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# PROPERTIES OF MONOCYTE-DERIVED DENDRITIC CELLS LOADED WITH LYSATES OF CANCER CELLS EXPOSED TO CYTOTOXIC PEPTIDES

**Background**. This study is based on the idea of using tumor cell membrane lysis induced by diarylethene-containing analog of cytotoxic peptides (CPs) — gramicidin S to create a new approach for obtaining dendritic cells (DCs)-based anticancer vaccine. It is supposed that cancer cells undergoing immunogenic cell death release the damage-associated molecular patterns (DAMPs), and thus enhance immunogenic maturation and activation of DCs. The **aim** of this study is to analyze the phenotypic and functional characteristics of the generated monocyte-derived DCs loaded with CPs-treated lysates of tumor cells. **Materials and Methods**. The triple-negative human breast cancer cell line MDA-MB-231 was used in the study. DCs were generated from peripheral blood monocytes using a recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). Tumor cells were treated with LMB033 CPs containing a diarylethene fragment (photoswitch) in two ring forms — "closed" with low activity and toxicity and "open" with high activity. The obtained lysates of tumor cells were co-incubated with human monocyte-derived DCs. The analysis of the phenotypic characteristics of DCs was performed by a flow cytometry using monoclonal antibodies to CD83, CD86, CD11c, HLA-DR, and HLA-ABC. The expression level of mRNA of cytokine genes and indoleamine 2,3-dioxygenase (*IDO*) gene was determined using the quantitative real-time PCR. **Results**. The highest cytotoxic effect on MDA-MB-231 cells was detected after 6-h incubation with the open form of LMB033 at concentrations of 16 and 32 μM. The studied CPs even at the lower of the tested concentrations caused externalization of phosphatidylserine in almost 100% of apoptotic cells of MB-MDA-231 cells following 6-h incubation. Loading monocyte-derived DCs with lysate of MDA-MB-231 cells treated with LMB033 peptide in open or closed forms caused a different effect on the antigen-presenting properties of cells depending on the form of the peptide. Compared to DCs loaded with untreated lysate, a significant increase in the number of mature activated $CD83^+$ DCs was found after loading with lysates of cells treated with open (16 μM) or closed (32 μM) forms of LMB033. CPs-induced lysates of MDA-MB-231 cells did not cause significant changes in the expression of mRNA of Th1 polarizing cytokines TNF-α, IL-12, neither did these lysates activate the transcription of the genes of immunosuppressive cytokines and IL-10, TGF-β, and the *IDO* gene. This indicates the absence of the activation of the immunosuppressive properties of the generated DCs. **Conclusion**. The presented data open the prospects for developing an effective antitumor immunotherapeutic vaccine based on DCs using CPs LMB033.

**Keywords**: cytotoxic peptides, dendritic cells, maturation, mRNA expression, tumor cells lysate.

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The current cancer treatments including chemotherapy, radiotherapy, targeted, and immunotherapy have improved outcomes for cancer patients to a certain extent [1]. However, these treatments still have some limitations, for instance, chemotherapy and radiotherapy usually lead to therapeutic resistance as well as severe side effects, while immunotherapy and targeted therapy are not powerful enough to overcome tumor heterogeneity and therapeutic resistance. The most promising drugs for cancer immunotherapy — checkpoint inhibitors and cellular technologies based on dendritic cells (DCs) — have shown their effectiveness only in a limited number of cancer patients, primarily due to the immunological features of tumors, including a limited tumor mutational burden (TMB), poor infiltration by immune cells, and active immunosuppression [2, 3]. Therefore, scientists continue the search for agents that promote a more stable and highly active antitumor immune response, especially for advanced tumors. In particular, there is an active search for compounds that can transform cold tumors into hot ones increasing the level of infiltration by cytotoxic T cells and DCs, which increases the effectiveness of their response to immunotherapy [4]. The compounds capable of causing immunogenic tumor cell death (ICD) are promising in this regard. Certain anticancer modalities are known to induce ICD associated with the release of damage-associated molecular patterns (DAMPs) assisting in triggering the events leading to the development of antitumor immunity. Cancer cells undergoing ICD may themselves cause a tumor-specific immune response targeting live cancer cells and residual tumor tissue. As a result, patients can obtain long-term clinical benefits from a treatment response initiated by cytotoxic chemotherapy and physical induction.

ICD-associated DAMPs include several constitutively expressed molecules fulfilling housekeeping functions or released extracellularly upon loss of homeostasis triggered by cellular stress or death signals (cDAMPs): calreticulin (CRT) and ERp57, HSP70, and HSP90 heat-shock proteins, mitochondria-derived N-formylated peptides and DNA, the non-histone protein high-mobility group Box 1 (HMGB1), annexin A1 (ANXA1), ATP, and other nucleotides, dsRNA and dsDNA, and F-actin [5]. By analogy with PAMPs, cDAMPs bind to PRRs, toll-like receptor (TLR) family expressed on innate immune cells. These danger signals can exert many different effects, from facilitating the phagocytosis of dying cells to stimulating their activation and release of immunomodulatory cytokines. Moreover, the exposure of CRT on the surface of cancer cells succumbing to ICD also induces tumor antigen presentation and tumor-specific CTL responses. These DAMPs can provide "find-me", "eat-me", "present-me", and maturation signals with an immunostimulatory or immunosuppressive effect on the DCs [6]. After the engulfment of dying tumor cells and sensing of DAMPs, DCs will mature and upregulate the chemokine receptor CCR7, along with co-stimulatory molecules such as CD80 and CD86.

Together with cDAMPs, the secretion of inducible DAMPs by dying cancer cells undergoing ICD attracts myeloid and T cells to the tumor microenvironment and plays an essential role in sustaining immunogenicity and enabling tumor rejection. Therefore, these DAMPs are responsible for the "anticancer vaccine effect" of ICD.

The known inducers of ICD comprise the conventional and targeted anticancer therapies that include but is not limited to certain chemotherapeutics, proteasomal inhibitors, mitotic poisons, tyrosine kinase inhibitors, the epidermal growth factor receptor-specific monoclonal antibodies, cyclin-dependent kinase inhibitors, antitumor antibiotics, oncolytic viruses, and various physical/chemical interventions, such as irradiation, hypericin-based photodynamic therapy (PDT), and high hydrostatic [7, 8].

