Expression of tolerogenic potential-representing markers on clinical-grade therapeutic dendritic cell-based cancer vaccines

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Background. Currently a particular interest is given to specific active cancer immunotherapy (therapeutic cancer vaccination) strategies such as treatment with specially "educated" autologous dendritic cells (DCs). The purpose of these vaccines is to enhance antitumor immune responses against the existing tumor. DC vaccines are composed of tumor antigen-loaded mature DCs, which are produced for each cancer patient individually. However, recent data indicate that there are at least two types of mature DCs – immunogenic, which activate antitumor immune responses, and tolerogenic, which suppress immune responses, thereby interfering with effector mechanisms of protective antitumor immunity.

Hence, the aim of our study was to assess the expression of immunogenic and tolerogenic potential-representing markers on the surface of therapeutic DC vaccines generated using two different maturation approaches.

Materials and methods. Two different DC maturation approaches were investigated: Cocktail 1, composed of lipopolysaccharide (200 ng/mL) and interferon-γ (50 ng/mL), and Cocktail 2, composed of IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (10 ng/mL), PGE$_2$ (1 µg/mL). Secretion of two basic cytokines – the immunostimulatory IL-12p70 and the immunosuppressive IL-10 – has also been investigated.

Results. We show that a subset of immature DCs expressed tolerogenic markers. Importantly their expression profile considerably differed on mature DCs, depending on the maturation approach used.

Conclusions. In particular, our results indicate that Cocktail 1 is superior to Cocktail 2 for the production of clinical-grade therapeutic cancer DC vaccines, both in terms of immunophenotypical attributes of DC tolerogenicity and their cytokine secretory profile.

Key words: cancer immunotherapy, dendritic cell vaccine, immunogenic dendritic cells, tolerogenic dendritic cells

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INTRODUCTION

Tumor growth and progression are closely related to a diminished ability of the immune system to recognize and destroy cancer cells (1). Thus antitumor immunotherapy is applied in order to restore immune responses against malignant cells (2). Currently particular interest is given to specific active immunotherapy strategies such as therapeutic vaccination with tumor antigen-loaded and properly matured autologous dendritic cells (DCs) (3). The aim of these vaccines is to enhance antitumor immune responses against the existing tumor. Mostly DC vaccines are produced for each cancer patient individually using autologous monocytes isolated from peripheral blood.

Currently DC vaccination is widely applied worldwide with clinical responses achieved in up to 54% of patients (4, 5). Therefore DC immunotherapy should be individualized in order to select cancer patients who would most likely respond to this kind of treatment. One possible approach of individualization is a more detailed characterization of DCs in the vaccine preparation. Currently the suitability of DC vaccines for clinical application is mainly based on the evaluation of biomarkers representing DC maturation and activation (CD83, CD80, CD86, HLA-DR, etc.) and in some cases also on the results of functional assays, such as autologous or allogeneic mixed lymphocyte reaction (6). However, there is increasing evidence that a mere evaluation of these DC characteristics (especially their phenotype alone) may be insufficient or even misleading since there are at least two types of mature DCs – immunogenic, which induce and augment antitumor immune responses, and tolerogenic, which block the generation of antitumor immune responses. Thus additional research is needed for a more precise characterization of DC vaccines and prediction of their potential clinical efficacy. In this study we investigated the cytokine secretory profile and its association with the expression of biomarkers representing the immunogenic versus the tolerogenic potential of therapeutic DC vaccines individually produced for patients with urogenital tumors.

MATERIALS AND METHODS

Study population

32 patients with primary urogenital cancer (prostate adenocarcinoma, renal cell carcinoma or urinary bladder urothelial carcinoma) treated at the Institute of Oncology, Vilnius University were enrolled in the study during the period ranging from September 2011 to October 2012. All patients signed an informed patient consent approved by the local bioethics committee.

Collection of blood samples

For the research purposes, thirty two milliliters of peripheral blood were collected into CPT™ vacutainers (BD Biosciences, USA) before surgery. Vacutainers were gently vortexed to avoid blood coagulation and transported directly to the laboratory for the production of DC vaccines.

Generation of DC vaccines

Peripheral blood mononuclear cells (PBMC) were separated from the whole blood by density gradient centrifugation at $1\ 500 \times g$ for 20 min at room temperature. PBMC were washed twice and centrifuged at $250 \times g$ for 10 min. The cells were resuspended in *X-VIVO* medium (Lonza Walkersville, Inc., USA) and cultured in 6-well plates (Sigma, Germany) at a concentration of 5×10^6 cells/mL (3 mL per well) at 37 °C in a humified 5% CO_2 atmosphere. After 2 hours of incubation the medium with non-adherent cells was removed, whereas adherent monocytes were resuspended in *X-VIVO* medium containing 2% of fetal calf serum and supplemented with 50 ng/ml of the granulocyte-macrophage colony stimulating factor (GM-CSF) (eBioscience) and 50 ng/ml of the interleukin (IL)-4 (BD Biosciences, USA). Monocytes were incubated for 6 days at 37 °C in a humified 5% CO_2 atmosphere. On day 3, the cell culture medium was replaced with a fresh medium. The differentiation of monocytes into immature DCs was examined every day using a light microscope Nikon Eclipse TS10 (Nikon, Japan). On day 6, the generated immature DCs were resuspended in fresh *X-VIVO* medium and incubated for 4 hours with autologous tumor lysate, containing 30 μg of proteins. Tumor lysate was prepared by four freezing-thawing cycles from $1\ cm^2$ of the autologous tumor sample. After 4 hours of incubation with tumor lysate, cell culture medium

was supplemented with the maturation stimuli. Two maturation cocktails were used, including Cocktail 1 composed of IFN-γ (50 ng/mL) (eBioscience) and LPS (200 ng/mL) (BD Biosciences, USA) and Cocktail 2, composed of IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (10 ng/mL) and PGE$_2$ (1 μg/mL) (all from Calbiochem, USA). Immature DCs were incubated for 24 hours at 37 °C in a humified 5% CO$_2$ atmosphere. After the induction of maturation, DCs were harvested, washed, counted and used for the evaluation of surface marker expression by multicolor flow cytometry, using a LSR II cytometer (BD Biosciences, USA). In addition, 1.5×10^6 of mature DCs were resuspended in *X-VIVO* medium supplemented with CD40L (5 μg/mL; eBioscience) and incubated for 24 hours at 37 °C in a humified 5% CO$_2$ atmosphere. Cell culture supernatants were collected and stored at –70 °C until ELISA analysis was performed for the evaluation of IL-12p70 and IL-10 secretion by mature, CD40L-restimulated DCs.

