monocytes and granulocytes and respiratory burst of granulocytes was measured using *Staphylococcus aureus* and hydroxyethidium bromide by flow cytometry.

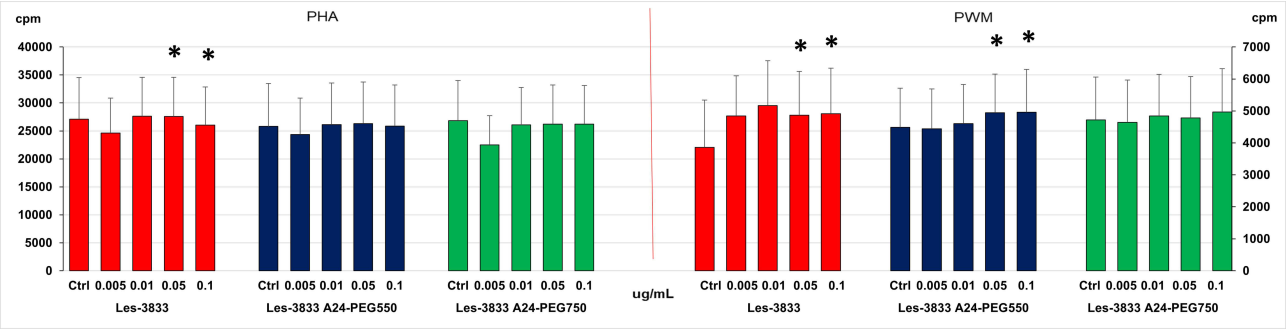

Figure 6 The effect of binding Les-3833 to the polymeric nanocarriers on proliferative activity of blood lymphocytes. The proliferative activity of lymphocytes in response to mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM) in the human peripheral blood in vitro exposed to Les-3833 – free drug in DMSO, complex Les-3833 A24-PEG550 and complex Les-3833 A24-PEG750 for 48h was measured as incorporation of [3H]-thymidine into replicating cells. Ctrl – control group (n = 6). Blood was exposed to 4 different concentrations: 0.005–0.1 μg/mL. Results are expressed as cpm (counts per minute)/ per culture. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean. Significance: *p < 0.05.

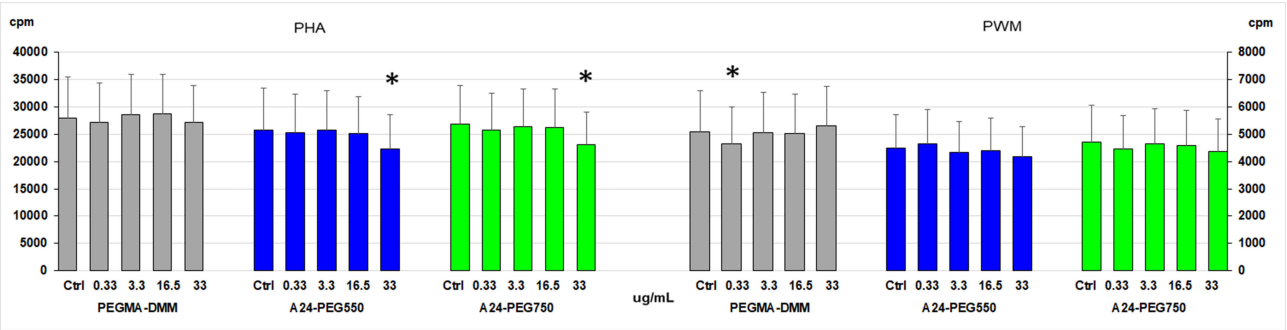

Figure 7 The effect of polymeric nanocarriers on proliferative activity of blood lymphocytes. The proliferative activity of lymphocytes in response to mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM) in the human peripheral blood in vitro exposed to polymeric nanocarrier PEGMA-DMM, A24-PEG550 and A24-PEG750 for 48h was measured as incorporation of [3H]-thymidine into replicating cells. Ctrl – control group (n = 6). Blood was exposed to 4 different concentrations: 0.33–33 μg/mL. Results are expressed as cpm (counts per minute)/ per culture. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean. Significance: *p < 0.05.

The Effect of Binding Les-3288 to the PNCs

Phagocytic activity of monocytes, granulocytes and respiratory burst of granulocytes derived from human blood and exposed to 4-thiazolidinone derivative Les-3288 and its complexes with PNCs A24-PEG550 and A24-PEG750 did not differ from unexposed control (Figure 8).

The Effect of Binding Les-3833 Derivative to the PNCs

No changes in phagocytic activity of granulocytes exposed to Les-3833 free substance were recorded (Figure 9). The effect of Les-3833 free substance on phagocytic activity of monocytes and respiratory burst of granulocytes manifested as random significant differences without clear dose-dependence. Binding Les-3833 drug to both PNCs resulted in a decrease in phagocytic activity of granulocytes and respiratory burst almost in whole range of concentrations tested (Figure 9).