Among the experimental compounds that induce ICD, cytotoxic peptides of natural origin are distinguished, in particular cationic antimicrobial peptides (CAPs) [9]. Such compounds include cytotoxic peptides (CPs), which are part of the innate immune system, in particular, those isolated from bacteria, fungi, etc. Besides acting directly on cancer cells and causing oncolysis, some oncolytic peptides can also induce the exposure of tumor-associated antigen (TAA) and release of DAMPs from dying tumor cells, thereby triggering a powerful local and systemic anticancer immune responses. Oncolytic peptides that can induce membrane lysis-mediated cancer cell death and subsequent anticancer immune responses provided a new paradigm for cancer therapy. It should be taken into account that natural CPs can cause significant side

effects due to their excessive nonspecific cytotoxicity. Therefore, most of them have failed in preclinical trials, and their potential as activators of the immune system against tumors remains unexplored.

Gramicidin S (GS), belonging to this class of peptides, exhibits antitumor potential, but it is not used *in vivo* as an oncolytic peptide due to the nonspecific cytotoxicity at the therapeutic concentrations [10]. Recently, some photocontrolled diarylethene containing analogs capable of changing their bioactivity upon exposure to light of different wavelengths have been created [11]. Their high cytotoxic activity have been shown in different experimental models *in vitro* and *in vivo* including cancer cells spheroids and cancer allografts [12].

This study was based on the idea of using lysis of tumor cell membranes induced by diarylethene-containing analogs of GS to develop a new approach for obtaining a DCs-based anticancer vaccine. Cancer cells undergoing ICD can be used for co-incubation with monocyte-derived DCs, which is thought to enhance their immunogenic maturation and activation. The aim of the study is to analyze the phenotypic and functional characteristics of the generated monocytic DCs loaded with lysates of MDA-MB-231 tumor cells treated with a GS analog LMB033 *in vitro*.

## Materials and Methods

***Culturing MDA-MB-231 cells***. The triple-negative human breast cancer cell line MDA-MB-231 (ATCC, USA) was selected for the study. A complete culture medium was used, consisting of RPMI-1640 (Gibco, USA), 10% fetal calf serum (FCS) (Gibco, USA), 2 mM L-glutamine (Gibco, USA), and a mixture of 100 U penicillin/0.1 mg/mL streptomycin (Gibco, USA). Cells were cultured in a $CO_2$ incubator at 37 °C and 5% $CO_2$.

***Treatment of MDA-MB-231 cells with cytotoxic peptides***. LMB033 for the study was kindly provided by Lumobiotics (Germany). We used two samples of a photoswitchable LMB033 containing a diarylethene fragment in two ring forms — "closed "(hereinafter clLMB033) with low activity and toxicity and "open" (hereinafter opLMB033) characterized by a high activity [9]. The working solutions were prepared in deionized water (from concentrated CPs stock solutions containing 100% DMSO) and used at concentrations of 16 μM and 32 μM.

The aqueous solutions of either LMB033 isoform at concentrations of 16 μM and 32 μM or water (in controls) in a volume of 0.1 mL were added to the cells according to the experimental design. To determine the incubation time at which the optimal cytotoxic effect is achieved, the plate was incubated for 6 h from the moment of CPs addition, and the samples were analyzed by Annexin V flow cytometry at time points of 30 min, 3 h, and 6 h.

Preparation of tumor cell lysates. The tumor cell concentration was adjusted to 107/mL with RPMI 1640 medium («Sigma», USA) and subjected to 5 freeze/thaw cycles (–20 °C/+37 °C). The resulting lysate was centrifuged at 8,000 rpm for 15 min. The supernatant was used for DCs loading. The RNA and protein concentration were determined by spectrophotometry using a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, USA). Thus, in the DC culture medium, in the supernatant of tumor cell lysates, the final concentration of proteins was 1.36 ± 0.10 mg/mL, of RNA — 370 ± 25.9 μg/mL.

***Generation of DCs from peripheral blood monocytes***. DCs were generated from peripheral blood monocytes (10 mL) of practically healthy people (all manipulations were performed in compliance with the rules of aseptic techniques). Leukocytes were separated in a Ficoll density gradient ($p$ = 1.077 g/cm$^3$), after which the cells were resuspended in RPMI-1640 medium (Sigma, USA) with the addition of 2 mM/L L-Gly, 100 μg/mL streptomycin, and 100 U/mL penicillin and incubated in a plastic bottle at 37 °C, 5% $CO_2$ for 2—3 h. After that, the cells were shaken slightly, and those that did not adhere were removed by washing them off. The cell concentration was adjusted to $0.5 \times 10^6$/mL with culture medium and 1% autologous plasma, and 100 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leucomax Novartis, India/Schering-Plaugh, USA or ICN, USA) and 20 ng/mL interleukin-4 (IL-4) (Sigma, USA) were added. Growth factors were also added to DCs on the 3rd day of culture. Thus, immature DCs were obtained for further maturation with lysates of MDA-MB-231 cells pretreated with both functional forms of LMB033. The lysates were added on the 6th day of DCs culture. On the

7th day of maturation, 100 ng/mL LPS (Sigma, USA) and IFN alpha-2b (Laferobion, Biopharma, Ukraine) were added to all DCs samples in the test and control samples. On the 8th day, the viability of the generated DCs was assessed using flow cytometry.

Loading of DCs with MDA-MB-231 cell lysates. On the 6th day of cultivation, the supernatants of MB-MDA-231 tumor cell lysates prepared as described above were added to the obtained immature DCs: untreated (for negative control) and treated with LMB033 CPs in closed and open forms and concentrations of 16 μM and 32 μM. To the positive control samples of mature DCs, only RPMI-1640 culture medium was added in an equivalent volume.

***Flow cytometry***. To determine the number of devitalized cells, we used the Dead Cell Apoptosis Kit with Annexin V PE & SYTOX™ Green for Flow Cytometry (Invitrogen, USA), which allowed us to determine the number of dead cells and those in the early stages of apoptosis. The analysis was performed according to the manufacturer's recommendations using a Navios EX flow cytometer, Beckman Coulter, USA.

Analysis of the phenotypic characteristics of human DCs was performed by flow cytometry using monoclonal antibodies to markers CD83, CD86, CD11c, and HLA-ABC, labeled with FITC, and antibodies to HLA-DR, labeled with phycoerythrin (Beckman Coulter, USA). The measurement results were analyzed using the Navios EX software.

***Molecular genetic studies***. Total RNA was isolated from DCs by acid-phenol extraction. The isolated RNA was treated with DNase using DNase, DNase-buffer (Ambion, USA), and RNase inhibitor (Applied Biosystems, USA) reagents according to the manufacturer's protocol. The RNA concentration was determined spectrophotometrically using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA), and the RNA sample was adjusted to a concentration of 200 ng/μL. High-capacity cDNA (Applied Biosystems, USA) was used for reverse transcription. The cDNA obtained as a result of the reverse transcription reaction was diluted 2-fold with a DNA buffer for subsequent PCR setup (20 μL of DNA buffer was added to 20 μL of cDNA solution).

