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Comparative Efficiency of Different Techniques for *In Vitro* Maturation of Dendritic Cells.

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ABSTRACT

In antineoplastic therapy, to effectively eliminate neoplastic cells based on the use of CD8+ cytotoxic lymphocytes, it is necessary to acquire a large population of mature antigen-presenting dendritic cells (DC). These cells are required for activation of CD8+ T lymphocytes against surface antigens of neoplastic cells. Since dendritic cells represent a rare subpopulation of peripheral blood mononuclear cells, the most common approach is collection of peripheral blood monocytes and their ex vivo differentiation into dendritic cells with subsequent maturation in the presence of cancer antigens. There are different maturation techniques, though their relative effectiveness is not fully evaluated. In this paper, we conducted a direct comparison of the two methods of inducing dendritic cells maturation: induction with a cytokine based cocktail (IL1-b, TNF-alpha, INF-gamma) and induction through the use of bacterial lipopolyssacharide (LPS). In order to evaluate the effectiveness of each method of generating tumor-specific CD8+ cytotoxic lymphocytes (CTLs), we produced fluorescent protein-expressing cancer cell line, which was used as a target for CTLs. To assess efficiency of each maturation technique, we monitored elimination of fluorescent cancer cells. It was shown that the availability of bacterial lipopolyssacharide in the culture medium contributed to a more effective maturation, production of tumor-specific T lymphocytes and subsequent elimination of CTL cancer cells.

Keywords: dendritic cells, cytotoxic T lymphocytes, lipopolyssacharide.

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INTRODUCTION

Today, in the treatment of cancer, the approaches based on the use of the patient's own immune capacity are becoming increasingly popular. These types of treatment are combined in one big group called cancer immunotherapy. The rapid growth of the popularity of these methods is due to the high degree of safety for the patient compared with conventional treatment methods such as radiotherapy and chemotherapy. The immunotherapeutic methods of malignant neoplasms therapy consist in either stimulating the immune system of the patient through systemic administration of cytokines or interleukins, the administration of agents that make neoplastic cells more accessible to components of the immune system, or in vitro cultivation and expansion of immune cells of the patient and their subsequent introduction in order to enhance immune response against neoplastic cells.

The immunotherapeutic agents either cause enhancement of the immune response or can block "masking" receptors on the surface of the cancer cells. This method does not lead to any toxic effects affecting all body cells and allows the immune system to identify and selectively destroy only cancer cells. Cellular vaccines based on the use of dendritic cells, as well as directive activation of cytotoxic T lymphocytes (CTLs) against either specific cancer antigen or the total mixture of cancer cell antigens, are currently considered the two most promising immunotherapeutic approaches (June, 2007; Palucka and Banchereau, 2013). Both methods involve the use of dendritic cells as a key transmission element which is the antigen to excitatory cells of the immune system. The main point that contributes to quality outcome is the right choice of technique used for the maturation of dendritic cells. Currently, the most common maturation technique of immature dendritic cells population is the use of a panel of several cytokines: IL1-b, TNF-alpha, IL-6 and INF-γ (Li et al., 2012). Also the species of the cytoderm of gram-negative bacteria – lipopolyssacharide (LPS) or products of viral origin – double-stranded viral RNA (dsRNA, double-stranded RNA) can be used for the dendritic cells maturation (Granucci et al., 1999; Ruscanu et al., 2012). In the process of transformation (from immature to mature), dendritic cells undergo a series of phenotypic and functional changes, such as the rearrangement of the molecules of major histocompatibility complex (MHC), reorganization of the cytoskeleton, enhancement of expression of chemokines, cytokines and several proteases, as well as expression of cell adhesion molecules and chemokine receptors (Tan and O'neill, 2005; Jensen and Gad, 2010; Shin et al., 2006). After maturation, dendritic cells become able to migrate to lymphoid organs, where they introduce obtained antigens to helper or cytotoxic T lymphocytes (Behrens et al., 2004; Belz et al., 2002). Thus, the efficiency of the immune response, and, as a result, the whole immunotherapy depends on the quality of dendritic cells maturation process. In this work we have tested different methods of *in vitro* maturation of dendritic cells. It is shown that bacterial lipopolyssacharide (LPS) has the greatest activity in terms of stimulating the maturation of dendritic cells, while the panel of cytokines IL1β, IL6, and TNF-α are less effective.

