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Staphylococcus aureus WOOD 46 CYTOPLASMIC MEMBRANE EXTRACT AS A FACTOR FOR THE MATURATION OF THE DENDRITIC CELLS

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The aim of the research was to examine the effect of *Staphylococcus aureus* Wood 46 cytoplasmic membrane extract on the maturation and functional activity of generated *in vitro* dendritic cells from monocytes in the peripheral blood of healthy patients and patients with malignancies. We found that the cytoplasmic membrane extract in the minimum of the studied concentrations (0.2 μg/ml) significantly promoted the increased expression of CD86 and HLA-DR molecules, mRNA of cytokines IFN-γ and TNF-α, and CC chemokine generated from CCR7 in dendritic cells. The results showed that the cytoplasmic membrane extract contributed to T1-helper polarization of dendritic cells and can be used to generate them in the dendritic cell vaccines production for cancer patients immunotherapy.

Key words: dendritic cell, extract of cytoplasmic membranes, dendritic cell vaccines.

The advancement of molecular biology, immunology, and biotechnology led to solid prospects of better results for the treatment of patients suffering from malignant growths. Certain hopes are excited by the developing biotherapy techniques aimed at correction of specific immune response to the tumors with the help of vaccines.

The implementation of dendritic cells (DC) for antineoplastic immunity became possible only recently due to the detection of antigen-presenting function of these cells, in particular, due to the discovery of the unique ability of DC to process tumor-associated antigens and present them together with the molecules of the major histocompatibility complexes (classes I and II) to the immune system cells, which bolsters the formation of antitumor immune response (or the response to these antigens). This is why the DC were put to use as natural adjuvants to create efficient next-generation anticancer vaccines [1, 2].

The plasticity of functional activity of immature DC lets one, by modulating the conditions of *in vitro* culture, to obtain cells with desired properties, mainly the ability to present antigens and activate specific immune reactions employing cell-mediated immunity. In order to do this, various stimuli are used, for example, anti-inflammatory cytokines, c-KitL and FLT3L (ligands of tyrosine kinase receptor), ligands of the receptor CD40, substances belonging to pathogen- or microbe-associated molecular patterns (two-chained RNA, lipopolysaccharides – LPS) and CpG-sequences of bacterial DNA) [3]. It should be noted that the nature of the activating stimulus and the way of tumor antigen loading have a substantial effect on the functional activity of DC.

Immunobiological properties of the biopolymers of the bacterial cell wall are fairly well-known, while the immunomodulatory potential of its cytoplasmic membranes and specific molecules are currently investigated [4]. The superficial biopolymers of some bacteriae, particularly the components of the extract of cytoplasmic membranes (ECM), are viewed as highly immunogenic substances and potential components of vaccine preparations [5].

Non-fractionated preparations of superficial structures of bacteriae are considered to have higher immunomodulatory potential than their isolated and purified components [6]. Immunomodulatory properties of ECM are largely based on the presence (depending on the isolation method) of lipoteichoic acid [7].

Meanwhile, the most optimal combinations of inductors of DC maturation are not yet established, and so the search for the most efficient combinations of factors to obtain functionally mature DC, able to be used for antineoplastic vaccines, continues to remain an urgent problem. Besides that, the range of properties of maximally therapeutically efficient DC is not yet defined [8, 9].

The aim of our work was to study the effect of ECM of *Staphylococcus aureus* Wood 46 on the maturation and functional activity of DC generated from the peripheral blood monocytes, used to produce anticancer vaccines.