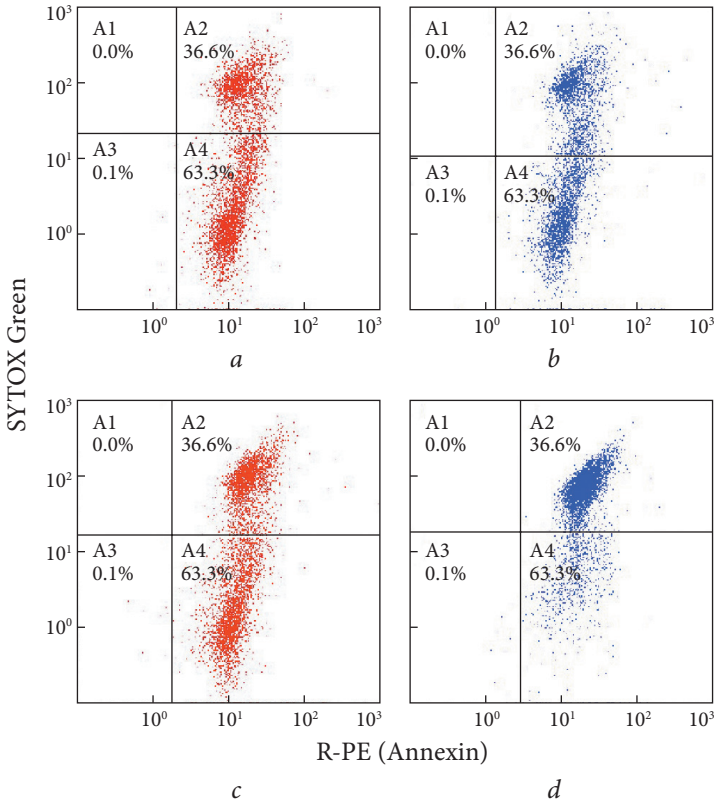
The expression level of cytokine genes and indoleamine 2,3-dioxygenase gene (*IDO*) was deter-

*Table 1.* **Primer sequences and PCR conditions for studying the expression of cytokine genes and *IDO* gene**

| cDNA | Primer sequences | Annealing temperature (Tm), °C |
|---|---|---|
| *TGF-β* | forward GGACATCAACGGGTTCACTA<br>reverse CCGGTTCATGCCATGAATGG | **60** |
| *TNF-α* | forward CCCAGGCAGTCAGATCATCTTC<br>reverse AGCTGCCCCTCAGCTTGA | **60** |
| *IDO* | forward TCAATGCCCCATACAACAAA<br>reverse TGGCAAGACCTTACGGACATCTC | **60** |
| *IL-10* | forward TGGACAACATACTGCTAACC<br>reverse GGATCATTTCCGATAAGGCT | **60** |
| *GAPDH* | forward GCCAAGGTCATCCATGACAACTTTGG<br>reverse GCCTGCTTCACCACCTTCTTGATGTC | **57** |

*Table 2.* **Primer and TaqMan probe sequences for determining the level of *IL-12* gene expression**

| Gene | *GAPDH* | *IL-12p35* | *IL-12p40* |
|---|---|---|---|
| Forward primer | 5'TCACCCACACTGT-GCCCATCTACGA3' | 5'CTCCTGGACCACCT-CAGTTTG3' | 5'CGGTCATCTGCCGCAAA3' |
| Reverse primer | 3'CAGCGGAACCGCT-CATTGCCAATGA5' | 3'GGT-GAAGGCATGGGAA-CATT5' | 3'TGCCCATTCGCTCCAA-GA5' |
| TaqMan probe | 6-VIC-AT-GCCCTCCCCCATGC-CATCCTGCGT-TAMRA | 6-FAM-CCAGAAACCTCCCC-GTGGCCA-TAMRA | 6-FAM-CGGGCCCAGGACC-GCTACTATAGC-TAMRA |



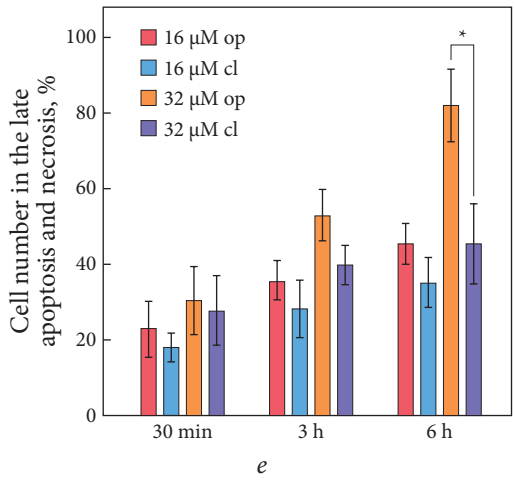




***Fig. 1.*** Cytotoxic activity of CPs on MB-MDA-231 cells after 6-h treatment: *a*) closed form of LMB033 peptide, 16 μM; *b*) open form of LMB033 peptide, 16 μM; *c*) closed form of LMB033 peptide, 32 μM; *d*) open form of peptide LMB033, 32 μM; *e*) relative cell number in the late apoptosis and necrosis after treatment of LMB033 peptide (M±σ). * The difference is significant, $p \leq 0.05$ (when comparing the action of the open form of peptides to the closed one)

mined using quantitative PCR with real-time detection on a 7500 Real-Time PCR Systems instrument (Applied Biosystems, USA) using specific primers (0.25 μM each) and SYBRGreen fluorochrome. Primer sequences were selected using Primer Express® Software v3.0 from "Applied Biosystems" (USA) and synthesized by "Applied Biosystems" (USA) (Tables 1 and 2).

Primers were used at a concentration of 0.3 μM, and probes — 0.2 μM. The following temperature cycle was used: start of amplification at 95 °C — 5 min, then accumulation of the amplification product during 50 cycles: 94 °C 15 s, 60 °C 1 min. To control the mRNA expression level of the studied genes, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured. After the end of the amplification reaction, the results were recorded according to the recommendations of the device manufacturer. The level of cytokine gene expression was estimated using the ΔΔCt method with normalization relative to the expression of the *GAPDH* gene.

***Statistical analysis***. Statistical analysis was performed using the Statistica 10.0 software package (Stasoft Inc., USA). Normality of distribution was checked using the Shapiro — Wilk test. To compare data in two groups, Student's *t*-test was used for parametric data and the Mann — Whitney test for nonparametric data. When comparing more than two groups, analysis of variance with post-hoc Tukey test was used for normally distributed data, and the Kruskal — Wallis test for nonparametric data. The difference was considered statistically significant at $p < 0.05$.