MATERIALS AND METHODS

Preparation of the peripheral blood monocytes (PBMC) fraction

The peripheral blood monocytes fraction was prepared from 50 ml of blood of a healthy donor by centrifugation in ficoll density gradient. The donor's blood was collected in sterile vacuum tubes containing Li-heparin anti-coagulant agent (Greiner Bio-one, Austria). After sampling, the blood samples were combined and diluted with PBS solution in a ratio of 3:1 (PBS-blood). Further, 35 ml of diluted blood was layered on 15 ml ficoll solution (Life Technologies, USA). The samples were centrifuged at 400g for 30 minutes. The supernatant was removed, the buffy coats above the ficoll solution, containing the mononuclear fraction of blood cells, were transferred into separate tubes, and washed three times with 40 ml of PBS solution followed by centrifugation at 400g for 10 minutes. After that, the cells were centrifuged at 200g over 10 minutes. The washing procedure was repeated three times. Further, the mononuclear fraction was expanded in RPMI-1640 medium plus 10% of fetal bovine serum (FBS) in an amount of $5x10^7$ cells.

Dendritic cells plating and maturation

The mononuclear fraction in the amount of $5x10^7$ was plated in RPMI-1640 nutrient medium containing 10% fetal bovine serum (FBS). The cells were incubated at 37°C in 5% CO_2 atmosphere for 2 hours. After incubation, suspended cells were removed and transferred to another culture dish of 75 cm^2. The RPMI-

1640 (10% FBS) nutritional medium containing 1 ng/ml of GM-CSF and 2 ng/ml of IL-4 (PeproTech, USA) was added to adherent cells (Mody et al., 2015). On the third day of plating, fresh medium RPMI-1640, containing GM-CSF and IL-4 in the same concentration, was added to the cells. On the fifth day, differentiated immature dendritic cells were removed and transferred to 24-well plate at a concentration of $5x10^5$ cells/ml (1 ml/well). Then lysate of RKO line cells (colon carcinoma) was added to dendritic cells as a source of antigen. Cell lysate was prepared by sequential freezing and thawing technique. Freezing of the sample was carried out in liquid nitrogen for 3 minutes, thawing was conducted at 50°C. The procedure was repeated 3 times. The number of RKO cells was taken based on the ratio of 1(DC):10(RKO). Plating with the neoplastic lysate was performed over 24 hours in RPMI-1640 nutrient medium (10% FBS, 1 ng/ml GM-CSF, 2 ng/ml IL-4).

Further, dendritic cells were divided into three groups: in group *a* maturation of dendritic cells was performed by adding bacterial lipopolyssacharide (LPS); in group *b* the maturation was performed using a mixture of IL1-b, TNF-alpha, INF-gamma cytokines; while group *c* included control immature dendritic cells.

Plating and activation of cytotoxic T lymphocytes (CTLs)

The suspension cells obtained at the stage of selection of immature dendritic cells were used as a source of cytotoxic T lymphocytes. Cytotoxic T lymphocytes were isolated by the immune magnetic separation technique, using the EasySep™ Human CD8+ T Cell Enrichment Kit (StemCell, USA) in accordance with the manufacturer's advice. Produced lymphocytes population was expanded in RPMI-1640 (10% PBS) medium. Lymphocytes proliferation was activated by the addition of phytohemagglutinin (PHA) into nutritional medium at a final concentration of 10 µg/ml. The cells were incubated at 37°C in 5% CO_2 atmosphere. After four days of cultivation, lymphocytes were collected, centrifuged at 400g for 10 minutes and re-suspended in 2 ml of RPMI-1640 (10% FBS) nutritional medium. Cell numbers were counted after trypan blue staining on Fuchs-Rosenthal chamber. Subsequently, the primary CTL culture and mature DCs were transferred to 12-well plate in the amount of $5x10^5$ DCs per well and $5x10^6$ CTLs per well that corresponded to the cell ratio of 1(DC):10(CTLs). Joint cultivation was performed for 48 hours in 1.5 ml of RPMI-1640 medium with 10% FBS and a mixture of IL-2 (5 ng/ml) and IL-7 (5 ng/ml) cytokines. The efficiency of dendritic cells maturation process and antigen presentation was tested by comparing the ability of lymphocytes to eliminate transgenic cells of RKO line, expressing fluorescent tagRFP protein. This was achieved by co-cultivation of RKO-tagRFP and activated cytotoxic T lymphocytes in several ratios: 1:10, 1:50, and 1:100 (RKO-tagRFP: CTLs). The efficiency of elimination of cancer cells of RKO line was checked by measuring cell proliferation level using the CyQUANT Cell proliferation Assay and measuring the fluorescence yield on a Microplate Reader Triad LT (Dynex Technologies, USA).