Materials and Methods

Cell culture. The DC were generated from monocytes of the peripheral blood of 10 practically healthy patients and 7 who had malignant growths (all manipulations were carried out aseptically). The leucocytes were separatively divided in Fikoll density gradient (ρ = 1.077 g/cm^3), after which the cells were resuspended in the RPMI–1640 medium (Sigma, CIIIA) with the addition of 2 mM/l L-Gly, 100 μg/ml streptomycin and 100 unit/ml penicillin and incubated in a plastic flask at 37 °C, 5% CO_2 for 2–3 hours. The cells were then gently shaken and those which weren't attached to the surface washed off. The concentration of cells was adjusted to 0.5×10^6/ml using culture medium and 1% of autologous plasma was added, as well as 100 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leucomax Novartis, India / Schering-Plaugh, CIIIA or ICN, USA), 20 ng/ml interleukin-4 (IL-4) (Sigma, USA). The growth factors were also added to the DC on the third day of culturing. On the seventh day of maturation, DC received 100 ng/ml of LPS (Sigma, USA) or ECM and 2αb-IFN ("Laferobion", "Biofarma", Ukraine) at the concentration of 10 thousand IU/ml. The ECM used in our study, produced by *Staphylococcus aureus* Wood 46, did not contain lipoteichoic acid (LTA), and was isolated by the Department of Microbiology and General Immunology of the National Taras Shevchenko University of Kyiv. The presence of LTA impurities in ECM preparation might substantially influence its immunomodulatory properties. With this in mind, we tested the presence of LTA in the extracts we obtained, by electrophoresis on denaturing gel after Lemley (SDS-PAGE) in 15% gel. To visualize LTA the gel was dyed with alcian blue (Santa Cruz) in 50% ethanol with addition of 10% acetic acid after the standard method [10]. As a positive control, we used a commercial preparation of LTA of *S. aureus* (Sigma, USA). The results of the analysis showed there was no LTA in the studied extracts of ECM.

Since previous research showed the efficiency of ECM *in vitro* in the concentration range of 0.2 to 2 μg/ml [11], we selected four concentrations for our research: 0.24; 0.5; 1.0, and 2.0 μg/ml.

Immunological methods. The analysis of functional and phenotypic characteristics of DC was carried out by flow cytofluorometry with monoclonal antibodies to markers CD83, CD86, CD11c, marked with fluorescein isotiocyanate (FITC) and to HLA-DR, marked with phycoerythrin (Becton Coulter, USA). Phagocytic activity (PA) of the DC was determined by flow cytometry based on quantitative estimates of the number of bacteriae of *S. aureus*, marked with FITC that they consumed.

Sample analysis was carried out on a flow cytofluorometer FACSCalibur (Becton Dickinson, USA) with CellQuest-PRO software (Becton Dickinson, USA).

Quantitative PCR. Total RNA from DC was isolated using reagents from "Ribozol-A" ("AmpliSens", Russia) according to the manufacturer's instructions. To carry out reverse transcription we used PCR test "Reverta-L-100" ("AmpliSens", Russia).

The level of expression of mRNA genes of cytokines IL-12p35, IL-12p40, INF-γ, IL-10, TNF-α and chemokine CCR7 was determined by quantitative PCR with results being detected in real time on 7500 Real-Time PCR Systems (Applied Biosystems, USA), using specific primers and the fluorochrome SYBRGreen (Thermo Scientific, USA). Primer sequences were selected using Primer Express® Software v3.0 and synthesized in Applied Biosystems (USA). To control the level of expression of mRNA of the studied cytokines, we measured the expression of mRNA gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The level of gene expression for cytokines was determined by ΔΔCt normalized to the expression of the control gene.

Statistical methods. The data were statistically treated using Statistica 6.1

(Stasoft Inc., USA). To compare the data in two groups we used Student's t-criterion and Mann-Whitney test. The difference was considered statistically significant for $P < 0.05$.

Results and Discussion

Mass production of DC *in vitro* became possible due to cytokine "cocktails", added to induce their differentiation from progenitor cells. The "golden standard" for the generation and differentiation of DC is considered the combination of GM-CSF and IL-4 or GM-CSF and tumor necrosis factor (TNF-α). Generally, culturing lasts for 5–6 days, after which the immature DC are loaded with antigens, their maturation is induced for 2 days, and then they are used for vaccines. DC maturation factors include LPS and various cytokines: IL-1, IFN-α, GM-CSF and TNF-α [12]. After receiving the signal through TLR, the DC sharply change the expression of membrane molecules. The expression of the major histocompatibility complexes I and II classes, and in molecules of co-stimulation (CD80, CD83, CD86), undergoes a major boost. As the result, the cells obtain the factors necessary to efficiently present antigens – the DC mature. Meanwhile, the expression of anti-inflammatory chemokines CCR1, CCR5 and CXCR1 is decreased, while the amount of CCR7 and CXCR4 grows, which helps DC migration to lymphoid organs [13]. Bearing this in mind, we compared the effect of LPS and ECM on the expression of superficial markers of the generated DC.