## Results and Discussion

***Cytotoxic effect of CPs on MB-MDA-231 cells.*** The highest cytotoxic effect on MDA-MB-231 cells was recorded when using the opLMB033 at concentrations of 16 and 32 μM after 6 h of incubation, which was in line with our expectations (Fig. 1). At the same time, the action of clLMB033 at concentrations of 16 and 32 μM at this time point showed a slightly lower cytotoxic effect compared to the open form, which coincided with the data on the higher safety of clLMB033 [11]. This trend was also observed at time points of 30 min and 3 h, while the cytotoxic effect of opLMB033 at a concentration of 32 μM at the 6th hour of incubation was 1.8 times higher ($p < 0.05$) than the action of clLMB033 at a similar concentration. Considering

these results, we chose a 6-h incubation regimen of cancer cells with LMB033 CPs as optimal for further use in experiments with DCs loading.

It should be noted that CPs even at the lower of the tested concentrations, under the 6-h cultivation, caused an externalization of phosphatidylserine in the apoptotic MB-MDA-231 cells. The total number of the dead cells and cells in the early stages of apoptosis reached almost 100%. It is known that the remains of apoptotic and necrotic tumor cells are absorbed equally effectively by DCs. However, it has been proven that necrotic cells are more immunogenic in natural conditions, since necrosis is associated with inflammation and DAMPs. The absorption of apoptotic cells can have different consequences: DCs do not activate and mature when absorbing apoptotic cells, or DCs loaded with such antigenic material more effectively stimulate CTLs to produce IFN-γ and secrete themselves higher levels of IL-12p70, induce a specific T-cell response, and thus stimulate Th1. Some studies have shown that apoptotic cells can independently stimulate the development of inflammation *in vivo* due to the activation of caspases and the induction of IL-1β and IL-18 production, which subsequently stimulate the production of other cytokines that activate DCs. Although it is believed that fewer DAMPs are released during classical apoptosis than during necrotic cell death, apoptotic cells also exert immunogenic properties, especially via the products of the late stages of apoptosis [13].

Given the controversial mechanism of the action of gramicidin S and its analogs, the question about the ability of LMB033 peptides to induce powerful ICD of tumor cells remains open. Therefore, an assessment of the key DAMPs release inherent for the ICD process induced by peptides may be important for understanding the mechanisms of the effect of CPs on tumor cells and DCs maturation. This aspect is very important, since the maturity of DCs and their ability to present tumor antigens to T cells and polarize their response along the Th1 or Th2 pathway, induce tolerance, or develop an antitumor immune response significantly depend on the strategy of tumor cell devitalization and the efficiency of tumor antigen loading [14].

***Effect of LMB033-treated MDA-MB-231 cell lysates on the expression of surface markers by generated DCs***. An assessment of DCs viability is important, because CPs LMB033 remain in the lysate that was added to the generated DCs. The viability of the generated monocytic DCs in all experimental samples was about 80% at the time of assessment. It follows that the addition of LMB033 of both isoforms at final concentrations of 0.16 and 0.32 μM into the DCs culture did not have strong cytotoxic effects on the generated DCs. Our studies showed that 7-day cultivation including 2 days in the presence of CPs contributed to the acquisition of typical morphological features of DCs by monocytes. Since DCs maturation is associated with the expression of the CD83 molecules, as well as increased expression of the HLA-DR, CD86, and CD38 molecules, the assessment of these markers served as the basis for phenotypic analysis.

The phenotype of the mature DCs is characterized by the high expression of the MHC class II antigen-presenting complexes (HLA-DR) and CD86 and CD83 markers. CD11c is an integrin considered a canonical marker of DCs. Although CD11c is also expressed on monocytes and macrophages (moderate and low levels of expression, respectively), this marker is important for assessing the phenotypic characteristics of DCs. Since HLA-A, -B and -C are "classical" MHC class I molecules expressed on the surface of most types of human nucleated cells, the number of cells expressing HLA-ABC is not a specific marker for assessing the phenotype of mature DCs. However, an increased density of HLA-ABC expression on DCs may predict their ability to effectively cross-present to killer T cells, which is important for the initiation of effective antitumor responses [15].

When analyzing the percentage of generated DCs that simultaneously express CD86 and HLA-DR markers (Fig. 2, *a*), no significant differences were found between the experimental samples and the control group of mature DCs. However, compared to the control of mature DCs, there were observed the tendencies to reduction of the number of $CD86^{+}HLA\text{-}DR^{+}$ DCs in DC samples loaded with the lysate of the peptide-untreated tumor cells and the samples treated with lysate obtained under the action of 32 μM opLMB033 and to increase of this parameter in the case of using 32 μM of LMB033 in both forms at a concentration of 16 μM (Fig. 2, *a*). Therefore, loading DCs generated from peripheral blood monocytes *in vitro* with lysate of MDA-MB-231 cells treated with either of the two forms of LMB033 caused a slightly different effect