The Effect of PNCs

No alterations were found in phagocytic activity of monocytes in peripheral blood cultures administered with non-cytotoxic concentrations of all tested PNCs (Figure 10). Phagocytic activity and respiratory burst of granulocytes

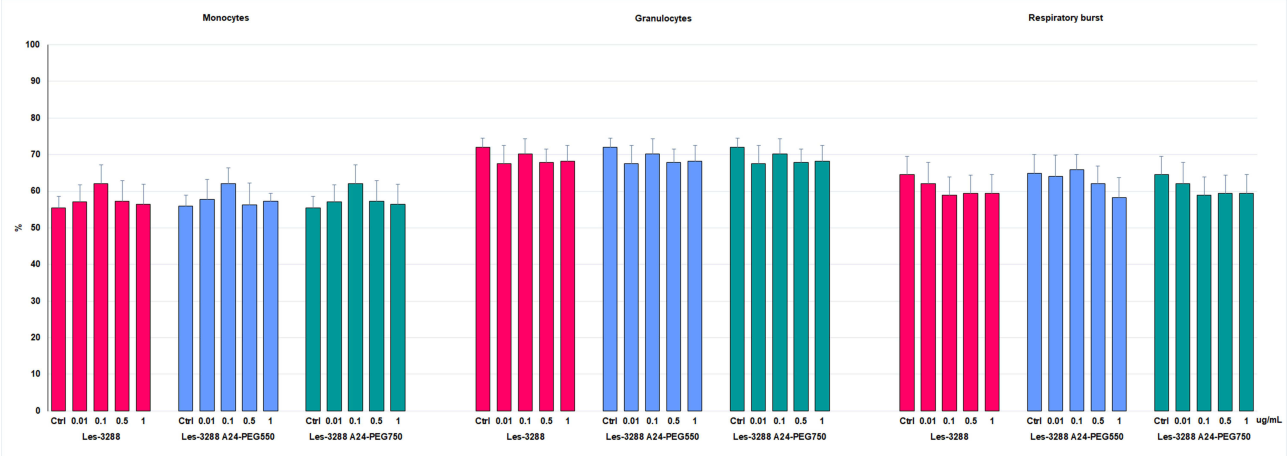

Figure 8 The effect of binding Les-3288 to the polymeric nanocarriers on phagocytic activity and respiratory burst of leukocytes. Phagocytic activity of monocytes, granulocytes and respiratory burst of granulocytes in the human peripheral blood (n = 8) in vitro exposed to Les-3288 – free drug in DMSO, complex Les-3288 A24-PEG550 and complex Les-3288 A24-PEG750 for 48h was evaluated using ingestion of fluorescein-labeled *Staphylococcus aureus*. Respiratory burst was monitored using hydroethidine by flow cytometry; Ctrl - control group. Blood was exposed to 4 different concentrations: 0.01–1 μg/mL. Results are expressed as the percentage of phagocytic activity and respiratory burst. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean.

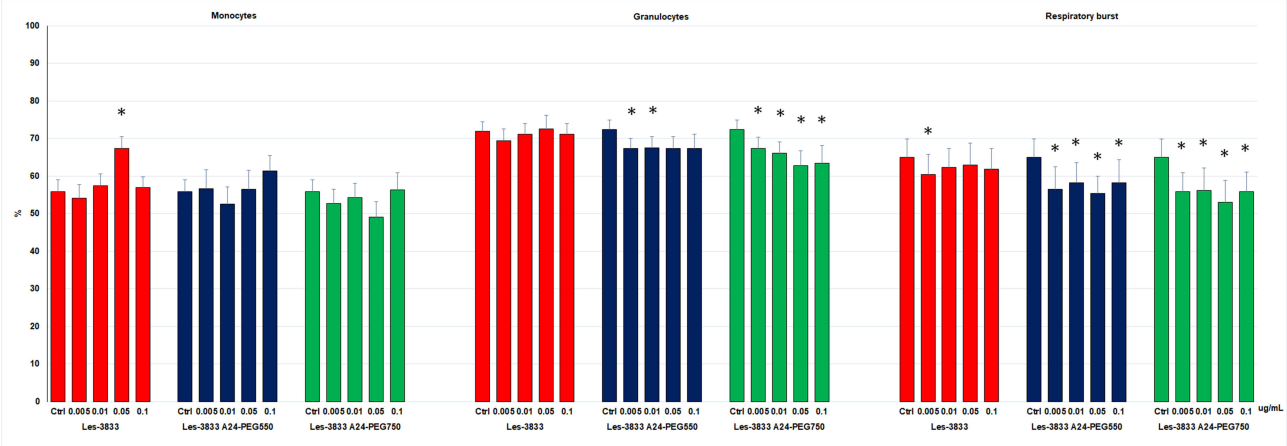

Figure 9 The effect of binding Les-3833 to the polymeric nanocarriers on phagocytic activity and respiratory burst of leukocytes. Phagocytic activity of monocytes, granulocytes and respiratory burst of granulocytes in the human peripheral blood (n = 8) in vitro exposed to Les-3833 – free drug in DMSO, complex Les-3833 A24-PEG550 and complex Les-3833 A24-PEG750 for 48h was evaluated using ingestion of fluorescein-labeled *Staphylococcus aureus*. Respiratory burst was monitored using hydroethidine by flow cytometry; Ctrl - control group. Blood was exposed to 4 different concentrations: 0.005–0.1 μg/mL. Results are expressed as the percentage of phagocytic activity and respiratory burst. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean. Significance: *p < 0.05.