As the results show, ECM at the lowest studied concentration (0.2 μg/ml) helps a statistically significant increase in the expression of CD86 and HLA-DR compared to the control (unstimulated DC), with $P = 0.038$ and $P = 0.037$, respectively (Fig. 1).

The level of co-expression of these molecules was higher than in DC, generated in the presence of LPS, by 10%. Meanwhile, this concentration of ECM did not influence the level of expression of CD83 (characteristic of the maturation degree of DC) or CD11c (characteristic of migratory activity of DC). It should be stated that the effect of ECM at other concentrations on the phenotypic characteristics of DC was no different from LPS (Table 1).

The functional state of DC is known to change with its maturation. Thus, when the DC are immature, they efficiently engulf antigens, mostly by endocytosis and micropinocytosis, but their ability to stimulate T-cells is limited. As they mature, their phagocytic activity decreases. In our study we showed that ECM substantially influenced the generated DC (Fig. 2) in a dose-dependent manner: with the ECM content increasing from 0.2 μg/ml to 2 μg/ml, DC phagocytic activity decreased from (36.00 ± 5.50)% to (29.00 ± 5.20)%.

Notably, if ECM was used at the concentrations of 0.2, 0.5 and 1.0 μg/ml, PA of DC decreased by 20, 31 and 36%, respectively compared to the control values. For the concentrations of ECM of 0.5, 1.0 and 2.0 mg/ml, these changes were statistically significant relative to control and to DC, generated in the presence of LPS, $P < 0.05$.

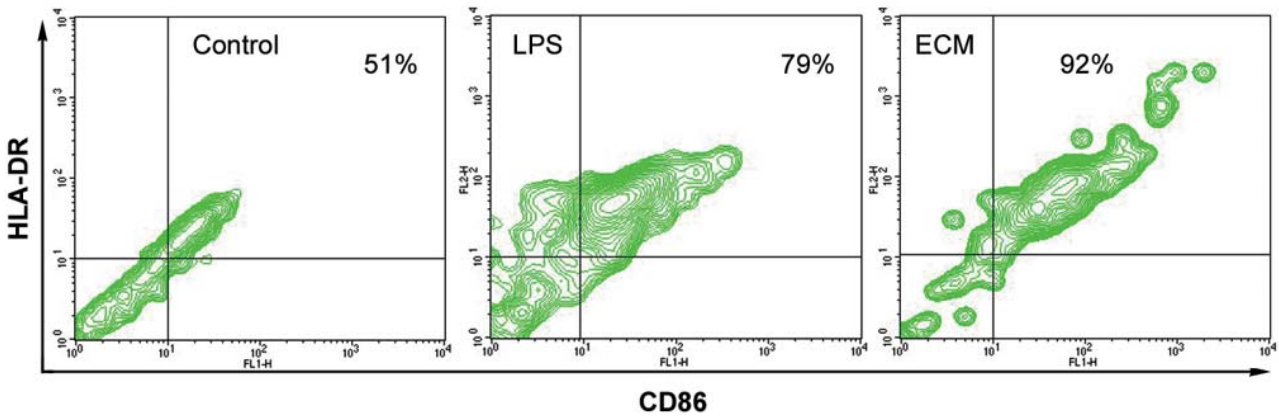

Fig. 1. **Level of expression of co-stimulatory molecules CD86 and HLA-DR on the surface of *in vitro* generated DC of a practically healthy patient**

Table 1. **Characteristics of *in vitro*-generated DC of in fact healthy patients**

Experimental conditions	Expression level, %				
	CD86	HLA-DR	CD86+/ HLA-DR+	CD83	CD11c
Control DC	57.25 ± 8.19	65.5 ± 9.35	56.50 ± 8.26	40.67 ± 76.53	70.33 ± 8.28
DC+LPS	71.25 ± 6.02	79.00 ± 5.35	70.00 ± 8.35	55.50 ± 7.60	79.50 ± 5.91
DC+ECM (0.2 μg/ml)	76.75 ± 7.65*	84.00 ± 6.10*	76.75 ± 8.91	54.00 ± 11.25	73.00 ± 7.45
DC+ECM (0.5 μg/ml)	66.00 ± 10.45	76.05 ± 9.40	67.40 ± 11.71	46.25 ± 10.29	68.75 ± 9.07
DC+ECM (1 μg/ml)	73.25 ± 8.38	80.75 ± 8.14	73.60 ± 9.60	43.00 ± 7.52	70.50 ± 10.00
DC+ECM (2 μg/ml)	69.75 ± 7.92	75.00 ± 6.96	71.60 ± 9.89	48.75 ± 8.03	67.50 ± 7.58