Plasmids and preparation of lentiviral particles

To achieve a sustainable RKO cell line, expressing the tag-RFP gene sequence, we used a delivery system based on lentiviral particles. Packing of lentiviral particles was carried out using a recombinant lentiviral vector pLA-CMV-tagRFP and auxiliary plasmids pVSV-g, pCMV-GAG, pCMV-Rev. Recombinant lentiviral vector pLA-CMV-tagRFP was created on the basis of universal lentiviral vector pLA-CMV-PL3, previously obtained in our laboratory. Cloning the sequence of tagRFP fluorescent protein gene was performed by *XbaI* and *BamHI* sites located in the 3'-terminal region relative to CMV-promoter (Fig. 1).

Previously, the tagRFP gene sequence was produced by the polymerase chain reaction (PCR) using primers with the laid restriction sites:

tagRFP Direct XbaI 5' 3 AGAGATCTAGAATGGTGTCTAAGGGCGAA' ,
tagRFP BamHI Reverse 5' AGAGAGGATCCTTAATTAAGTTTGTGCCCCAGTT 3'.

The tagRFP sequence was produced using a commercial plasmid pTagRFP-C vector (Evrogen, Russia).

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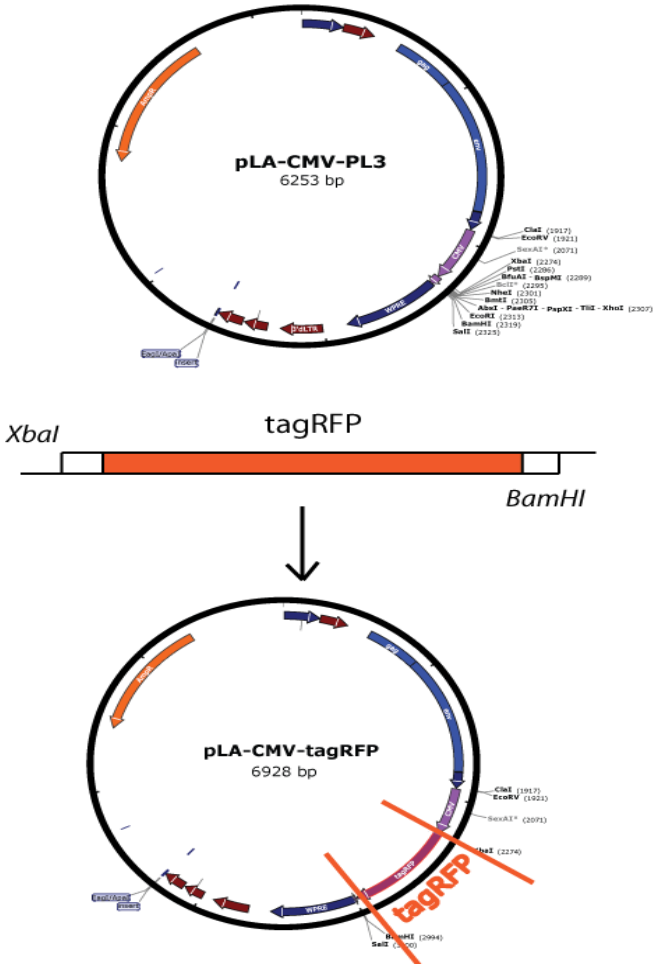

Figure 1: The diagram of tagRFP gene sequence cloning into pLA-CMV-PL3 plasmid DNA.

The resulting pLA-CMV-tagRFP plasmid contains tagRFP gene sequence cloned by XbaI-BamHI sites.

Packing of the recombinant lentiviral particles was performed in the HEK 293T cell line. Transfection of the HEK 293T cell line was performed using the TurboFect cationic reagent (Life Technologies, USA). Viral supernatants were collected within 48 and 72 hours after transfection. To precipitate viral particles we used polyethylene glycol 6000 (50% solution). After collection of culture medium we added 25% sterile solution of 50% PEG 6000, 10% 4M NaCl and 10% PBS. Samples were incubated for 10 hours at 4°C and then centrifuged at 7000g for 30 minutes. Further, the supernatant was removed, and the precipitate was dissolved in PBS, taken in an amount of 10% of original volume. Since, to some extent PEG 6000 is toxic to cells, we carried out a more thorough purification of viral stocks. For a more complete purification against PEG 6000, viral particles were concentrated by ultracentrifugation at 40,000 rpm over 40 min on a Beckman TL100 (USA) ultracentrifuge, followed by re-suspending in PBS volume taken in the amount equal to the original.