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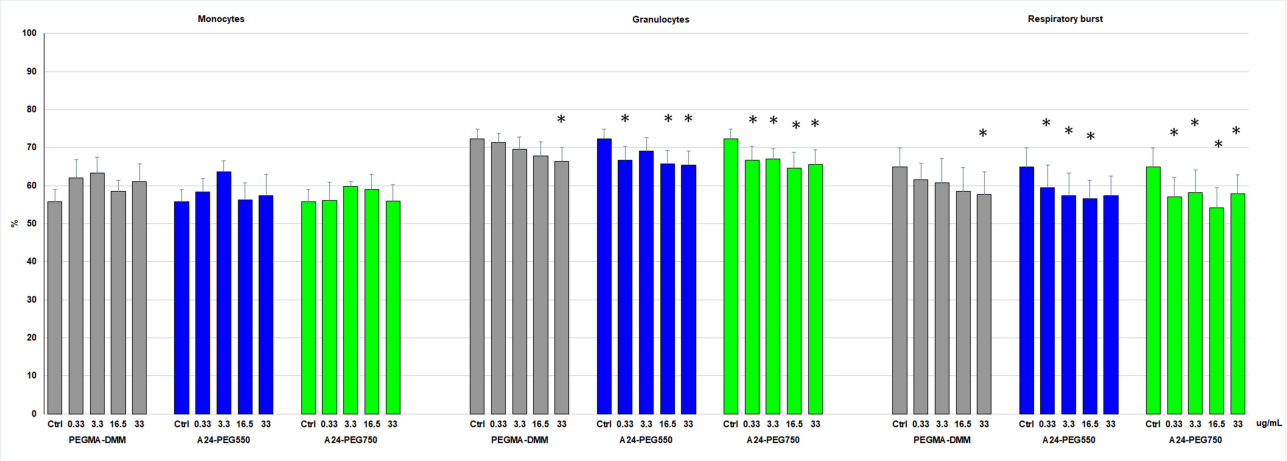

Figure 10 The effect of polymeric nanocarriers on phagocytic activity and respiratory burst of leukocytes. Phagocytic activity of monocytes, granulocytes and respiratory burst of granulocytes in the human peripheral blood (n = 8) in vitro exposed to polymeric nanocarrier PEGMA-DMM, A24-PEG550 and A24-PEG750 for 48h was evaluated using ingestion of fluorescein-labeled *Staphylococcus aureus*. Respiratory burst was monitored using hydroethidine by flow cytometry; Ctrl - control group. Blood was exposed to 4 different concentrations: 0.33–33 μg/mL. Results are expressed as the percentage of phagocytic activity and respiratory burst. Bars indicate the mean group activity of blood cells (mean + SEM); SEM - standard error of mean. Significance: *p < 0.05.

exposed to both pegylated nanoparticles were decreased in whole range of concentrations applied (Figure 10). The least affected cells were those with PEGMA-DMM nanopolymer; phagocytosis of granulocytes and respiratory burst were suppressed in high dosed cells only.

In vitro Production of Cytokines

The Effect of Binding Les-3288 Compound to the PNCs

In vitro production of cytokines IFN- TNF-α, IL-6 and IL-4 was measured using ELISA method in human blood cell culture supernatants exposed to PNCs and 4-thiazolidinone compounds. Results were expressed as percentage of control untreated cells. Original data on in vitro production of cytokines measured in pg/mL are shown in Supplementary material (Tables S1-S3).

The effect of Les-3288 free compound was manifested as a significant reduction in production of all cytokines at a high (sometimes medium) concentration (Figure 11). Binding of both PEG polymers significantly enhanced TNF-α production to control levels. The decrease in IFN-γ production was also improved by binding of molecule Les-3288 to the polymers, more significantly to PEG750. Levels of IL-6 and IL-4 reduced in cultures given Les-3288 free substance remained suppressed also after exposure to both Les-3288 PEG-containing compounds (Figure 11).

The Effect of Binding Les-3833 to the PNCs

No dramatic alterations between exposed and control cultures were found in levels of IFN- γ and IL-6 in supernatants exposed to the either Les-3833 free compound or complexes with PNCs (Figure 12). Isolated significances did not exceed a deviation of 5% from the control and represent rather biological variability. The decrease in TNF-α production found in cultures exposed to Les-3833 free compound was improved by binding of molecule Les-3833 to the polymers, more significantly to PEG750. Significant suppression in the production of cytokine IL-4 was observed in cultures exposed to Les-3833 free substance in whole range of concentrations tested (Figure 12). Binding of drug to the PEG750 polymer significantly improved the production of IL-4 comparable to control levels.

The Effect of PNCs

The effect of PEGMA-DMM nanopolymer was the most pronounced as a dose-dependent suppression of IL-6 production (Figure 13). Levels of TNF-α were significantly diminished in culture supernatants exposed to high concentration only. Production of IFN- γ and IL-4 was not affected.