Note: * — $P = 0.04$ relative to control DC, generated without maturation factors; ^{0} — $P = 0.02$ relative to DC, generated under the presence of LPS.

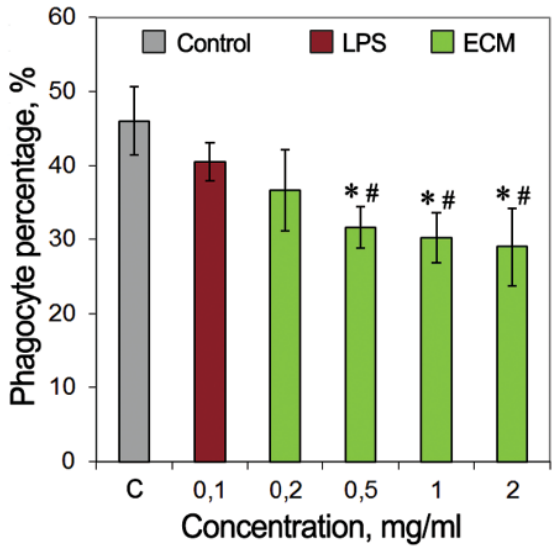

Fig. 2. **Phagocytic activity of the generated *in vitro* DC of in fact healthy patients:** control — DC, generated without maturation factors; LPS — DC, generated in the presence of LPS; ECM — DC, generated in the presence of ECM at different concentrations; * — $P = 0.03$ relative to control; # — $P = 0.05$ relative to LPS

The DC, usable as the cell base for creation of anticancer vaccines, must meet the requirements that reflect their ability to present antigens and stimulate T-cells. Besides a high maturity level, co-stimulatory molecules and adhesion molecules, they have to secret cytokines, which are able to activate T1-helper (Tx1)-mediated immune response, as well as to express the chemokine receptor CCR7, which is necessary for DC migration into lymph nodes [14]. Therefore the next stage of our work centered on studying the effect of ECM on the levels of cytokine genes expression which determine the direction and efficiency of immune reactions in the organism. It is known that the development of the Tx1-type immune response is induced by high levels of IL-2, IL-12, and the differentiation into the Tx2-type is supported by IL-5, IL-10 [15]. In our following research we used as the DC generation maturation factor ECM (0.2 μg/ml), since we showed it to be the most efficient.

We established that the level of the expression of mRNA of cytokine IL-12, which consists of two sub-units (IL-12p35 and IL-12p40), under the influence of ECM remains at the same level as for the DC, generated in the presence of LPS, while the level of expression of mRNA IL-10 decreases 1.5-folds, respectively (Fig. 3, *A*). Analyzing the balance of pro- and anti-inflammatory cytokines, we established that DC, generated in the presence of ECM, have more prominent Tx1-polarizing properties compared to those obtained with LPS.

Our research showed that if ECM was used, the DC exhibit a significant increase in the expression of mRNA IFN-γ compared to control and cells, generated in the presence of LPS, $P = 0.002$ (Fig. 3, *B*). The increase in the production of IFN-γ might indirectly suggest

the prevailing stimulation of Tx1-pathway when DC interact with lymphocytes, and the activation of NK-cells.

It was established that anti-inflammatory cytokines are the most important agents to mobilize DC. Moreover, they serve as intermediary messengers to activate DC migration. The most fully studied of such mediators are interleukine-1b (IL-1b) and TNF-α. Numerous experiments where the cytokines were neutralized, with the genes for their receptors IL-1RI and TNF-aRII knocked-out together with caspase-1, necessary for the realization of the IL-1b's activity, showed that the effect of these cytokines is a necessary and sufficient stimulus to mobilize the movement of DC from the place of their primary localization [16]. It was also established that a substantial increase in the expression of TNF-α in activated DC not only supported the domination of Tx1-cytokines in the DC population, but also boosted up their survival potential, endorsing the regulation of expression of BAK, BCL-2 and FLIPL [17]. The data showed that adding ECM to DC *in vitro* leads to an increase in the levels of expression of cytokine TNF-α 6.6 times compared to control ($P = 0.002$) and 1.4-folds compared to LPS ($P = 0.008$).