Morphological evaluation of DCs
A sample of DCs for morphological analysis was randomly taken from 2 wells of a 6-well plate. DCs were dessicated, fixed directly with 96% ethanol and stained using a modified Pap (Papanicolaou) staining method according to the instructions of the manufacturer. The cells were analyzed under the light microscope Nikon Eclipse TS10 (Nikon, Japan) using ×100 and ×400 magnifications.

Analysis of DC surface marker expression by multicolor flow cytometry
Sample staining
For the 6-color flow cytometry-based identification of DCs, a combination of the following monoclonal antibodies was used: CD83-PE-Cy5, HLA-DR-Horizon V500, CD80-Horizon V450, CD85k-PerCP-eFluor 710, CD273-PE, CD274-FITC (all from BD Biosciences, USA). Isotype controls for each antibody (IgG1/IgG2a/IgG2b) (BD Biosciences, USA) were used to determine non-specific detection antibody binding and fluorescence background.

Samples containing 1×10^6 cells were incubated with 20 μl of each monoclonal antibody for 20 minutes at room temperature in the dark. Cells were washed twice with Cell WASH solution (BD Biosciences, USA) and fixed with CellFIX solution (BD Biosciences, USA).

Flow cytometric analysis
Data acquisition was performed on a LSR II flow cytometer (BD Biosciences, USA) equipped with three lasers (VioFlame 405 nm, Sapphire blue 488 nm and HeNe 633 nm) and capable of 8-color analysis. Instrument was calibrated weekly following manufacturer's instructions. Unstained and single color control samples were collected to calculate the compensation matrix. For each multi-stained sample 100 000 events were acquired. Forward scatter (FSC) and side scatter (SSC) signals were plotted in a linear mode and fluorescent signals were plotted in a logarithmic mode. The green fluorescence (FITC) was collected through a 530/30 nm bandpass filter, orange / red (PE) through a 585/42 nm bandpass filter, violet (Horizon V500) through a 525/50 nm bandpass filter, red (Alexa Fluor 647) through a 660/20 nm bandpass filter, blue (PE-Cy5) through a 670/14 nm bandpass filter, red (PerCP-eFluor 710) through a 750/60 nm bandpass filter. Flow Cytometry Standard (FCS) files were analyzed using BD FACSDiva 6.0 software (BD Biosciences, USA).

Gating strategy
Debris and dead cells were gated out. Dendritic cells were gated by their characteristic forward and side scatter. Maturation marker CD83 was used to detect immature DCs (CD83 low) and mature DCs (CD83 high). Each group was exposed for further analysis of their immunogenic and tolerogenic potential-representing markers.

Analysis of DC cytokine secretory profile by ELISA
Concentrations of IL-12p70 and IL-10 were measured in culture supernatants of mature DCs by the Enzyme-Linked Immunosorbent Assay (ELISA) according to the instructions of the manufacturers (Invitrogen Human IL-10 and Invitrogen Human IL-12+p40).

Statistical analysis
The results were analyzed using descriptive and comparative statistical methods. Average of age at diagnosis and concentration of interleukins, as well as 95% confidence intervals (CI) of the average were calculated. Significance testing of average equality was performed using an unpaired Student's

t-test considering that standard deviations are unequal. The inequality of standard deviations was confirmed using a F-test. All the calculations were done using the data analysis and statistical software package Stata 11.

Statistical analyses of flow cytometry data were performed using a non-parametric Mann-Whitney *U* test.

RESULTS

Patient's characteristics

During the study period ranging from September 2011 to October 2012 thirty two patients (24 men and 8 women) with primary urological cancer (18 with prostate adenocarcinoma, 12 with renal cell carcinoma, and 2 with urinary bladder urothelial carcinoma) were enrolled in the study. The average age of the patients was 63.45 (95% CI 60.17–66.73) years. The majority of patients (n = 21; 66%) had stage I and II disease. Patient characteristics are shown in the Table.

Cell yields

The average concentration of PBMC after density gradient centrifugation was 28.86×10^6/mL (95% CI 22.86–34.86). The final concentrations of fully mature DCs were 6.73×10^6/mL (95% CI 1.84– 11.62) using maturation Cocktail 1 (LPS, 200 ng/ mL + IFN-γ, 50 ng/mL) and 5.57×10^6/mL (95% CI 2.38–8.75) using maturation Cocktail 2 (IL-1β, 10 ng/mL + IL-6, 10 ng/mL + TNF-α, 10 ng/ mL + PGE$_2$, 1 μg/mL). No statistically significant differences in the concentrations of fully mature DCs were found between the two maturation methods (p > 0.05).

Further in the article DCs matured with Cocktail 1 are defined as DC-1, whereas DCs matured with Cocktail 2 are defined as DC-2.

Evaluation of DC morphology

Morphological evaluation of unstained DCs was performed on day 3 (immature DCs) and day 7 (mature DCs). In addition, mature (day 7) DCs were stained using a modified Pap method for a more accurate assessment of their morphology. On day 3, the presence of non-adherent (loosely adherent) immature DCs, scattered between adherent monocytes, was observed (Fig. 1). On day 7, typical morphology of mature DCs, characterized by larger amount and longer dendrites protruding out from the cell surface, was observed. Importantly, the morphological features characteristic of mature DCs were apparently more pronounced in DCs matured with Cocktail 1 compared with those matured with Cocktail 2 (Fig. 2).