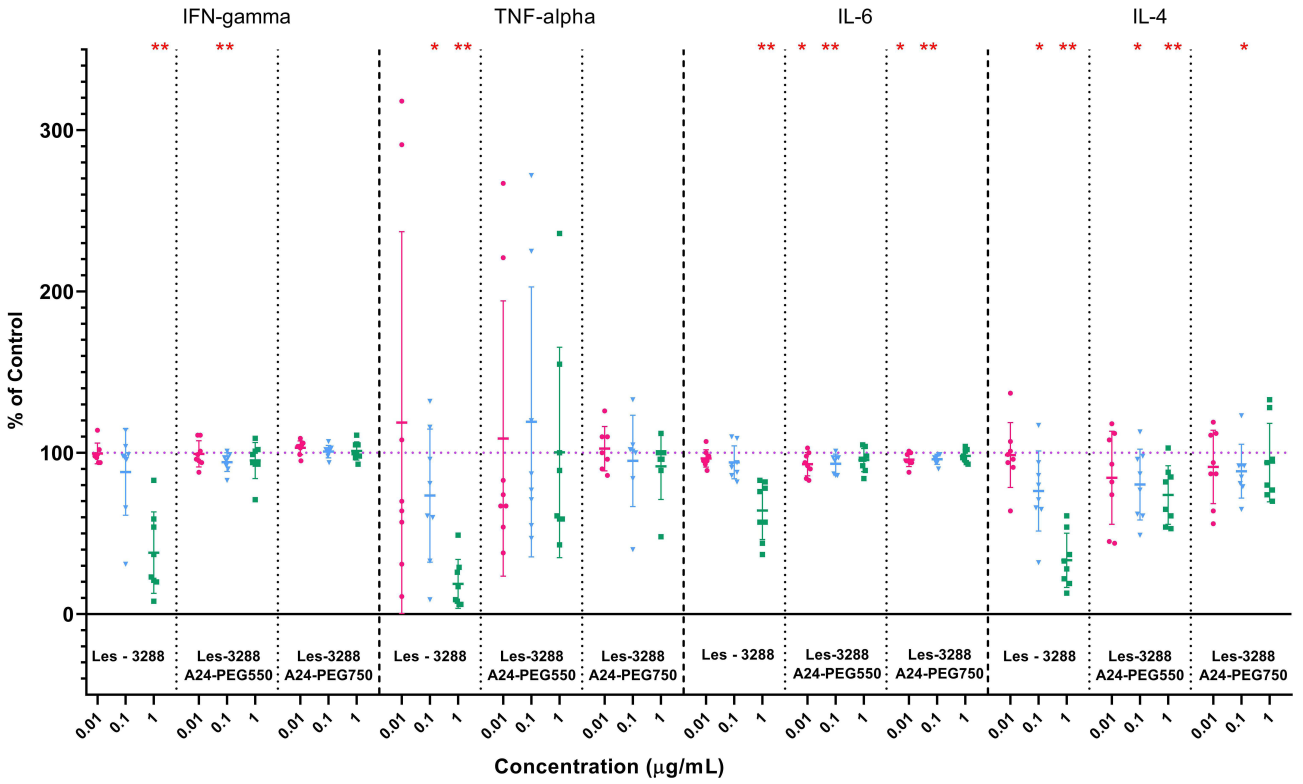

Figure 11 The effect of binding Les-3288 to the polymeric nanocarriers on production of cytokines IFN-γ, TNF-α, IL-6 and IL-4 in cell culture supernatants. Cytokines were measured in phytohemagglutinin stimulated blood cell culture supernatants (n = 8–9) using the ELISA method. Human peripheral blood was exposed to Les-3288 – free drug in DMSO, complex Les-3288 A24-PEG550 and complex Les-3288 A24-PEG750 for 48h. Results are expressed in % of Control. Significance: *p < 0.05, **p < 0.01.
Abbreviations: IFN – interferon, TNF- tumor necrosis factor, IL-interleukin.

The A24-PEG550 polymer showed the most prominent suppressive effect in production of all selected cytokines. Mainly, IL-4 was decreased in all range of concentrations tested. The levels of TNF-α were reduced already after the application of a low dose. Production of IL-6 and IFN- γ were declined in middle concentration of A24-PEG550; IL-6 was diminished also in high one (Figure 13).

Second pegylated nanocarrier A24-PEG750 also significantly suppressed levels of IL-6. Although differences were significant, we can consider differences as a result of biological variability rather than biological relevance (98%, 97%, 97% of control). Levels of IL-4 were decreased in middle dosed supernatants. Production of IFN- γ and TNF-α was not changed (Figure 13).

Discussion

Pyrazoline-thiazolidinone isatins represent a promising class of compounds in cancer research exhibiting significant cytotoxic effects against various cancer cell lines. Recent research highlights their ability to target specific molecular pathways, including the PI3K/Akt and MAPK signaling cascades, which are critical in cancer cell proliferation and survival.[32–35] In addition to anticancer effects, they have antimicrobial potential.[36] Recent studies have also explored the anti-inflammatory potential of thiazolidinone derivatives,[37] and these compounds have shown promising results in inhibiting pro-inflammatory cytokines and enzymes. Several derivatives showed excellent inhibition of TNF-α and IL-6 expression in LPS-stimulated macrophages.[38] These diverse effects prompted our effort to evaluate the effects of our derivatives on the immune response. The main points of cellular and humoral immunity were studied. The immune response of lymphocytes, granulocytes, and monocytes to the PEG-polymeric nanocarriers with 4-thiazolidinone-based chemotherapeutics was determined using immune function assays. The proliferative capacity of T lymphocytes and T-dependent B lymphocytes was evaluated through lymphocyte proliferation activity assay. The function of granulocytes (mainly polymorphonuclear granulocytes) and monocytes was monitored using a test of phagocytic activity and