One should note that it is the CCR7 chemokine plays the leading part in DC migration into secondary lymphoid organs. We established that ECM treatment of DC incubated *in vitro* causes a substantial increase in the level of expression of mRNA CCR7 (Fig. 3, *B*). The level of expression in DC, generated in the presence of LPS, remained at the level of control values obtained without adding maturation factors, while ECM supported an increase in this chemokine by 2.3-folds ($P = 0.0007$).

In recent years, it was found that DC in the bodies of cancer patients can undergo quantitative and qualitative changes. Thus, DC, generated in patients with cancer complaints, are substantially different in phenotypic and functional properties from DC, obtained from monocytes from the peripheral blood of practically healthy patients. In particular, they can attain tolerogenic/regulatory activity [18, 19]. That is why our next task was to study the effect of ECM on DC generation *in vitro* from cells obtained from cancer patients. The results are given in Table 2. We established that DC, treated with ECM at the concentration of 0.2 μg/ml, are by their phenotypes not substantially different from DC treated with LPS.

Also when we were studying the phagocytic activity (PA) of generated *in vitro* DC of cancer patients, we found that ECM at the concentration of 0.2 μg/ml supports a significant decrease of their PA compared to DC, generated with LPS, from 41.86 ± 4.88% to 34.42 ± 3.96%, respectively ($P < 0.05$).

Thus, our results provide a case for using ECM of *Staphylococcus aureus* Wood 46 as maturation factor for DC production *in vitro*. We proved that ECM stimulates the differentiation and maturation of DC of donors and cancer patients and can be used for their generation.

DC generation *in vitro* is a complex technological process which demands

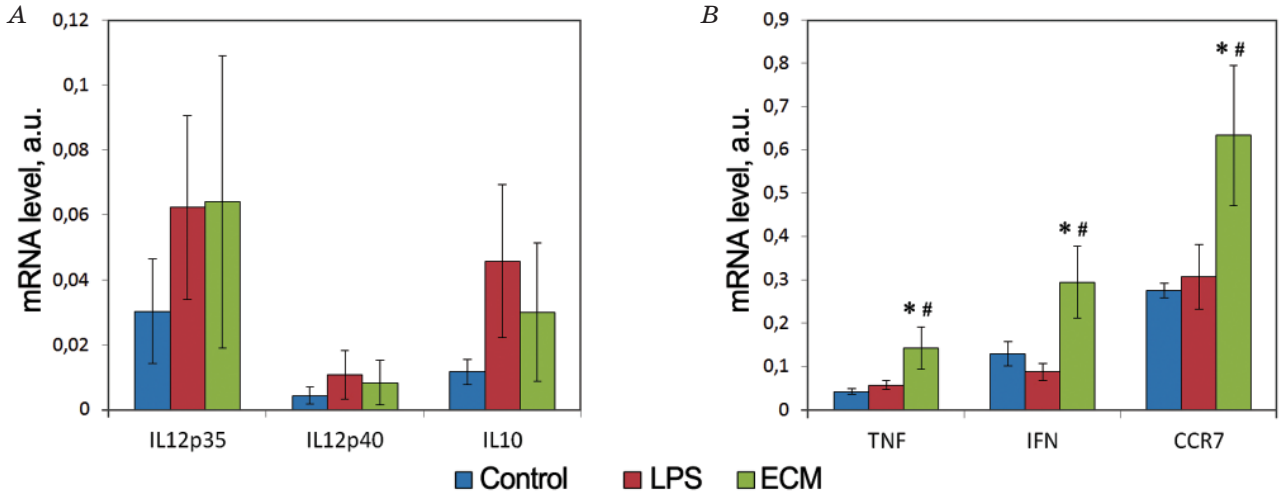

Fig. 3. **Level of expression of mRNA of cytokines in the *in vitro*-generated DC:**
ECM — DC generated in presence of ECM at the concentration of 0.2 μg/ml
* — $P < 0.05$ relative to control; # — relative to LPS