Table. Patient distribution according to the localization and stage of the primary cancer

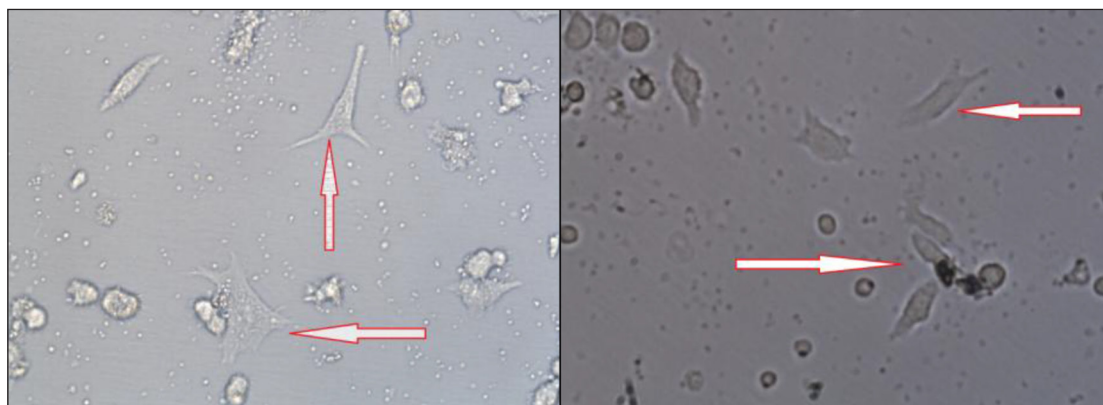| Stage / localization	Stage I	Stage II	Stage III	Stage IV	Unspecified
Renal cell carcinoma	6	–	2	3	1
Prostate cancer	4	11	1	–	2
Bladder cancer	–	–	1	1	–

Fig. 1. Unstained immature DCs (shown by arrows) on the 3rd culture day of monocytes in *X-VIVO* medium, supplemented with 50 ng/ml GM-CSF and 50 ng/ml IL-4. ×400 magnification

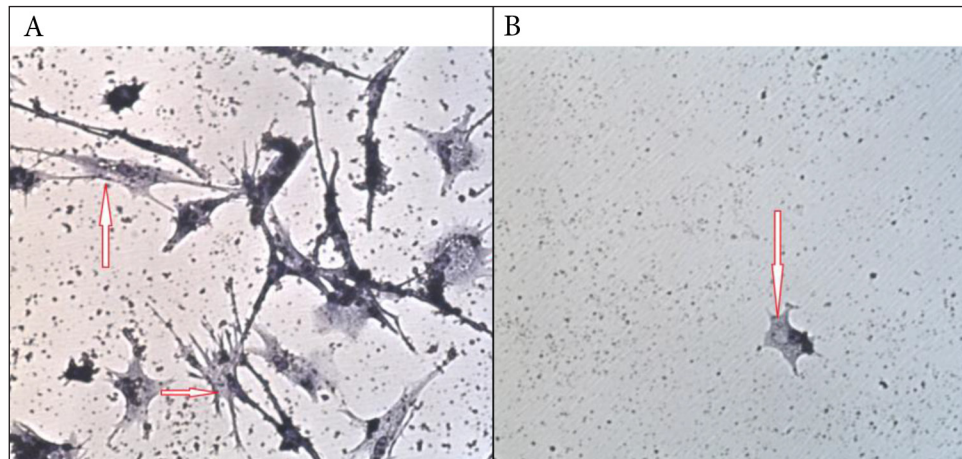**Fig. 2.** Mature DCs stained using a modified Pap stain, ×400. 7th cultivation day; A – DCs matured with Cocktail 1, B – DCs matured with Cocktail 2

Analysis of DC surface markers

Expression of immunogenic (CD80, CD86, HLA-DR) and tolerogenic (CD273, CD274, CD85k) properties-representing biomarkers was analyzed on the surface of immature (CD83low) and mature (CD83high) DCs by multicolor flow cytometry. In addition, surface expression of the migratory potential-representing chemokine receptor CCR7 was analyzed on both immature and mature DCs (data not shown).

Expression of CD83 on DCs

High surface expression of CD83 is a selective characteristic of human DC maturation (7). Consistent with this notion, we found that before the induction of DC maturation expression of CD83 on their surface was low (CD83low), whereas after DC incubation with the maturation-inducing stimuli, a considerable proportion of DCs showed high surface expression of CD83 (CD83high), indicating the appearance of mature DCs. Notably, our results showed that a significantly greater proportion of mature CD83high DCs was generated using maturation Cocktail 1 compared with Cocktail 2 (88.5 ± 20.3% and 67.2 ± 15.3%, respectively, p = 0.041).

Surface expression of immunogenic potential-representing biomarkers (CD80, CD86, HLA-DR) on immature (CD83low) and mature (CD83high) DCs

As expected, we found that before the induction of maturation only a negligible proportion (10.4 ± 2.4%) of immature CD83low DCs showed high surface expression of immunogenic potential-representing markers. Since these DCs do not express the main maturation marker CD83, but express other activation / maturation markers it is very likely that they represent a small population of DCs in the transitional differentiation stage between immature and mature DCs. In addition, we also investigated CCR7 expression on both immature and mature DCs (data not shown). We found that CCR7 was either absent or low on immature CD83low DCs with low expression levels of immunogenic markers. However, significantly greater proportion of DCs with intermediate or even high CCR7 expression was found in a minor population of CD83low DCs with high expression of immunogenic markers, further endorsing our notion that these cells represent a subset of transitional differentiation stage DCs.