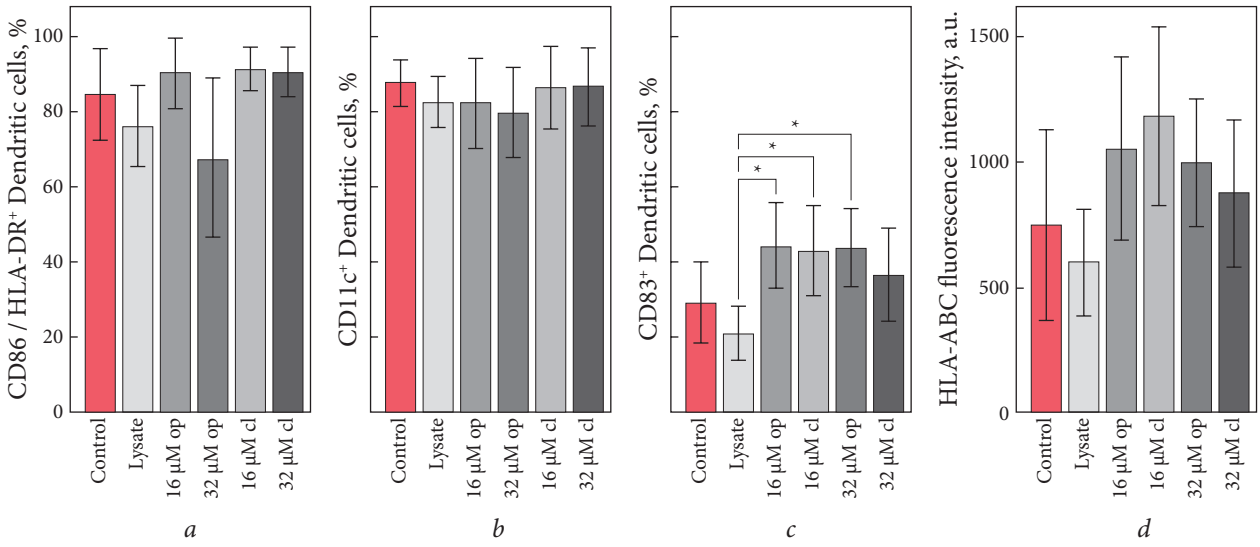



***Fig. 2.*** The number of DCs expressing *a*) CD86 and HLA-DR; *b*) CD11c; *c*) CD83, and *d*) the density of HLA-ABC expression on DCs generated *in vitro* after loading with lysate of intact and LMB033 peptide-treated MDA-MB-231 cells, op — open form of LMB033 peptide; cl — closed form of LMB033 peptide (M±σ). * The difference is statistically significant, $p \leq 0.05$

on the antigen-presenting properties of cells depending on the peptide form.

The CD11c molecule is an α-integrin and, in combination with other adhesion molecules, promotes the migration of activated DCs to the secondary lymphoid tissues. Another important role of CD11c is to mediate the phagocytosis of complement-inactivated C3b-opsonized particles; the ligands of this receptor are LPS, complement components iC3b, fibrinogen, and ICAM-1 molecules [16]. In our study, the percentage of $CD11c^+$ DCs in none of the culture samples differed significantly from the control and between themselves (Fig. 2, *b*). However, the lowest value was observed when analyzing samples with the addition of the lysate/opLMB033 at a concentration of 32 μM. These results indicate that the given experimental conditions did not affect significantly the numbers of $CD11c^+$ DCs, which were similar to the activated DCs control.

Membrane CD83 is expressed on various activated immune cells, although the highest expression is observed on mature DCs. Membrane CD83 is an informative marker of DCs maturation [17]. CD83 has been shown to enhance the expression of MHC class II and CD86 by blocking the association of MHC class II with the ubiquitin ligase MARCH1 [18].

The results of flow cytometry demonstrated (Fig. 2, *c*) that the percentage of cells expressing CD83 after loading with tumor cell lysate treated with opLMB033 at a concentration of 16 μM was 2.12 times higher than the corresponding indicator in samples with the addition of untreated lysate, $p < 0.05$. At the same time, maturation of DCs under the influence of lysate treated with clLMB033 at a similar concentration also contributed to the 2.10-fold increase in the percentage of $CD83^+$ DCs, compared to the samples with addition of untreated lysate, $p < 0.05$. The number of $CD83^+$ DCs in the case of using lysate treated with 32 μM opLMB033 also increased by 2.07 times compared with the DCs samples generated under the influence of lysate untreated with the peptides, $p < 0.05$. However, none of the experimental samples differed significantly by the indicators compared to the control of mature DCs. It is worth noting that the expression of CD83 on human DCs correlates with an increased ability to stimulate T cells *in vitro* [19].

Our studies have shown that the relative number of HLA-ABC molecules (molecules of the major histocompatibility complex MHC type I) on generated DCs changes slightly under the influence of CPs. Using both forms of LMB033, an increase in the expression level of HLA-ABC molecules was determined by measuring the average fluorescence intensity (mean index) (Fig. 2, *d*). It should be noted that the largest increase in the mean value by 1.6 times compared to the control values was observed when using 32 μM LMB033, thus indicat-