Infection of the RKO cell line was carried out in DMEM serum-free culture medium with the addition of polybrene at a final concentration of 8 µg/ml. After 24 hours, cells were transferred into fresh culture medium DMEM (10% FBS). The efficiency of the fluorescent protein expression was checked within 72 hours after viral infection using a fluorescence microscope. The cells, possessing the required level of expression of the fluorescent marker, were separated by sorting on a BD FacsVantage SE cell sorter (BD, USA).

RESULTS AND DISCUSSION

The availability of the tagRFP gene sequence insertion segment was analyzed by PCR and restriction analysis (Fig. 2). The vector was also sequenced using the following primers:

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Direct 5' CAAAAACAAATTACAAAAATTCAAAATTTT 3'
Reverse 5' CAAATTTTGTAATCCAGAGGTTGATT 3'

Sequencing was carried out at the Evrogen Company (Russia).

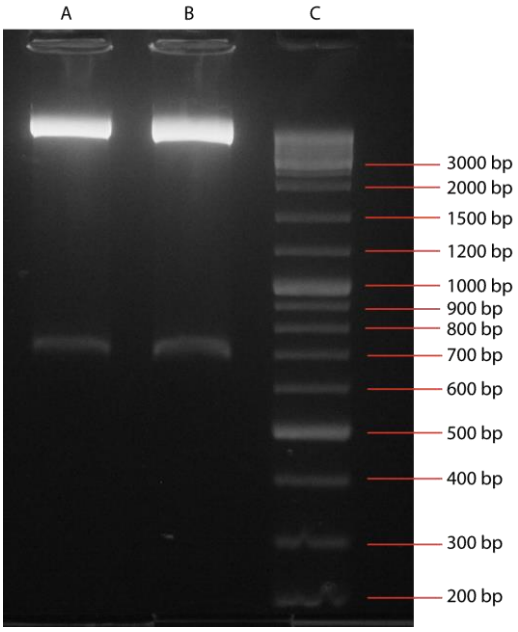

Figure 2: Analysis of lentiviral vector pLA-CMV-tagRFP:

A) PCR of the tagRFP gene; *B)* Restriction of the pLA-CMV-tagRFP plasmid at *XbaI* and *BamHI* sites; *C)* The molecular length marker GeneRuler DNA ladder mix 100-10000 bp (Invitrogen).

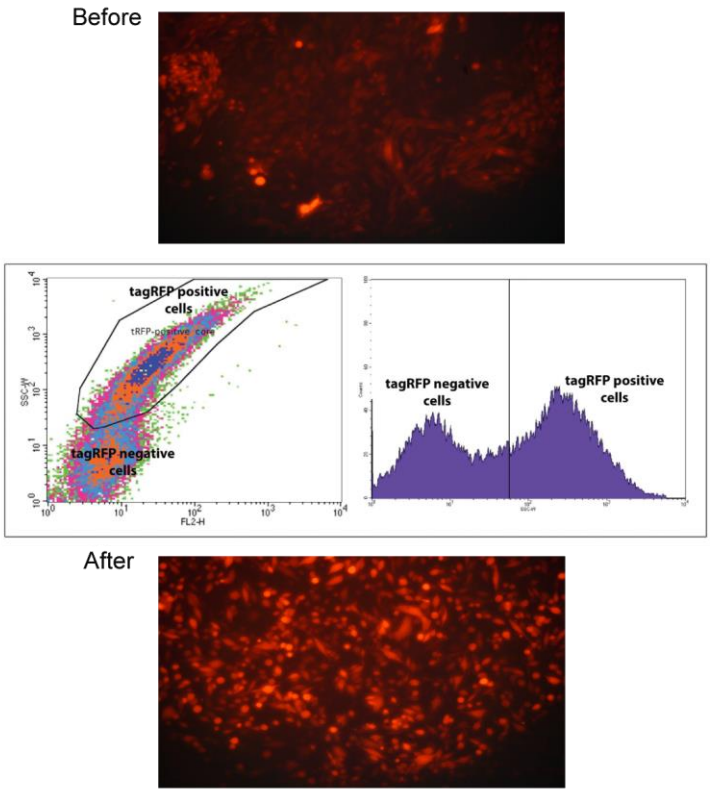

Figure 3: Selection of the RKO line cells expressing tagRFP fluorescent protein. The figure shows the selection outcome (before and after) of transgenic RKO line cells. Selection was performed using BD FacsVantage SE cell sorter (Beckton Dickinson, USA)