After the induction of maturation, a considerable proportion of CD83high DCs expressed high levels of immunogenic potential-representing biomarkers. Importantly, we found that high surface expression of these biomarkers was significantly higher on DC-1 compared with DC-2 (88.4 ± 2.6% and 77.5 ± 7.4%, respectively, p = 0.043). Therefore it is evident that after the induction of maturation not all DCs have undergone maturation and there still were DCs with low expression of CD83, namely 12.6% of DC-1 and 22.5% of DC-2 were CD83low. Interestingly, in these remaining CD83low cells, there were significantly more DCs expressing high levels of at least two immunogenic biomarkers (transitional DCs) in DC-1 compared with DC-2 (74.8 ± 20.2% versus 37.4 ± 10.2%, respectively, p = 0.027). Collectively these results strongly imply
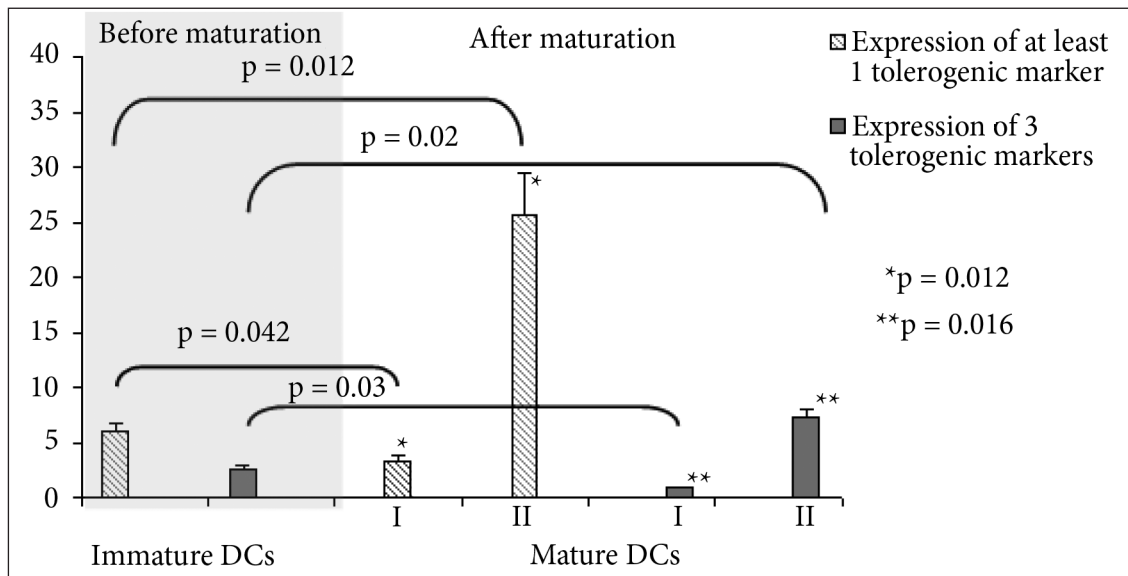
Fig. 3. Expression of tolerogenic markers on immature DCs before maturation and on mature DCs with high expression of immunogenic markers after maturation. I – maturation Cocktail 1 (LPS + IFN-γ), II – maturation Cocktail 2 (TNF-α + IL-1β + IL-6 + PGE$_2$)

that a combination of LPS and IFN-γ is superior to the so-called "standard" cocktail (IL-1β, IL-6, TNFα, PGE$_2$) for the induction of immunogenic DC maturation.

Surface expression of tolerogenic potential-representing biomarkers (CD85k, CD273, CD274) on immature (CD83^{low}) and mature (CD83^{high}) DCs
The expression of tolerogenic properties-representing biomarkers was analyzed in combination with immunogenic properties-representing biomarkers at a single cell level on both CD83^{low} and CD83^{high} DCs.

We found that before the induction of DC maturation there were 6 ± 1.1% of cells expressing at least one tolerogenic marker and 2.7 ± 0.4% of cells expressing all three tolerogenic markers on immature CD83^{low} DCs with low expression levels of immunogenic markers (Fig. 3). Similarly, in a minor population of CD83^{low} DCs with high expression levels of immunogenic markers (transitional DCs), there were 2.6 ± 0.3% of cells expressing at least one tolerogenic marker and 1.23 ± 0.15% of cells expressing all three tolerogenic markers (Fig. 4).

We found that after the induction of DC maturation, surface expression of either one or all three tolerogenic markers was significantly higher on both the predominant CD83^{high} DCs and the remaining CD83^{low} matured with Cocktail 1 compared with Cocktail 2 (Figs. 3, 4). Notably we found that a substantial proportion of CD83^{high} DCs with high expression of immunogenic markers (the desired cells in the vaccine preparation) also expressed at least one tolerogenic potential-representing marker. Most importantly, this was preferentially observed in DC-2 compared with DC-1. In particular, 3.4 ± 0.7% and 0.9 ± 0.05% of DC-1 expressed at least one tolerogenic marker and all three tolerogenic markers, respectively. However, there were even 25.6 ± 4.7% of DC-2 expressing at least one tolerogenic marker and 7.4 ± 0.8% of DC-2 expressing all three tolerogenic markers (Fig. 3). The same pattern of significantly higher expression of tolerogenic potential-representing markers was observed on DC-2 versus DC-1 in CD83^{low} populations with both high and low expression of immunogenic potential-representing markers in the final DC vaccine preparation (Fig. 4).