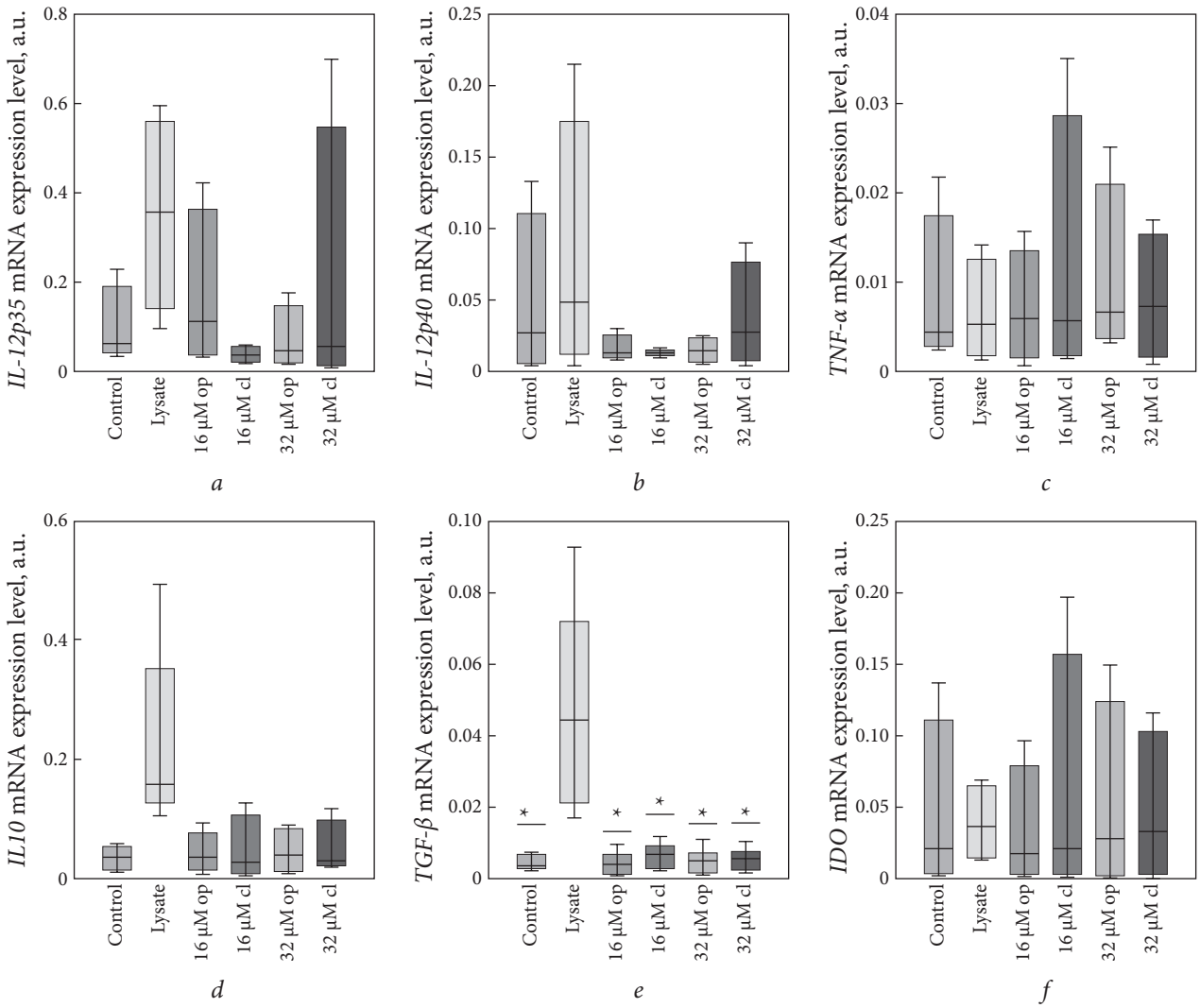



***Fig. 3.*** Normalized to *GAPDH* mRNA expression level of *a*) *IL-12p35*; *b*) *IL-12p40*; *c*) *TNF*-α; *d*) *IL-10*; *e*) *TGF-β*, *f*) *IDO* by generated *in vitro* DCs loaded with lysate of intact and LMB033 peptide-treated MDA-MB-231 cells: op — open form of LMB033 peptide; cl — closed form of LMB033 peptide. * The difference is statistically significant, $p \leq 0.05$ (when comparing to the DC loaded cell lysate)

ing an increase in the density of HLA-ABC molecules on the DCs membrane, and indirectly — the activation of cross-presentation of tumor antigens. The lowest average fluorescence intensity per cell among all experimental samples was found in the case of the addition of untreated lysate.

***Assessment of mRNA expression of cytokines and IDO genes by DCs loaded by lysates of MDA-MB-231 cells treated with LMB033***. DCs stimulation is accompanied by cytokine secretion, with the balance between pro-inflammatory and suppressor cytokines determined by the microenvironment. Apoptotic cell-induced DCs suppression has been linked to the key mediators that support these functional effects, the IFN-γ-dependent induction of IDO and TGF-β. It has been revealed that the apoptotic environment can suppress LPS-stimulated DCs responses [20]. Thus, analysis of cytokine mRNA expression can indirectly indicate the degree of immunogenicity of MDA-MB-231 lysates under the chosen processing method.

IL-12 is considered a key cytokine that causes polarization of T-helper cells to type 1. IL-12 secreted by DCs is a heterodimer consisting of the p40 and p35 subunits, and the activation of DCs by PAMPs or DAMPs can lead to an increase in the transcript levels of both subunits [21]. Our studies have shown that loading monocytic DCs with lysate of MDA-MB-231 cells treated with either of two LMB033 forms did not significantly affect the expression level of mRNA of IL-12 subunits (Fig. 3 *a*, *b*). Interestingly, we observed a trend to-

ward an increase in the expression level of mRNA of IL-12p35 and IL-12p40 subunits in DCs samples obtained with the addition of untreated lysate compared to the control, and tendencies to a slight increase in IL-12p35 and a decrease in IL-12p40 mRNA levels in DCs samples treated with opLMB033 at a concentration of 16 μM.

It is known that cells that secrete bioactive IL-12p70 also secrete free IL-12p40 subunit in excess compared to the IL-12 heterodimer, demonstrating constitutively higher levels of IL-12p40 expression, which is critical for directing IL-12p35 trafficking and IL-12p70 production. Jalah and colleagues [22] demonstrated that the formation of IL-12p40 homodimers is slow and inefficient, whereas IL-12p40/IL-12p35 heterodimers are formed at a rate dictated by the availability of IL-12p40. Thus, the presence of IL-12p35 in the same cell results in efficient production of the heterodimer.

Autocrine TNF-α signaling promotes monocyte survival and differentiation into DCs [23]. TNF-α, together with IL-1β, has been shown to be essential for DCs migration [24]. According to the results of our studies, all experimental DCs samples showed no significant changes in TNF-α mRNA expression levels compared to the control group of mature DCs, although high variability of values between samples within the experimental groups can be noted (Fig. 3, *c*).

The IL-10 mRNA expression levels in experimental samples did not differ significantly from the control mature DCs (Fig. 3, *d*) but showed a tendency to increase in the case of use of lysate of untreated MDA-MB-231 cells. The enhanced production of the immunoregulatory cytokine IL-10 is associated with an immature DCs phenotype and functional impairment of T-cell priming, which is manifested in a lower stimulatory capacity and direct suppressive effect [25].

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine that plays a specific role in the regulation of T-cell development and induction of immunological tolerance of DCs [26]. It promotes immunosuppression by inducing the formation of regulatory T cells (Tregs), which is important in suppressing autoimmune reactions but is undesirable in inducing antitumor immunity, and therefore, in developing DCs antitumor vaccines. Hence, the possibility of inhibiting DCs TGF-β mRNA expression and the role of LMB033 peptides in the implementation of this mechanism are interesting aspects for further studies in the context of finding new antitumor therapeutic approaches. It is worth noting that the assessment of the relative mRNA expression levels does not allow for the accurate conclusions about the expression of cytokines and *IDO* genes, since we do not take into account post-translational modifications of the mRNAs under study. This is a limitation of the chosen method. Therefore, to confirm the results, additional assessment of translation products, followed by folding and post-translational modifications, is necessary.

As we found (Fig. 3, *e*), none of the groups of DCs samples loaded with lysate using peptides showed significant changes in *TGF*-β mRNA expression levels compared to the control group of mature DCs but just a tendency to a slight increase under the influence of opLMB033 at a concentration of 32 μM. A significant difference in mRNA expression of this suppressive cytokine was observed in DCs loaded with untreated MDA-MB-231 lysate — by 10.5 times compared to the control group of samples, $p < 0.05$. This indicator was also significantly higher than the corresponding values in experimental DCs samples where the peptides were used.

IDO is a cytosolic heme-containing enzyme that catalyzes the breakdown of tryptophan to kynurenine. Tryptophan deprivation activates the ribosomal kinase GCN2, which detects the binding of uncharged tRNA to ribosomes. The activated GCN2 triggers an integrated stress response cascade that results in the transcriptional silencing of most genes and promoting cellular autophagy. This cascade blocks cell cycle entry of activated T cells, activates resting Tregs, and induces a tolerogenic tumor microenvironment [27]. In our experiment, none of the samples demonstrated a significant difference in the levels of *IDO* mRNA expression relative to the control of mature DCs (Fig. 3, *f*).