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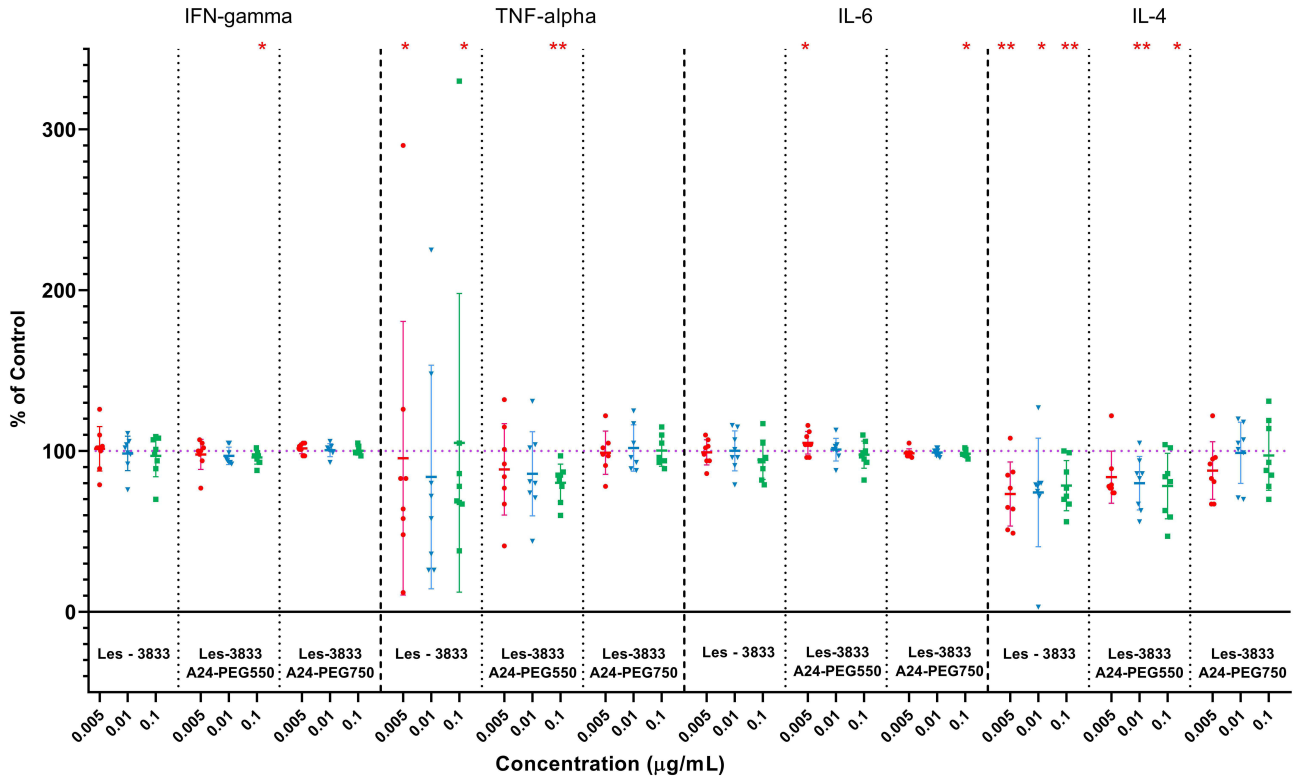

Figure 12 The effect of binding Les-3833 to the polymeric nanocarriers on production of cytokines IFN-γ, TNF-α, IL-6 and IL-4 in cell culture supernatants. Cytokines were measured in phytohemagglutinin stimulated blood cell culture supernatants (n = 8–9) using the ELISA method. Human peripheral blood was exposed to Les-3833 – free drug in DMSO, complex Les-3833 A24-PEG550 and complex Les-3833 A24-PEG750 for 48h. Results are expressed in % of Control. Significance: *p < 0.05, **p < 0.01.
Abbreviations: IFN – interferon, TNF- tumor necrosis factor, IL-interleukin.

respiratory burst of leukocytes. Additionally, in vitro cytokine production assays, which monitor the intricate cell–cell communication among immune cells, were employed. These assays are complex and facilitate the assessment of functional immune interactions, thereby enhancing our understanding of immune cell cooperation.

The basic requirement for the (nano)safety of new drugs, including oncotherapeutics, is low or no cytotoxicity to immune cells unless the immunosuppressive effect is intentional. In general, synthesized PNCs proved low cytotoxicity, with the best results for the A24-PEG750 and PEGMA-DMM nanoparticles. First 4-thiazolidinone derivative Les-3288 was neither cytotoxic in free-form nor conjugated with PNCs within the whole range of tested concentrations 0.01 μg/mL - 1 μg/mL. Regardless the form, second anticancer molecule Les-3833 displayed more significant cytotoxicity to PBMCs. Changes in the structure of the molecule substitutes caused a significant shift towards worsening cytotoxicity even in comparison with Dox.

Subsequently, non-cytotoxic concentrations of the test substances were used for the functional immune assays. In the first step, the functional ability of lymphocytes to divide upon mitogen stimulation was measured using a proliferation assay. High doses of both PEG-contained polymeric carriers (A24-PEG550 and A24-PEG750) significantly diminished proliferation of T cells, while the T-dependent B cell response remained unaffected. Diverse effect on T and B cells might indicate higher sensitivity of T lymphocytes to the toxicity of PEG polymers. PEG polymers are commonly used to design new targeted therapeutics and vaccines. The data on the effect of PEG alone on the T cell response described no effect. However, studies are extremely rare.[39] The effect of PEG functional nanocomplexes on T lymphocyte response depends on the intended use of designed nanocomplex. In the case of cancer therapy, PEG hydrogel cross-linked with two fibronectin-derived peptides, decorated with gold nanoparticles functionalized with the activating antibody CD3 showed T cell activation (CD69 expression and IL-2 secretion).[40] Also, pegylated poly (lactic-co-glycolic acid) PLGANPs encapsulated tumor-associated antigen showed stimulation of IL-12 production, T cell proliferation and IFN-γ production by T cells in vitro.[40] In vaccine development, complex triblock polymer with PEG enhanced CD4+ T and CD8+ T cell activation, improved the lymphocyte proliferation efficiency, and increased the secretion of different