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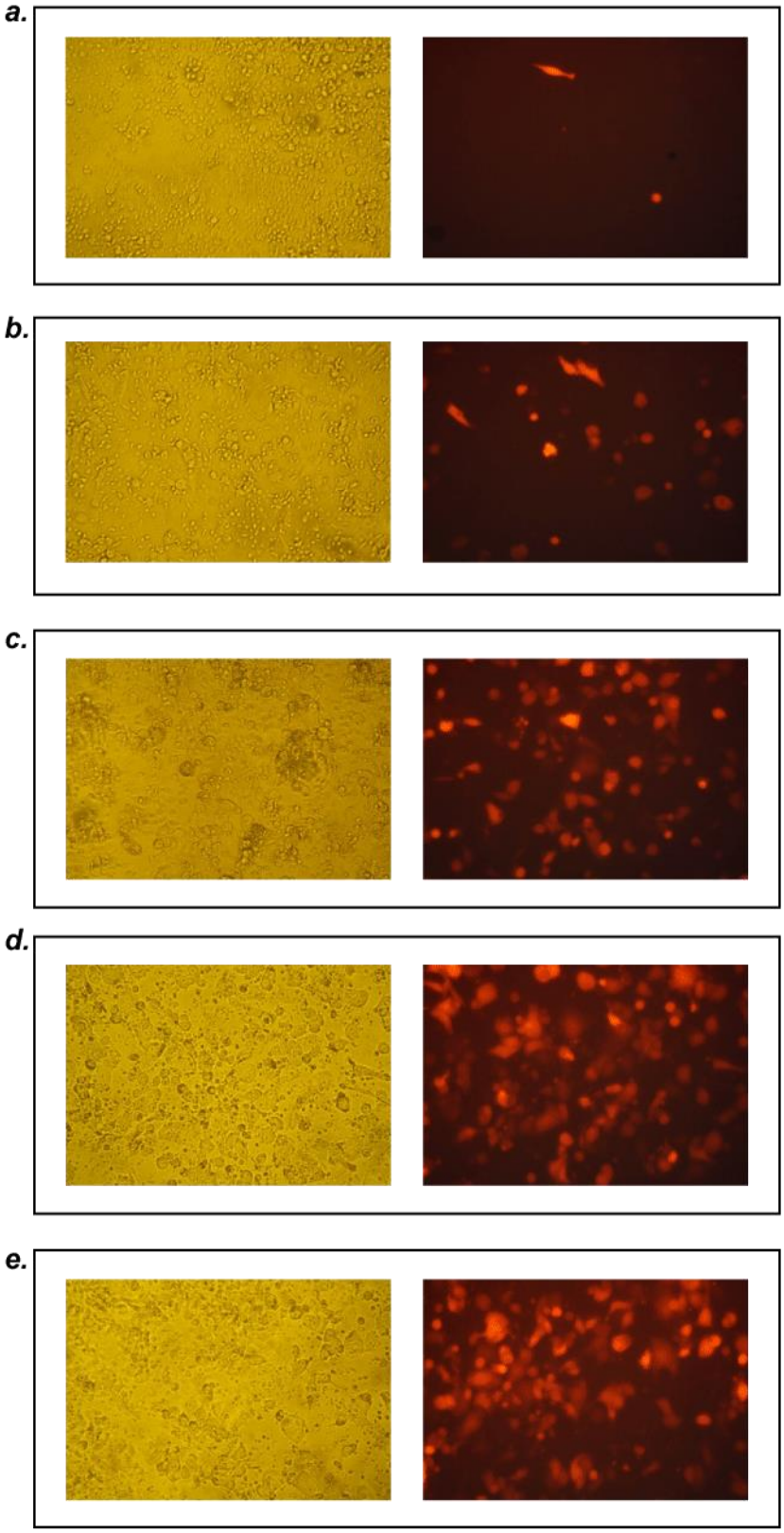

Figure 4: Fluorescent photomicrography of RKO-tagRFP culture after co-plating with cytotoxic T-lymphocytes, produced using dendritic cells activated by different techniques:

a) maturation of dendritic cells was carried out using bacterial lipopolyssacharide (LPS); *b)* maturation was performed using the cytokine based cocktail of IL-1β, TNF-alpha, and INF-gamma; *c)* control culture – T lymphocytes planted with non-maturated dendritic cells; *d)* control – T lymphocytes non-cultured with dendritic cells;
e) population of the RKO line cells without addition of the T lymphocytes.