However, the *IDO* mRNA expression levels in DCs samples loaded with untreated lysate or with lysate of cells treated with the closed form of LMB033 at a concentration of 32 μM tended to slightly increase.

The proinflammatory cytokines IFN-γ, TNF-α, IL-6, and IL-1β, as well as TLR9 and TLR4 agonists (CpG DNA and LPS, respectively), are potent agonists of IDO transcription, which can prevent excessive immune responses. There is much evidence

for a role for IDO in tumor "escape" from immune surveillance; suppressive effects on T-cell responses in different compartments of the tumor microenvironment have been documented in several studies. DCs constitutively express IDO, but an additional set of trigger signals is required to enhance the activity of this enzyme during antigen presentation. IDO has also been shown to promote tolerance through noncatalytic signaling by inducing the release of TGF-β by DCs [27].

Our study demonstrated that the expression of the key costimulatory molecules on DCs (CD86, CD11c, HLA-ABC, and HLA-DR) during maturation under the influence of MDA-MB-231 lysates treated with LMB033 at the specified concentrations did not undergo the significant changes compared to the control. At the same time, compared to DCs loaded with untreated lysate, a significant increase in the number of mature, activated $CD83^+$ DCs was found under the influence of lysates treated with either of two forms of LMB033 at a concentration of 16 μM and opLMB033 at a concentration of 32 μM.

We did not detect significant changes in the levels of mRNA expression of Th-1 polarizing cytokines TNF-α and IL-12 under the influence of CPs-treated lysates of MDA-MB-231 cells. At the same time, activation of transcription of genes of immunosuppressive cytokines and IL-10, TGF-β and the enzyme IDO did not occur either, which indicates the absence of the activation of the immunosuppressive properties of generated DCs. Moreover, DCs loaded with CPs-untreated lysate of MDA-MB-231 demonstrated a statistically significant increase in the levels of mRNA associated with the tolerogenic profile of TGF-β, however, the use of CPs LMB033 in all concentrations contributed to the leveling of this effect.

Overall, the obtained results indicated the successful maturation and activation of DCs under the influence of MDA-MB-231 lysates treated with open and closed forms of LMB033 *in vitro*, with additional stimulation with LPS and IFN-alpha 2b. Cancer cells succumbing to ICD foster a proficient interface with DCs, enabling their immunogenic maturation and engagement of adaptive immunity against cancer [28]. The presented data open the prospect of developing an effective antitumor immunotherapeutic vaccine based on DCs using CPs LMB033.

REFERENCES

1. Winter RC, Amghar M, Wacker AS, et al. Future treatment strategies for cancer patients combining targeted alpha therapy with pillars of cancer treatment: external beam radiation therapy, checkpoint inhibition immunotherapy, cytostatic chemotherapy, and brachytherapy. *Pharmaceuticals*. 2024;17:1031. https://doi.org/10.3390/ph17081031
2. Lao Y, Shen D, Zhang W, et.al. Immune checkpoint inhibitors in cancer therapy—How to overcome drug resistance? *Cancers*. 2022;14:3575. https://doi.org/10.3390/cancers14153575
3. Hato L, Vizcay A, Eguren I, et. al. Dendritic cells in cancer immunology and immunotherapy. *Cancers*. 2024;16:981. https://doi.org/10.3390/cancers16050981
4. Liu YT, Sun ZJ. Turning cold tumors into hot tumors by improving T-cell infiltration. *Theranostics*. 2021;11(11):5365-5386. https://doi.org/10.7150/thno.58390
5. Serrano-del Valle A, Anel A, Naval J, Marzo I. Immunogenic cell death and immunotherapy of multiple myeloma. Front. C*ell Dev Biol*. 2019;7:50. https://doi.org/10.3389/fcell.2019.00050
6. Garg AD, Agostinis P. Cell death and immunity in cancer: From danger signals to mimicry of pathogen defense responses. *Immunol Rev*. 2017;280(1):126-148. https://doi.org/10.1111/imr.12574
7. Galluzzi L, Vitale I, Warren S, et al. Consensus guidelines for the definition, detection and interpretation of immunogenic cell death. *J Immunother Cancer*. 2020;8(1):e000337. https://doi.org/10.1136/jitc-2019-000337
8. Kepp O, Senovilla L, Vitale I, et al. Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunology*. 2014;3(9):e955691. https://doi.org/10.4161/21624011.2014.955691
9. Tornesello AL, Borrelli A, Buonaguro L, et al. Antimicrobial peptides as anticancer agents: functional properties and biological activities. *Molecules*. 2020;25(12):2850. https://doi.org/10.3390/molecules25122850.
10. Okamoto K, Tomita Y, Yonezawa H, et al. Inhibitory effect of gramicidin S on the growth of murine tumor cells *in vitro* and *in vivo*. *Oncology*. 1984;41(1):43-48. https://doi.org/10.1159/000225789
11. Babii O, Afonin S, Schober T, et al. Peptide drugs for photopharmacology: how much of a safety advantage can be gained by photocontrol? *Future Drug Discov*. 2020;2(1):FDD28. https://doi.org/10.4155/fdd-2019-0033
12. Khranovska N, Skachkova O, Gorbach O, et.al. Anticancer immunogenic potential of oncolytic peptides: recent advances and new prospects. *Exp Oncol*. 2024;46(1):3-12. https://doi.org/10.15407/exp-oncology.2024.01.003