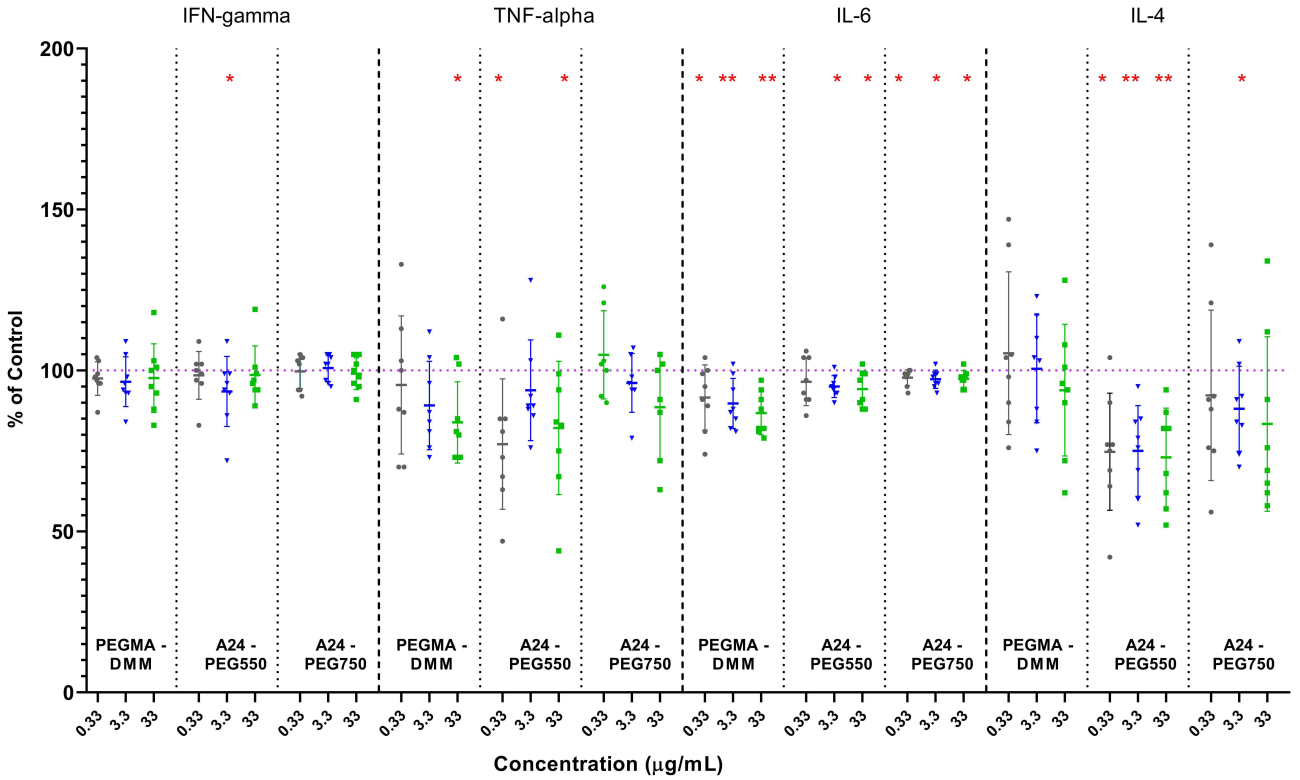

Figure 13 The effect of the polymeric nanocarriers on production of cytokines IFN-γ, TNF-α, IL-6 and IL-4 in cell culture supernatants. Cytokines were measured in phytohemagglutinin stimulated blood cell culture supernatants (n = 8–9) using the ELISA method. Blood was exposed to polymeric nanocarriers PEGMA-DMM, A24-PEG550 and A24-PEG750 for 48h. Results are expressed in % of Control. Significance: *p < 0.05, **p < 0.01.
Abbreviations: IFN – interferon, TNF- tumor necrosis factor, IL-interleukin.

cytokines.[41] PEGylated polysaccharide vaccine nano-adjuvant enhanced CD4+ and CD8+ T cells-derived memory (CD44high CD62Lhigh), and effector (CD44high CD62Llow) cells as well as functional phenotypes.[42] Cationic PEG polymeric micelle-based vaccine also potently enhanced T cell proliferation and the secretion of IL-5 and IFN-γ.[43] On the other hand, nanoplatform of PD-1 antibody and TEPP-46 integrated by PEG modified PLGA designed for treatment of autoimmune uveitis inhibited early activation and proliferation of effector T cells.[44]

The most significant positive result of the binding of polymer conjugates to anti-cancer molecules was observed as an alleviation of suppressive effect of Les-3288 to the lymphocytes. After binding of free Les-3288 to polymers, the original immunosuppressive effect of Les-3288 free drug on proliferative response of the T lymphocyte and T-dependent B cells was significantly improved. A marked increase in proliferation almost to control response values was observed. The intention to reduce toxicity by binding the drug to the polymer succeeded.

The effect of Les-3833 on lymphocyte function was completely different. In the case of Les-3833, 10 times lower doses were used for testing. The lower doses did not suppress the function of lymphocytes, but instead stimulated them. Unexpected stimulatory effects of Les-3833 free drug and compound bound to the smaller polymer carrier on T-dependent B lymphocytes was observed in cultures in vitro stimulated to pokeweed mitogen. Our finding is similar with the observation of nonspecific immunostimulation with low doses of cytotoxic drugs Cyclophosphamide or Dox.[45–48] Liu et al found that Dox in combination with IOX1 (5-carboxy-8-hydroxyquinoline) greatly promotes T cell infiltration and activity and significantly reduces tumor immunosuppressive factors. Their liposomal combination reduces the growth of murine tumors and offers a long-term immunological memory function.[48]