The results of sequencing confirmed the availability of tagRFP gene insertion segment in lentiviral vector pLA-CMV-PL3. Further, using this plasmid we have obtained a sustainable RKO cell line, expressing the sequence of tagRFP fluorescent protein gene. To obtain sustainable line, we used lentiviral delivery technique of the target DNA sequence. This technique has considerable advantage over the transient introduction of the plasmid DNA due to the fact that lentiviral vectors allow quick integrating a significant amount of genetic material into the genome of host cells and obtaining sustainably expressing transgene cell line within a short time. For preparation of lentiviral particles we used the HEK 293T cell line. This line expresses a sequence of a large T antigen of SV40 virus, which is necessary for episomal replication of the vector plasmids containing lentivirus gene. After transfection of the HEK 293T cell line we collected nutritional medium and performed viral particles concentration. Then we infected the RKO cell line. The expression of tagRFP fluorescent protein sequence was checked using a fluorescence microscope. The level of tagRFP gene expression has reached its maximum within 36 hours of cells plating in the DMEM/F12 nutrient medium with 10% FBS. To remove cells not expressing fluorescent protein, as well as to produce the cell line, useful for visualization of the results of subsequent experiments, we carried out selection of the RKO line cells by cell sorting technique and selected cells, characterized by the most intense fluorescence within the red range that corresponded to the fluorescence of the tagRFP protein (Fig.3).

Dendritic cells were produced from blood monocytic fraction using a standard technique. For this purpose the population of blood monocytes was cultivated in a nutrient medium RPMI 1640 (10% FBS) with the addition of rhIL-4 and rhGM-CSF (Elkord et al., 2005).

On the first day of cultivation, the monocytic fraction was a homogeneous adherent population. After three days of cell plating on a nutrient medium with cytokines the number of suspension cells increased significantly. On the sixth day of cultivation, the whole population consisted of purely suspension culture with a small quantity of cytoplasmic outgrowths. After adding antigen and cultivation with maturity factors, dendritic cells still consisted of suspension cell culture. At that, the shape of cells, characterized by the presence of a large number of cytoplasmic outgrowths, was significantly changed that confirmed indirectly the maturation process. The measurement of the DC maturation efficiency was determined by the rate of elimination of cancerous cells by cytotoxic T lymphocytes. The fluorescence measurement results are presented in Fig. 4.

The results of co-plating of cytotoxic T lymphocyte and RKO-tagRFP cell line were visualized by light and fluorescent microscopy (40-fold magnification). In the conducted experiment the elimination rate of RKO-tagRFP cancer cell line depended on the efficiency of transmission of cancer antigens by dendritic cells to cytotoxic T lymphocytes. In turn, the effectiveness of the antigenic determinants presentation depends directly on the chosen dendritic cells maturation method. The elimination rate of cancer cells was calculated relatively to RKO-tagRFP control culture, cultivated without addition of cytotoxic T lymphocytes. At that, a low fluorescence yield indicates high level of cancer cells elimination. To obtain quantitative data on the elimination rate, we used two different methods – the determination of the proliferating cells number by a commercial kit CyQUANT Cell proliferation Assay, and direct measurement of sample fluorescent yield by a microplate fluorimeter. Data obtained using the CyQUANT Cell proliferation Assay poorly correlated with visualization data on the fluorescence microscope due to the contribution of cytotoxic T lymphocytes. On the contrary, direct measurements of the red fluorescence by a microplate fluorimeter correlated well with the visualization data. It was determined that the fluorescence yield of the conditional groups was as follows: *a)* (DCs maturation using LPS) - 10715 (12.77%); *b)* (DCs maturation using a cytokine based cocktail) - 20522 (24.47%); *c)* (use of non-maturated DCs) - 38137 (45.46%); *d)* (co-plating with non-activated T lymphocytes) - 69883 (83%); *e)* (control culture of RKO-tagRFP) - 84659 (100%).