13. Montico B, Nigro A, Casolaro V, Dal Col J. Immunogenic apoptosis as a novel tool for anticancer vaccine development. *Int J Mol Sci*. 2018;19:594. https://doi.org/10.3390/ijms19020594
14. Galea-Lauri J, Wells JW, Darling D. Strategies for antigen choice and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell-based cancer vaccination. *Cancer Immunol Immunother*. 2004;53(11):965-977. https://doi.org/10.1007/s00262-004-0542-8
15. Sakamoto T. Koya T. Togi M, et al. Different *in vitro*-generated MUTZ-3-derived dendritic cell types secrete dexosomes with distinct phenotypes and antigen presentation potencies. *Int J Mol Sci*. 2022;23:8362. https://doi.org/10.3390/ijms23158362
16. Wu J, Wu H, An J, et al. Critical role of integrin CD11c in splenic dendritic cell capture of missing-self CD47 cells to induce adaptive immunity. *Proc Natl Acad Sci U S A*. 2018;115(26):6786-6791. https://doi.org/10.1073/pnas.1805542115
17. Li Z, Ju X, Silveira PA, et al. CD83: activation marker for antigen presenting cells and its therapeutic potential. *Front Immunol*. 2019;10:1312. https://doi.org/10.3389/fimmu.2019.01312
18. Tze LE, Horikawa K, Domaschenz H, et al. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *J Exp Me*d. 2011;208(1):149-165. https://doi.org/10.1084/jem.20092203
19. Prechtel AT, Turza NM, Theodoridis AA, Steinkasserer A. CD83 knockdown in monocyte-derived dendritic cells by small interfering RNA leads to a diminished T cell stimulation. *J Immunol*. 2007;178(9):5454-5464. https://doi.org/10.4049/jimmunol.178.9.5454
20. Williams CA, Harry RA, McLeod JD. Apoptotic cells induce dendritic cell-mediated suppression via interferon-gamma-induced IDO. *Immunology*. 2008;124(1):89-101. https://doi.org/10.1111/j.1365-2567.2007.02743.x
21. Gerber AN, Abdi K, Singh NJ. The subunits of IL-12, originating from two distinct cells, can functionally synergize to protect against pathogen dissemination *in vivo*. *Cell Rep*. 2021;37(2):109816. https://doi.org/10.1016/j.celrep.2021.109816
22. Jalah R, Rosati M, Ganneru B, et al. The p40 subunit of interleukin (IL)-12 promotes stabilization and export of the p35 subunit: implications for improved IL-12 cytokine production. *J Biol Chem*. 2013;288(9):6763-6776. https://doi.org/10.1074/jbc.M112.436675
23. You K, Gu H, Yuan Z, Xu X. Tumor necrosis factor alpha signaling and organogenesis. *Front Cell Dev Biol*. 2021;9:727075. https://doi.org/10.3389/fcell.2021.727075
24. Stoitzner P, Zanella M, Ortner U, et al. Migration of Langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1beta. *J Leukoc Biol*. 1999;66(3):462-470
25. Llopiz D, Ruiz M, Infante S, et al. IL-10 expression defines an immunosuppressive dendritic cell population induced by antitumor therapeutic vaccination. O*ncotarget*. 2017;8(2):2659-2671. https://doi.org/10.18632/oncotarget.13736
26. Esebanmen GE, Langridge WHR. The role of TGF-beta signaling in dendritic cell tolerance. *Immunol Res*. 2017;65(5):987-994. https://doi.org/10.1007/s12026-017-8944-9
27. Mellor AL, Lemos H, Huang L. Indoleamine 2,3-dioxygenase and tolerance: Where are we now? *Front Immunol*. 2017;8:1360. https://doi.org/10.3389/fimmu.2017.01360
28. 26 Janssens S, Rennen S, Agostinis P. Decoding immunogenic cell death from a dendritic cell perspective. *Immunol Rev*. 2024;321(1):350-370. https://doi.org/10.1111/imr.13301

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ВЛАСТИВОСТІ МОНОЦИТАРНИХ ДЕНДРИТНИХ КЛІТИН,
НАВАНТАЖЕНИХ ЛІЗАТАМИ ПУХЛИННИХ КЛІТИН,
ОБРОБЛЕНИХ ЦИТОТОКСИЧНИМИ ПЕПТИДАМИ

**Стан питання**. В основу цього дослідження покладено ідею використання мембранолізу пухлинних клітин, індукованого діарилетенвмісним аналогом цитотоксичних пептидів (ЦП) — Граміцидину S, для створення нового підходу до отримання протипухлинної вакцини на основі дендритних клітин (ДК). Передбачається, що ракові клітини, які піддаються ICD, вивільняють молекулярні структури, пов'язані з пошкодженням (DAMPs), і таким чином посилюють імуногенне дозрівання та активацію ДК. Метою цього дослідження є аналіз фенотипових і функціональних характеристик генерованих моноцитарних ДК, навантажених лізатами пухлинних клітин, індукованими ЦП. **Матеріали та методи**. Для дослідження була використана лінія клітин потрійно негативного раку грудної залози людини MDA-MB-231. ДК були генеровані з моноцитів периферич-

ної крові з використанням рекомбінантного гранулоцитарно-макрофагального колоніестимулюючого фактора людини (ГМ-КСФ) та інтерлейкіну-4 (ІЛ-4). Пухлинні клітини обробляли цитоксичними пептидами LMB033, що містять діарилетеновий фрагмент (фотоперемикач) у двох кільцевих формах — «закритій» з низькою активністю та токсичністю та «відкритій», що характеризується високою активністю. Отримані лізати пухлинних клітин використовували для коінкубації з ДК. Аналіз фенотипових характеристик ДК людини проводили методом проточної цитометрії з використанням моноклональних антитіл до маркерів CD83, CD86, CD11c, HLA-DR та HLA-ABC. Рівень експресії мРНК генів цитокінів та ферменту IDO визначали за допомогою кількісної ПЛР у реальному часі. **Результати**. Виявлено найвищу цитотоксичну дію на клітини MDA-MB-231 при використанні відкритої форми LMB033 у концентраціях 16 і 32 мкМ після 6 годин інкубації. Досліджувані ЦП навіть при нижчій із досліджуваних концентрацій за 6-годинного культивування спричиняли екстерналізацію фосфатидилсерину майже в 100% апоптичних клітин лінії MB-MDA-231. Встановлено, що навантаження ДК, генерованих з моноцитів периферичної крові *in vitro*, лізатом лінії пухлинних клітин MDA-MB-231, оброблених пептидом LMB033 у відкритій або закритій формах, викликає різноспрямований вплив на антиген-презентуючі властивості клітин залежно від форми пептиду. Порівняно з ДК, завантаженим необробленим лізатом, виявлено статистично значуще збільшення кількості зрілих, активованих $CD83^+$ ДК під впливом лізатів, оброблених відкритою та закритою формами LMB033 у концентрації 16 мкМ та відкритою формою ЦП у концентрації 32 мкМ. Отримані результати свідчать про успішне дозрівання та активацію ДК під впливом лізатів MDA-MB-231, оброблених відкритою та закритою формами LMB033 *in vitro*. Статистично значущих змін рівнів експресії мРНК Th-1 поляризуючих цитокінів TNF-α, IL-12 під впливом ЦП-індукованих лізатів клітин MDA-MB-231 не виявлено. При цьому активація транскрипції генів імуносупресивних цитокінів та IL-10, TGF-β і ферменту IDO також не відбулася, що свідчить про відсутність активації імуносупресивних властивостей генерованих ДК. **Висновок**. Представлені дані відкривають перспективу для створення ефективної протипухлинної імунотерапевтичної вакцини на основі ДК з використанням ЦП LMB033.

**Ключові слова**: цитотоксичні пептиди, дендритні клітини, дозрівання, експресія нРНК, лізат пухлинних клітин.