Our results of evaluating the phagocytic activity and respiratory burst of leukocytes showed that Les-3833 bound to both PEG nanocarriers significantly decreased phagocytic activity and respiratory burst of granulocytes almost in whole range of concentrations tested. Given that these granulocyte's functions remained unchanged after the addition of the free-form of the Les-3833 to the cells, we suggest that the suppression of phagocytic and respiratory functions was directly due to the

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polymeric nanoparticles. Indeed, the effect of Les-3833 on phagocytosis and respiratory burst follows the same suppressive trend observed after exposure to pure PNCs PEG550 and PEG750. Based on published data on the ability of polymeric nanoparticles to block phagocytosis of further particles, we suppose that blockage of the phagocyte system by polymeric nanoparticles A24-PEG550 and A24-PEG750 might be responsible for suppressed phagocytosis of *S. aureus* seen in our study.[49,50] Our assumption is supported by the finding of Guo et al[51] that bacteria competed with the nanoparticles for uptake via phagocytosis. Together with the findings of Kelley et al[52] that PEGylation of model drug carriers enhances phagocytosis by primary human neutrophils, this information might help to explain our results. Strategies of deliberate blockade of the phagocyte system with PEGylated liposomes, peptide-labeled liposomes, exosomes are used to pro-long the circulation half-life of drugs.[52,53] Approach to preemptively suppress RES phagocytic function with PEGylated liposomes was used to enhance therapeutic efficacy of paclitaxel-containing nanoparticles (Liu et al, 2015).[49]

Data on the interaction of PEG polymeric nanoparticles with phagocytes and their function to uptake and kill bacteria are provided by several authors,[50,52,54] however, data on the effect of pure PEG polymers are not available. In contrast to our results, studies with polymers have shown that association of PEG-based nanoparticles was greater with monocytes than with granulocytes.[55] This could explain the preference to focus studies on monocyte/macrophage function. For example, the ability of primary murine macrophages to take up and kill Escherichia coli following treatment with magnetic nanoparticles functionalized with PEG chain showed resistance to treated macrophages without significant loss of function.[55] Poly (ethylene glycol-amine)-derivatized graphene oxide nanosheets were efficiently taken up by mice peritoneal macrophages inducing a significant increase of *C. albicans* phagocytosis by both M1 proinflammatory macrophages and M2 reparative macrophages.[56] Among other polymers, amino-functionalized polystyrene nanoparticles inhibited phagocytosis of *Escherichia coli* by both M1 and M2 human macrophages.[56]

More rare studies on neutrophils showed that human polymorphonuclear granulocytes preferentially phagocytose PEGylated versus carboxylated polystyrene microspheres.[52] Silk fibroin PEGylated 5-fluorouracil nanoparticles did not affect phagocytic activity of the human granulocytes.[57] Polyacrylic acid (PAA) coated and non-coated iron oxide nanoparticles triggered oxidative burst in human neutrophils.[58] PAA-coated ZnO nanoparticles decreased neutrophil viability and PAA-TiO2, CeO2, Fe2O3, ZnO NPs all induced an increase in neutrophil respiratory burst in goldfish.[59]

Many "polymer" studies were dedicated to the potential applications of polymeric delivery systems to increase the selectivity of antibiotics for phagocytic cells and enhance therapeutic efficiency in the treatment of intracellular infections.[60] Mainly, PLGA nanoparticles were found to provide high cellular accumulation of antibiotics and effectively kill intracellular *S. aureus*.[60–62] PEG polymers were also found to be helpful. PEGylation improved the pharmacokinetic and therapeutic properties of capsule depolymerase delivered for the treatment of mice infected with *B. anthracis*.[63] Macrophage targeted pegylated iron oxide nanodecoys loaded with rifampicin enhanced the killing efficiency of intracellular *Mycobacterium tuberculosis* in in vitro macrophages, and also significantly reduced the mycobacterial burdens in the lung of infected mice with alleviated pathology.[64] Poly(ethylene)glycol-poly(β-amino esters) micelles with conjugated antimicrobials effectively killed Streptococcus mutans in mice and men.[65] PLGA-PEG nanoparticles grafted with antimicrobial peptide accelerates killing kinetics for *P. aeruginosa* and *S. aureus*.[66] PEG particles displayed the lowest overall mononuclear phagocyte system accumulation of the polymers tested (PEG, poly(N-(2-hydroxypropyl) methacrylamide) and poly(methacrylic acid).[55]

The unanswered question remains why Les-3288 bound to identical polymeric nanoparticles did not affect phagocytosis at all. One possible explanation is the different size or and/or configuration of the Les-3288 molecule compared to Les-3888 compound bound to the PNCs. Functionalization with specific polymers or ligands and changes in the size, shape, or surface of particles have important effects on their recognition and internalization by professional and nonprofessional phagocytes and have a major influence on their fate and safety.[67]

The monitoring of in vitro cytokine production showed predominantly suppressive effect or no effect of anticancer drugs and PNCs on the levels of IFN-γ, TNF-α, IL-6 and IL-4 in blood supernatants. Functional complexes configured for cancer therapy should not affect production of IL-6 and IL-4 cytokines at all, except for IFN-γ and TNF-α whose increased production could be beneficial in some cancers.[68] IFN-γ a typical anticancer cytokine produced by activated T cells and natural killer cells, facilitates anticancer immunity via recruiting highly immunogenic cells such as $CD4^+$ and $CD8^+$ T cells, and infiltrates M1-phase tumor associated macrophages.[69] Several nanocomplexes with biodegradable

polymer nanoparticles (PEG, PLGA-PEG, PELGE, DHPD, poly caprolactone) were designed and used to increase IFN-γ levels in animal or in vitro models.[70–75] The slight reduction in IFN-γ production (93–99%) caused by complexes and PNCs was statistically significant but it can be considered biologically insignificant. The worst observed dose-dependent suppression of IFN-γ was caused by the free molecule Les-3288; however, binding to PEG550 and PEG750 polymeric carriers significantly improved IFN-γ production to control values. From this point of view, complexes of Les-3288 with PNCs can be considered safer molecules.