Evaluation of IL-12p70 and IL-10 secretion by mature DCs
For further evaluation of the immunostimulatory and immunosuppressive potential of mature DCs, a secretion profile of IL-12p70 and IL-10 was measured in mature, CD40L-restimulated DC cultures by ELISA.
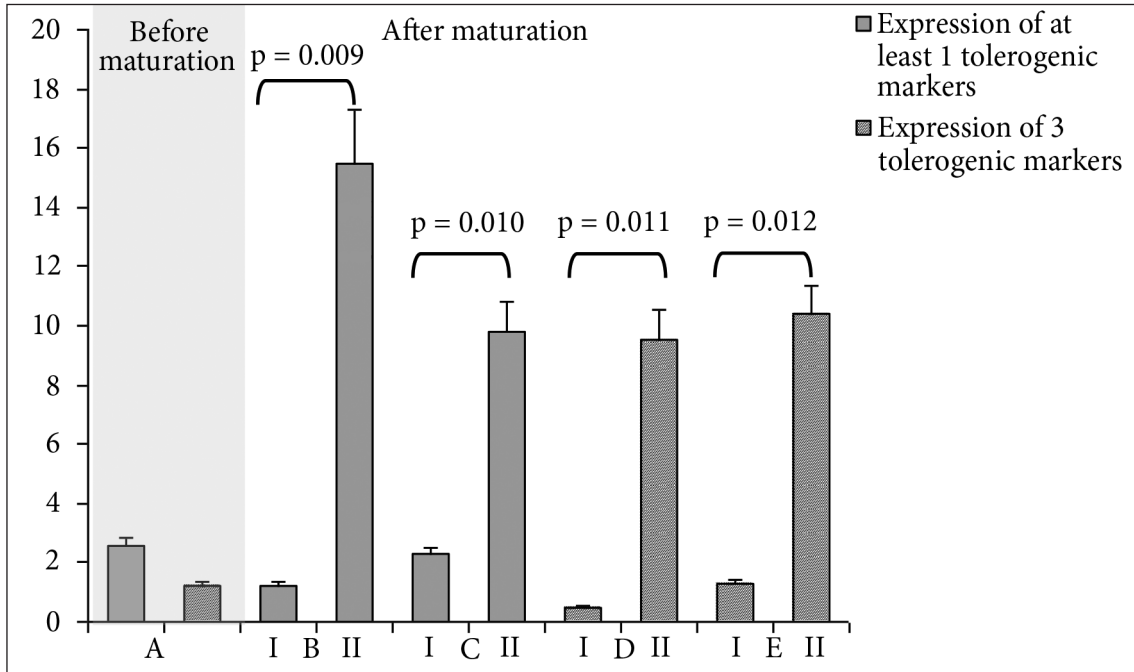
Fig. 4. Expression of tolerogenic markers on immature DCs before and after the induction of DC maturation. A, B, D – immature DCs with high expression of at least two immunogenic markers, C, E – immature DCs with low expression of at least two immunogenic markers, I – maturation Cocktail 1 (LPS + IFN-γ), II – maturation Cocktail 2 (TNF-α + IL-1β + IL-6 + PGE$_2$)

Secretion of the immunostimulatory cytokine IL-12p70 by mature DCs

Average concentrations of the immunostimulating, Th1-polarizing cytokine IL-12p70 were 9.51 pg/mL [95% CI 3.68–15.34] and 5.26 pg/mL [95% CI 0.52–11.05] in the supernatants of DC-1 and DC-2, respectively. Therefore the secretion of IL-12p70 by mature DCs was 40% higher in DC-1 compared with DC-2. Even more apparent differences in IL-12p70 secretion between DCs matured with the two different cocktails were observed in a group of patients. Namely, in 8 of 32 (25%) patients the secretion of IL-12p70 was 3.6 fold higher by DC-1 versus DC-2 (Fig. 5). In these 8 patients the average
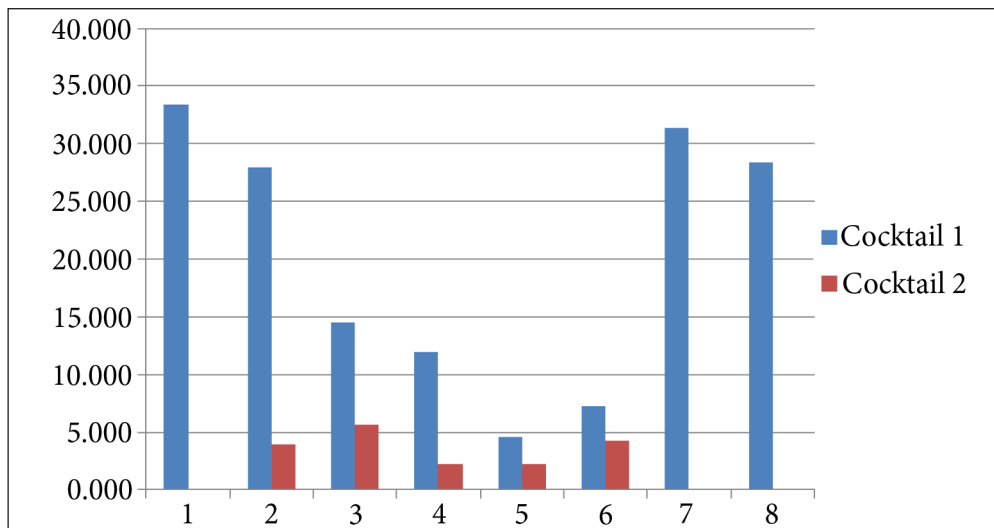

