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ORIGINAL ARTICLES

Comparison of two different protocols for the induction of maturation of human dendritic cells *in vitro*

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Background. *Dendritic cells (DC) have been used for immunotherapy of malignant tumors, different kinds of infections, and other clinical conditions. For that purpose, optimal conditions for the generation of functionally mature DC in vitro are required. Two different protocols for the induction of maturation of monocyte-derived DC (MDDC) were compared in this study.* ***Methods.*** *MDDC were generated in vitro by cultivating adherent monocytes of healthy volunteers with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) during 6-days period. The immature DC thus prepared were induced to mature using two protocols. DC were stimulated for 2 days with lipopolysaccharide (LPS), or with a cocktail of proinflammatory mediators (PM) containing IL-1β, IL-6, tumor necrosis factor α (TNFα), and prostaglandin E_2 (PGE_2), respectively. Phenotypic characteristics of MDDC and their endocytic activity were studied by flow cytometry. Allostimulatory activity of these cells was tested in the mixed leukocyte reaction (MLR), whereas the production of cytokines was determined by ELISA kits.* ***Results.*** *MDDC matured with PM (PM-DC) were predominantly non-adherent cells, while about 30% of LPS-matured DC were adherent cells. In comparison with LPS-DC, PM-DC expressed higher levels of CD86 and CD83, had lower endocytic activity, produced higher levels of IL-10 and lower levels of IL-12, and more strongly stimulated proliferation of allogeneic lymphocytes.* ***Conclusion*** *The protocol based on the combination of proinflammatory cytokines and PGE_2 is better for the induction of maturation of human MDDC in vitro than the protocol using LPS alone.*

Key words: **dendritic cells; inflammation mediators; cytokines; clinical protocols.**

Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) with the unique ability to initiate a primary immune response to protein antigens by the activation of naive T cells (1, 2). During the course of DC differentiation from precursors, two major stages can be identified. An immature stage of DC is characterized by a high functional capability in taking up and processing antigens. In contrast, mature DC decrease the antigen uptake capability and express chemokine receptors for migration to peripheral lymphoid tissues, where they exert potent APC function. Moreover, the maturation of DC is additionally enhanced during interactions with T cells by signals provided by T cells themselves (3, 4). Maturation process is central for optimal functional DC and enables a single cell type to perform different highly specialized functions (2, 4).

DC have been successfully generated *in vitro* from their precursors such as bone marrow, umbilical $CD34^+$ stem cells, or monocytes (5–7). In recent years, there has been a growing interest in using human DC prepared *ex vivo* for immunotherapy. For such purposes, optimization of culture conditions is needed for the preparation of large

numbers of functionally potent DC to be transferred *in vivo*. Immunotherapy with mature DC is required for the induction of anti-tumor immunity, or the stimulation of the immune response against intracellular microorganisms (8–12). In contrast, tolerant semi-mature DC could be used for the treatment of autoimmune diseases and for the prevention of transplant rejection (13, 14).

In this study immature monocyte-derived DC (MDDC) were generated using GM-CSF (100 ng/ml) and low concentrations of IL-4 (5 ng/ml), as previously described (15, 16). Immature MDDC were treated with two protocols using lipopolysaccharide (LPS) and a cocktail of proinflammatory mediators (PM), respectively, in order to check their potential to induce phenotypic and functional maturation of these cells.

Methods

DC cultures

Monocytes (purity 75–85%) were purified from buffy coats of healthy volunteers by Lymphoprep gradient (Nycomed, Oslo, Norway), and by plastic adherence, as described (15, 16). Buffy coats were prepared at the Institute of Transfusiology of the Military Medical Academy, Belgrade. To induce the differentiation of immature DC, monocytes were cultivated for 7 days in 6-well tissue culture plates (Flow, Irvine, Scotland) in 2 ml of complete RPMI-1640 medium with the addition of 10% heat-inactivated fetal calf serum (FCS) (ICN), streptomycin, gentamycin, penicillin and 2-mercaptoethanol (Sigma). The medium was supplemented with 100 ng/ml of human recombinant GM-CSF (Leucomax, spec. activity 4.44×10^6 IU, Sandoz-Schering Plough, Basel, Switzerland), and 5 ng/ml of IL-4 (Roche Diagnostics GmbH, Mannheim, Germany). After 3 days, cultures were fed with 1 ml of fresh medium with cytokines. At day 6, non-adherent, immature MDDC were collected, washed twice with RPMI medium, and replated in 24-well plates (5×10^5 cells/ml/well). MDDC were induced to mature using two different protocols. Cells were stimulated with LPS (1 μg/ml) (Sigma), or with a cocktail of PM (TNFα, IL-6, IL-1β, all at concentrations of 10 ng/ml and PGE_2, 1 μmol/l). Cytokines were purchased from R&D Company (Minneapolis, USA), whereas PGE_2 was from Sigma. After two additional days of cultivation, MDDC were collected and centrifuged. In most experiments, both non-adherent and adherent cells were collected together. Adherent cells were detached by vigorous pipeting or by the treatment of cells with 1 mmol/l EDTA for 10–15 min. Supernatants were collected and frozen at -20 °C until the levels of cytokines were determined. DC from the pellet were washed twice with RPMI medium, counted and than used for morphological, phenotypic, and functional assays. For morphological analysis, cytospins were prepared from cultivated DC using a cytocentrifuge (MPW-350 R, Warsaw, Poland), and stained with May-Grunwald Giemsa.