Table 2. **Features of *in vitro*-generated DC in cancer patients**

Experimental conditions	Level of expression, %				
	CD86	HLA-DR	CD86+/HLA-DR+	CD83	CD11c
DC + LPS	58.25 ± 10.09	63.25 ± 11.48	59.63 ± 6.88	30.38 ± 10.59	73.13 ± 14.14
DC + ECM (0.2 μg/ml)	58.25 ± 10.75	56.75 ± 10.62	55.63 ± 11.00	29.75 ± 13.21	69.00 ± 9.88

implementation of expensive reagents which we import from abroad. As the result of our research, we found that using ECM allows to partially decrease the cost of DC-vaccines by substituting some of the reagents. The proposed technology of DC production will favor an efficient and economically rational immunotherapy of cancer patients.

Thus, ECM at the minimal of the studied concentrations (0.2 μg/ml) leads to a significant boost in the expression of molecules of CD86 and HLA-DR on the generated DC. We established that ECM substantially impacts phagocytic activity of the generate DC in a dose-dependent manner: increasing ECM concentration led to decreasing PA. ECM favous increases in the levels of expression of mRNA of Tx1-polarizing cytokines IFN-γ and TNF-α in the generated DC. Using ECM as a maturation factor for DC helps to substantially increase the level of chemokine CCR7's expression, which suggests an increase in migratory activity of DC and ability to initiate immune response.

Thus, treating DC *in vitro* with the ECM of *Staphylococcus aureus* Wood 46 stimulates differentiation and maturation of DC of donors and cancer patients and can be used to generate them during immunotherapy for cancer patients.

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ЕКСТРАКТ ЦИТОПЛАЗМАТИЧНИХ МЕМБРАН *Staphylococcus aureus* Wood 46 ЯК ФАКТОР ДОЗРІВАННЯ ДЕНДРИТНИХ КЛІТИН

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Метою дослідження було вивчити вплив екстракту цитоплазматичних мембран *Staphylococcus aureus* Wood 46 на дозрівання та функціональну активність дендритних клітин, генерованих *in vitro* з моноцитів периферичної крові практично здорових пацієнтів і хворих на злоякісні новоутворення. Встановлено, що екстракт цитоплазматичних мембран у мінімальній із досліджуваних концентрацій (0,2 мкг/мл) достовірно посилює експресію молекул CD86 і HLA-DR та мРНК цитокінів IFN-γ і TNF-α, а також хемокіну CCR7 в генерованих дендритних клітинах. Результати свідчать, що екстракт цитоплазматичних мембран сприяє Т1-хелперній поляризації дендритних клітин і може бути використаний для їх генерації під час виготовлення дендритноклітинних вакцин для імунотерапії онкологічних хворих.

Ключові слова: дендритні клітини, екстракт цитоплазматичних мембран, дендритноклітинні вакцини.

ЭКСТРАКТ ЦИТОПЛАЗМАТИЧЕСКИХ МЕМБРАН *Staphylococcus aureus* Wood 46 КАК ФАКТОР СОЗРЕВАНИЯ ДЕНДРИТНЫХ КЛЕТОК

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Целью исследования было изучение влияния экстракта цитоплазматических мембран *Staphylococcus aureus* Wood 46 на созревание и функциональную активность дендритных клеток, генерированных *in vitro* из моноцитов периферической крови практически здоровых пациентов и больных со злокачественными новообразованиями. Установлено, что экстракт цитоплазматических мембран в минимальной из исследованных концентраций (0,2 мкг/мл) способствует достоверному увеличению экспрессии молекул CD86 и HLA-DR и мРНК цитокинов IFN-γ и TNF-α, а также хемокина CCR7 в генерированных дендритных клетках. Результаты свидетельствуют, что экстракт цитоплазматических мембран способствует Т1-хелперной поляризации дендритных клеток и может быть использован для их генерации при изготовлении дендритноклеточных вакцин для иммунотерапии онкологических больных.

Ключевые слова: дендритные клетки, экстракт цитоплазматических мембран, дендритноклеточные вакцины.