Fig. 5. Differences in the secretion of the immunostimulatory cytokine IL-12p70 by DCs matured with Cocktail 1 and Cocktail 2
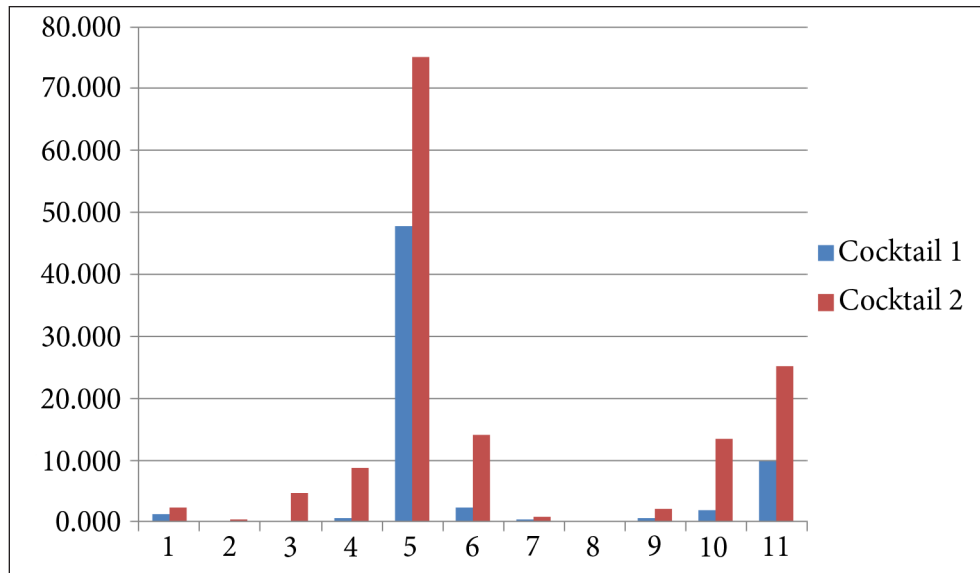
Fig. 6. Differences in the secretion of the immunosuppressive cytokine IL-10 by DCs matured with Cocktail 1 and Cocktail 2

concentrations of IL-12p70 in the supernatants of DC-1 and DC-2 were 13.26 pg/mL [95% CI 1.98–24.54] and 3.66 pg/mL [95% CI 1.86–5.46], respectively.

Secretion of the immunosuppressive cytokine IL-10 by mature DCs

We found that average concentrations of IL-10 were 8.52 pg/mL [95% CI –0.79–17.84] and 12.28 pg/mL [95% CI 0.50–24.06] in the supernatants of DC-1 and DC-2, respectively. Hence the secretion of this immunosuppressive cytokine was 30% lower in DCs matured with Cocktail 1 versus Cocktail 2.

Similarly as with IL-12p70, in a group of patients (12 of 32, 37.5%), IL-10 secretion was 2.2 fold higher by DC-2 compared with DC-1 (Fig. 6). In this patient cohort the average concentrations of IL-10 were 15.80 pg/mL [95% CI 2.44–34.05] and 7.07 pg/mL [95% CI 4.89–19.03] in the supernatants of DC-2 and DC-1, respectively.

DISCUSSION

The outgrowth and progression of cancer is mostly related to the immune system dysfunction which is generally induced by cancer cells, which aim at escaping the immunosurveillance (1). Various immunotherapeutical strategies have been developed for reprogramming the patients immune system from the state of tumor tolerance to an efficient antitumor immune reactivity resulting in complete or partial cancer destruction or stabilization of the disease (2). Among a variety of immunotherapeutical strategies, active specific immunotherapy (therapeutic cancer vaccination) is one of the most investigated and promising approaches which aims at inducing and / or augmenting long-lasting tumor antigen-specific immune responses (2, 3). Therapeutic cancer vaccination targets DCs, either in vivo or ex vivo. Both approaches have prons and cons and the superiority of one approach versus another remains to be elucidated in clinical trials. It also remains possible that an appropriate combination of both approaches may be the most optimal.

Using an ex vivo approach, DC vaccines are prepared from autologous peripheral blood monocytes, which are differentiated into immature DCs and subsequently loaded with tumor antigens (using various strategies, such as DC incubation with tumor lysate, mRNA transfection, generation of DC-tumor cell hybrids, etc.) and matured ones (8, 9). Importantly, a plethora of clinical trials have demonstrated that DC vaccines are safe and well tolerated by cancer patients (4–6).

Immunological responses to DC vaccination are observed in up to 70–80% of patients, whereas objective clinical responses (mostly partial and more rarely complete) are achieved in only 20–30% of the vaccinated patients (4, 6). It should be emphasized that clinical response (clinical benefit) is achieved in up to 54% of vaccinated patients when

a stable disease is regarded as a satisfactory outcome of immunotherapy rather than treatment failure (4, 6, 10, 11). In any case, it is evident that therapeutic cancer vaccination needs further optimization and individualization of its prescription in order to select cancer patients with the greatest likelihood of achieving clinical benefit. There are several possible ways for the individualization of therapeutic DC-based cancer vaccination, including a detailed characterization of various immune system parameters and tumor microenvironment as well as proper characterization of ex vivo-generated DC vaccines. With respect to characterization of DC vaccines, immunophenotyping, cytokine secretory profile and functional assays, such as autologous or allogeneic mixed lymphocyte reaction (MLR), can be applied in clinical practice. Current vaccine quality control mainly requires the evaluation of expression of several key markers, representing DC maturation / activation and immunogenic potential, including high expression CD83, CD80, CD86, HLA-DR, CCR7. However, recent data indicate that even mature DCs can be either immunogenic or tolerogenic, depending on the stimuli triggering their maturation (12–14). These results strongly imply that the evaluation of immunogenic potential-representing marker expression alone may be insufficient for a proper characterization of DC vaccines.