The second monitored cytokine in our study was TNF-α whose primary role is the regulation of immune cells. In our study, the most significant dose-dependent suppression of TNF-α production was caused by the free substance Les-3288; however, upon binding to both our PEG polymers, cytokine production returned to control. For Les-3833, PEG750 was more effective in this function. The role of TNF-α in cancer is different, depending on the type of cancer. TNF-α is used as an immunostimulant drug in the treatment of certain cancers, to overcome cancer multidrug resistance; other types of cancer cause its overproduction leading to cachexia.[72,76] Published studies showed that pegylated nanoparticles can affect the production of TNF-α.[72,76–78] PEGylated PLGA nanoparticles encapsulating astragalus polysaccharides and gold nanorods upregulated the production of TNF-α in tumor-bearing mice peaked on day 3 after focused ultrasound in vivo.[72]

IL-6 is one of the determining cytokines in the tumor microenvironment, predominantly produced by the tumor cells, resulting in their growth, proliferation and metastasis in multiple cancer types; therefore, proposed anticancer drugs must not trigger the production of this cytokine.[79] Clinically, increase of IL-6 serves as a negative prognostic marker in cancer, but on the other hand, while this cytokine is involved in T cell activation, it is still important to consider his pleiotropic effects on immune cell populations that are critical for tumor development.[80] In our study, complexes of anticancer drugs and pure polymers slightly but significantly suppressed levels of IL-6; the most significant suppression of IL-6 production was caused by high dose of free substance Les-3288 (65%), followed by a dose-dependent considerable decrease of IL-6 in cultures exposed to the PNC PEGMA-DMM (91–87%). The only observed stimulation of IL-6 production caused by the low dose of complex Les-3833 with A24-PEG550 (105%) can be considered as biologically non-significant. In experimental nanomedicine, pegylated nanoparticles designed to inhibit or reduce IL-6 production are being tested in the treatment of cancer in tumor-bearing mice, DSS-induced colitis in mice, cutaneous wound healing in diabetic rats or atopic dermatitis in mice.[79,81–85] On the other hand, some nanoparticles such as gold nanoparticles for cancer therapy were found to stimulate production of IL-6.[86,87]

Interleukin-4 (IL-4) is a pleiotropic cytokine and crucial immune system modulator. In relation to cancer, IL-4 and IL-13 can mediate tumor cell proliferation, survival, and metastasis in gastric, colon cancer, lymphoma, cancer of breast, lung and pancreas.[88–90] In our study, some of the tested anticancer molecules significantly reduced the production of IL-4, but no substance increased IL-4 cytokine production. Regarding the magnitude of decrease, IL-4 production was the most reduced by Les-3833 free substance ≥Les-3288 free substance. The positive effect of polymer binding in the sense of returning to control values was stronger for Les-3833 but only for that bound to PEG750. Among PNCs, A24-PEG550 suppressed IL-4 production most significantly. Complexes with polymeric nanoparticles known to reduce IL-4 have been used for experimental treatment of allergic asthma in mouse model.[91,92] On the other hand, efficient induction of IL-4 is desirable in vaccine development.[93,94]

Conclusion

The present study demonstrated that Les-3288 exhibited a cytotoxicity 10 times lower than that of Les-3833 when tested on human PBMCs. The binding of Les-3288 and Les-3833 to the PNCs A24-PEG750 and A24-PEG550 did not result in any improvement in the viability of human PBMCs. The binding of the polymer to the Les-3288 anticancer drug, with the objective of mitigating the immunosuppressive effects of the free drug on the proliferative activity of T lymphocytes and T-dependent B cells, demonstrated comparable efficacy for both A24-PEG750 and A24-PEG550 nanocarriers. In the case of Les-3833, the binding of drug to the smaller carrier A24-PEG550 was observed to preserve the stimulatory effect of the free drug on T-dependent B lymphocytes.

Neither form of Les-3288 affected phagocytic activity or respiratory burst of leukocytes. The binding of Les-3833 drug to both PNCs resulted in a decrease in phagocytic activity of granulocytes and the respiratory burst, in comparison with the unaltered granulocyte function in blood exposed to the free-form of the Les-3833 drug. It is hypothesized that

the blockage of the phagocyte system by polymeric nanoparticles A24-PEG550 and A24-PEG750 is responsible for the observed suppression.

The monitoring of cytokine production demonstrated that the Les-3288 drug-polymer complex markedly elevated the diminished levels of IFN-γ and TNF-α resulting from free Les-3288. Conversely, the levels of IL-6 and IL-4 remained suppressed. No significant effect of either form of Les-3833 on IFN-γ and IL-6 production was found. With regard to TNF-α and IL-4, the positive impact of polymer binding on the restoration of suppressed cytokine levels induced by the Les-3833 free drug was observed to be marginally more pronounced in the case of A24-PEG750.

Considering the previously demonstrated high therapeutic efficacy, low toxicity and absence of adverse immune responses associated with waterborne delivery systems utilizing novel PEGylated carriers of both water-soluble and water-insoluble drugs of various chemical structures, these systems may represent a significant area of interest for advancement of therapeutic strategies.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Slovak Medical University, Bratislava.

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Disclosure

The authors declare no conflicts of interest in this work.

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