Thus, based on the data obtained it can be concluded that the use of bacterial lipopolyssacharide is the most optimal DCs maturation technique that can be used in further studies on the application of DCs and CTLs in the field of immunotherapy. In addition, the use of bacterial lipopolyssacharide is most economically advantageous due to the low cost of the reagent that becomes especially noticeable when using immunotherapeutic approaches in vivo, because of the need for large amounts of DCs.

CONCLUSIONS

We have compared the effectiveness of different DCs maturation techniques for their use in immunotherapy towards production of tumor-specific fraction of cytotoxic T lymphocytes. To carry out such comparison we created RKO-tagRFP reporter cell culture expressing fluorescent protein that allowed us to accurately assess the efficiency of elimination of cancer cells by cytotoxic T lymphocytes, produced using various techniques. As a result of comparison of various DCs maturation techniques, it was revealed that the use of lipopolyssacharide allows achieving greater cytotoxic effect from the application of CTLs than the use of the cytokine based cocktail of IL-1β, TNF-alpha and INF-gamma.

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REFERENCES

[1] Behrens, G., Li, M., Smith, C.M., Belz, G.T., Mintern, J., Carbone, F.R., and Heath, W.R. 2004, "Helper T Cells, Dendritic Cells and CTLS Immunity," Immunology & Cell Biology, *82*, pp. 84-90.

[2] Belz, G.T., Carbone, F.R., and Heath, W.R., 2002, "Cross-Presentation of Antigens by Dendritic Cells," Critical Reviwes in Immunology, *22*, pp. 439-448.

[3] Elkord, E., Williams, P.E., Kynaston, H., and Rowbottom, A.W., 2005, "Human Monocyte Isolation Methods Influence Cytokine Production from in vitro Generated Dendritic Cells," Immunology *114*, pp. 204-212.

[4] Granucci, F., Ferrero, E., Foti, M., Aggujaro, D., Vettoretto, K., and Ricciardi-Castagnoli, P., 1999, "Early Events in Dendritic Cell Maturation Induced by LPS. Microbes & Infection, *1*, pp. 1079-1084.

[5] Jensen, S.S., and Gad, M., 2010, "Differential Induction of Inflammatory Cytokines by Dendritic Cells Treated with Novel TLR-Agonist and Cytokine based Cocktails: Targeting Dendritic Cells in Autoimmunity. Journal of Inflammation, *7*, pp. 37-40.

[6] June, C.H., 2007, "Principles of Adoptive T-Cell Cancer Therapy," Journal of Clinical Investigation *117*, pp. 1204-1212.

[7] Li, D.Y., Gu, C., Min, J., Chu, Z.H., and Ou, Q.J., 2012, "Maturation Induction of Human Peripheral Blood Mononuclear Cell-Derived Dendritic Cells," Experimental and Therapeutic Medicine, *4*, pp. 131-134.

[8] Mody, N., Dubey, S., Sharma, R., Agrawal, U., and Vyas, S.P., 2015, "Dendritic Cell-Based Vaccine Research against Cancer," Expert Review of Clinical Immunology, *11*, pp. 213-232.

[9] Palucka, K., and Banchereau, J., 2013, "Dendritic-Cell-based Therapeutic Cancer Vaccines," Immunity, *39*, 38-48.

[10] Ruscanu, S., Pascale, F., Bourge, M., Hemati, B., Elhmouzi-Younes, J., Urien, C., Bonneau, M., Takamatsu, H., Hope, J., Mertens, P.*, et al.,* 2012, The Double-Stranded RNA Bluetongue Virus InducesTtype I Interferon in Plasmacytoid Dendritic Cells via a MYD88-dependent TLR7/8-independent Signaling Pathway," Journal of Virology, *86*, pp. 5817-5828.

[11] Shin, J.S., Ebersold, M., Pypaert, M., Delamarre, L., Hartley, A., and Mellman, I., 2006, "Surface Expression of MHC Class II in Dendritic Cells is Controlled by Regulated Ubiquitination," Nature, *444*, pp. 115-118.

[12] Tan, J.K., and O'Neill, H.C., 2005, "Maturation Requirements for Dendritic Cells in T-Cell Stimulation Leading to Tolerance versus Immunity. Journal of Leukocyte Biology, 7*8*, pp. 319-324.