Hence the aim of our study was to investigate the expression of both immunogenic potential-representing markers (CD80, CD86, HLA-DR) and tolerogenic properties-representing markers (CD85k [ILT3], CD273 [PD-L2], and CD274 [PD-L1]) at a single cell level in order to phenotypically evaluate the immunogenic versus the tolerogenic potential of therapeutic DC vaccines individually prepared for patients with urogenital tumors. Autologous monocyte-derived DCs were matured using two maturation cocktails: i) Cocktail 1 composed of IFN-γ (50 ng/mL) and LPS (200 ng/mL); these DCs are defined as DC-1, and ii) Cocktail 2, composed of IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (10 ng/mL), and PGE$_2$ (1 μg/mL); these DCs are defined as DC-2. Consistent with the widely accepted concept (15–17), we found that the majority of immature CD83^{low} DCs express low levels of immunogenic markers CD80, CD86, and HLA-DR. Our data showed that only a negligible proportion of CD83^{low} DCs expressed high levels of immunogenic markers and we imply that these cells represent a small population of cells in a transitional differentiation stage between immature and mature DCs. In addition, we also investigated the expression of CCR7 expression on both immature and mature DCs (data not shown). We found that CCR7 was absent or low on immature CD83^{low} DCs with low expression levels of immunogenic markers. However, significantly greater proportion of DCs with intermediate or even high surface CCR7 expression levels was found in a minor population of CD83^{low} DCs with high expression of immunogenic markers, further reinforcing our notion that these cells represent a subset of transitional differentiation stage DCs.

We found that both maturation cocktails were effective in inducing high expression levels of immunogenic potential-representing biomarkers. However, the proportion of fully mature CD83^{high} DCs expressing high levels of immunogenic markers was significantly higher in DC-1 compared with DC-2 (88.4 ± 2.6% and 77.5 ± 7.4%, respectively, p = 0.043). These results would favor the use of Cocktail 1 versus Cocktail 2 for the production of clinical-grade DC vaccines. However, as we have already mentioned, the evaluation of immunogenic markers alone may be insufficient for a proper characterization of mature DCs since these cells may also express tolerogenic markers which may functionally interfere with the immunostimulatory potential of mature DCs and may be even involved in the induction of T-cell anergy or generation of regulatory T cells [12–14]. We found that before the induction of maturation there were 6% of immature CD83^{low} DCs expressing at least one tolerogenic marker and 2.7% of cells expressing all three tolerogenic markers (Fig. 3). Similarly, there were 2.6% of cells expressing at least one tolerogenic marker and 1.23% of cells expressing all three tolerogenic markers in a minor population of transitional DCs (Fig. 4). These results reveal that immature DC population already contains a subset of cells with phenotypical attributes of tolerogenicity. In general, all immature DCs are tolerogenic per se and assume the immunogenic function only after the induction of their maturation. However, it is also possible that a small subset of immature DCs with an imprinted tolerogenic potential exist in a general population of immature DCs. Since it has been shown that the functional

activity of DCs is shaped during the maturation process (18), it can be postulated that depending on the nature of DC maturation-inducing stimuli and local microenvironmental cues, the extent of this tolerogenic DC subset may either increase or decrease during their maturation. Indeed, we found that after the induction of DC maturation, the changes in the expression of tolerogenic potential-representing markers were observed on the surface of mature CD83^{high} DCs. Most importantly, the expression of these markers was significantly higher on DC-2 compared with DC-1 (Fig. 3). In particular, only 3.4 ± 0.7% of DC-1 and even 25.6 ± 4.7% of DC-2 expressed at least one tolerogenic marker (p = 0.012). Similarly 0.9 ± 0.05% of DC-1 and 7.4 ± 0.8% of DC-2 expressed all three tolerogenic markers (p = 0.016). It should be emphasized that after the induction of DC maturation a subset of DCs expressing tolerogenic markers significantly decreased in mature DC-1 population, but significantly increased in DC-2 population (Fig. 3). These data are of potential clinical relevance since they indicate that the use of Cocktail 1 is associated with a significant decrease of potentially tolerogenic subset in mature CD83^{high} DCs, whereas Cocktail 2 induces a three-fold expansion of this undesirable subset in mature DCs in comparison to their immature counterparts (Fig. 3). A very similar pattern of tolerogenic marker expression after the induction of DC maturation was also observed in the minor populations of the remaining immature and transitional CD83^{low} DCs (Fig. 4).

Collectively the data on the expression of immunogenic and tolerogenic markers on mature DCs indicate that Cocktail 1 (LPS + IFN-γ) is apparently superior to Cocktail 2 (IL-1β + IL-6 + TNFα + PGE$_2$) for the induction of DC maturation. Furthermore, the use of Cocktail 2 for the generation of clinical-grade DC vaccines may be even deleterious since mature DCs expressing high levels of both immunogenic and tolerogenic markers may activate T cells, however, due to insufficient delivery of the obligatory costimulatory signals these T cells become anergic or may even differentiate into immunosuppressive T cell subsets if the delivery of tolerogenic signals predominates (12, 19). From the clinical point of view it is very likely that the higher is the percentage of such potentially tolerogenic DCs in the vaccine preparation the lower is the potential of

therapeutic vaccine to induce effective antitumor immune responses. Moreover, administration of such vaccines may even be detrimental since they can potentially evoke immunosuppressive mechanisms, which further dampen antitumor immune responses.

It should be mentioned that the expression of tolerogenic markers on the surface of mature DCs was quite variable between individual patients irrespective of maturation stimulus (Cocktail 1 or Cocktail 2) used for the generation of mature DCs (data not shown). It may depend on individual characteristics of a patient, such as the extent of systemic tumor-induced immunosuppression, which may affect monocytes used for the preparation of DC vaccine ex vivo. Therefore, although the likelihood of generating a DC vaccine preparation containing a considerable proportion of potentially tolerogenic DCs is significantly higher using maturation Cocktail 2, it still cannot be refuted for DC vaccine preparation produced using Cocktail 1, and possibly various other maturation stimuli. Hence, based on our preliminary phenotypical analysis of mature clinical-grade DCs, it seems important to include the evaluation of tolerogenic marker expression as an obligatory quality control parameter determining the suitability of DC vaccines for clinical use irrespective of the maturation method applied.

We have also measured the secretion of two basic cytokines, critically involved in either the immunostimulatory, Th1-polarizing activity (IL-12p70) or the immunosuppressive activity (IL-10) of mature DCs. The secretion of these cytokines was measured after 24-hour restimulation of mature DCs with CD40L, mimicking the situation in vivo, taking into consideration that following DC migration from the peripheral tissues where they encounter antigens and undergo maturation, mature DCs are restimulated by Th cells in the lymph nodes (20, 21). Importantly, our results of the cytokine secretory profile parallel those of DC phenotypical analysis in terms of the predominance of the immunogenic versus tolerogenic potential of mature DCs depending on the maturation cocktail used. In particular, the secretion of the immunostimulatory cytokine IL-12p70 was significantly more pronounced and three-fold higher in DC-1 compared with DC-2, whereas the secretion of the immunosuppressive IL-10 was more

characteristic of DC-2 in comparison to DC-1. High secretion levels of IL-12p70 are one of the most important features of therapeutic cancer vaccines as it has clearly been demonstrated in various experimental and clinical studies (22–25). IL-12p70 drives and maintains the generation of Th1-polarized immune responses which are the key players in the detection and elimination of cancer (26). In contrast, IL-10 is one of the main immunosuppressive cytokines which dampen immune responses and / or induce the generation of immune cells with the immunosuppressive activity (27).

Collectively our data indicate that DC maturation Cocktail 2 (TNF-α + IL-1β + IL-6 + PGE$_2$) should not be used for clinical-grade DC vaccine production. It was the first DC maturation cocktail developed by the Jonuleit group in 1997 (28) and was defined as "standard" for a long time. However, recent evidence has clearly indicated that it no longer stands for such a definition since basically the only advantage of using this maturation cocktail is high migratory potential of mature DCs. Unfortunately, such DCs possess various negative features with respect to the induction and activation of effective antitumor immune responses (18, 21, 29–32). These results were obtained measuring various different parameters of mature DCs, including the secretion of immunostimulatory and immunosuppressive cytokines and chemokines, the ability of DCs to induce Th1-polarized responses, etc. However, to our knowledge the expression of tolerogenic potential-representing markers has not yet been investigated in clinical-grade DC vaccines. Consequently, the expression of tolerogenic markers is not generally assessed for the characterization of DC vaccine suitability for clinical use. Therefore current DC vaccine release criteria are generally based on the evaluation of immunogenic biomarkers alone and also on functional assays such as autologous or allogeneic MLR, however, without a more detailed phenotypical and functional analysis of the proliferating T cells in MLR. Our preliminary results indicate that the evaluation of parameters describing the tolerogenic potential of the generated DCs is reasonable and should be included into the release criteria of vaccine preparations in clinical trials. Only a detailed evaluation of various phenotypic and functional parameters will enable a complete charaterization of DC vaccines and might serve as one of tools for improving current vaccine production protocols and consequently clinical outcomes.

In conclusion, after evaluation of the results of our experiments, we could indicate that Cocktail 1 is superior to Cocktail 2 for the production of clinical-grade therapeutic cancer DC vaccines, both in terms of immunophenotypical attributes of DC tolerogenicity and their cytokine secretory profile.

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KLINIKINIO LYGMENS DENDRITINIŲ LĄSTELIŲ VAKCINŲ, SKIRTŲ VĖŽIO TERAPIJAI, TOLEROGENINIŲ ŽYMENŲ RAIŠKOS TYRIMAI

Santrauka

Įvadas. Pastaruoju metu daug dėmesio skiriama aktyvioms vėžio imunoterapijos strategijoms (terapinės vėžio vakcinos) bei gydymui specialiai „apmokytomis" autologinėmis dendritinėmis ląstelėmis (DL). Šių vakcinų veikimo principas – sustiprinti ir nukreipti organizmo imuninį atsaką prieš susiformavusį naviką. DL vakcinos yra gaminamos kiekvienam pacientui individualiai, jų pagrindą sudaro paciento DL, padengtos to paties paciento naviko antigenais. Naujausi tyrimų rezultatai rodo, kad egzistuoja mažiausiai dviejų rūšių subrendusios DL – imunogeninės, kurios aktyvina priešnavikinį imuninį atsaką, ir tolerogeninės, slopinančios imuninį atsaką, t. y. konkuruojančios su apsauginio priešnavikinio imuniteto veikimo grandimis.

Mūsų tyrimo tikslas – įvertinti klinikinio lygmens dendritinių ląstelių vakcinų, skirtų vėžio terapijai ir brandintų dviem skirtingais būdais, tolerogeninių žymenų raišką ląstelių paviršiuje.

Medžiaga ir metodai. Buvo vertinami du skirtingi DL brandinimo būdai: brandinimo mišinys I, kurio sudėtyje yra lipopolisacharido (200 ng/mL) ir interferono-γ (50 ng/mL), bei brandinimo mišinys II, kurio sudėtyje yra IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (10 ng/mL) ir PGE_2 (1 μg/mL). Papildomai buvo vertinama dviejų reikšmingų interleukinų sekrecija: imunostimuliuojančio IL-12p70 ir imunosupresinio IL-10.

Rezultatai. Mūsų atlikto tyrimo metu buvo nustatyta, kad ir nesubrendusios DL pasižymėjo tolerogeninių paviršiaus žymenų raiška. Subrendusių DL žymenų raiška buvo skirtinga priklausomai nuo pasirinkto brandinimo būdo.

Išvada. Tyrimo rezultatai atskleidė, kad, gaminant klinikinio lygmens dendritinių ląstelių vakcinas, skirtas vėžio terapijai, mišiniu I brandintos DL pasižymėjo geresnėmis savybėmis nei brandintos mišiniu II, vertinant DL pagal tolerogeninių paviršiaus žymenų raišką ir citokinų sekrecijos pobūdį.

Raktažodžiai: vėžio imunoterapija, dendritinių ląstelių vakcinos, imunogeninės dendritinės ląstelės, tolerogeninės dendritinės ląstelės