Flow cytometry

MDDC were washed in phosphate buffered saline (PBS) with 0.1% sodium azide and 2% FCS (PBS/FCS) and adjusted at concentrations of 1×10^5 cells/tube. Cells were incubated with the appropriate dilutions of the following monoclonal antibodies (mAbs) to: HLA-DR conjugated with phycoerythrin (PE), CD86-PE, CD83 conjugated with flourescein isothiocyanate (FITC), CD40-FITC, CD80, and CD54 unconjugated mAbs (Serotec) for 45 min at +4 °C. After washing in PBS/FCS, anti-mouse (Fab-2) Ig-FITC antibody (Serotec) with 5% normal human serum was added to the tubes stained with unconjugated mAbs, and the cells were further incubated for 30 min at +4 °C. The control consisted of the samples with the adequate irrelevant mouse mAbs. After washing, the cells were analyzed on EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany). At least 5 000 cells per sample were analyzed.

Allogeneic mixed leukocyte reaction (MLR)

Peripheral blood mononuclear (PBMN) cells were isolated from buffy coats using Lymphoprep gradient. Cells (2×10^5/well) were cultivated with different numbers of allogeneic MDDC, which were cultivated under different conditions in complete RPMI medium +10% FCS in 96-well flat-bottom cell culture plates for 5 days. Cells were pulsed with [^{3}H]-thymidine (1 μCi/well, Amersham, Books, U.K.) for last 18 hrs. Labeled cells were harvested onto glass fiber filters. Radioactivity was counted in a Beckman scintillation counter and expressed as cpm ± SD of triplicates.

Endocytosis assay

Endocytosis was measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. MDDC (2×10^5 per sample) were incubated in RPMI-1640 medium with the addition of FITC-dextran (1 mg/ml; mol. mass 40 000; Sigma) for 60 min at 37 °C or at +4 °C (for control binding). After incubation, cells were washed twice with PBS and fixed with 1% formaldehyde. The uptake of FITC-dextran by MDDC, previously cultivated with different protocols, was determined by flow cytometry. At least 5 000 cells per sample were analyzed.

Cytokine assays

Supernatants from MDDC cultivated with LPS or PM, as previously described, were assayed for the presence of IL-12 and IL-10. The levels of these cytokines were determined using a sandwich ELISA assay, following the instructions of the manufacturer (16). ELISA kits were from R&D.

Statistical analysis

Differences in parameters between various groups were evaluated using Student's t-test.

Results

Morphological characteristics of DC

Immature DC were stimulated with LPS or with a cocktail of PM for 2 days in order to induce their maturation. In the cultures treated with PM, a much higher percentage of non-adherent cells (NAC) was observed than in the cultures treated with LPS (Fig. 1). Under both conditions, NAC possessed typical dendritic morphology (Fig. 2). However, adherent cells in LPS-treated cultures, which comprised about 30% of total MDDC, showed firm adherence to plastic and fibroblast-like appearance. Adherent cells in PM-treated cultures (about 10% of total MDDC) were mainly loosely attached to plastic.

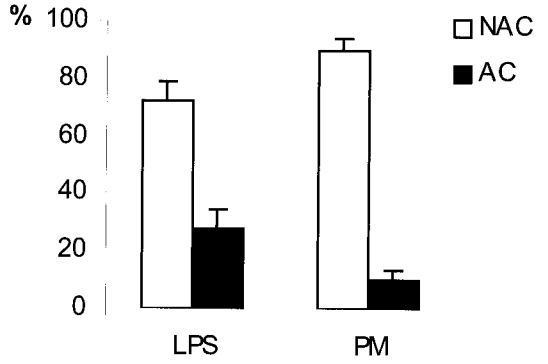

Fig. 1 – Proportion of adherent and non-adherent cells in MDDC cultures treated with two different protocols for induction of their maturation.

Immature MDDC were induced to mature by LPS or PM, as described in Methods. After 2 days, non-adherent cells (NAC) were collected. Adherent cells (AC) were removed by vigorous pipeting or by treatment with EDTA. The percentages of NAC and AC were determined on the basis of total number of recovered cells. Values are given as mean % ± SD for 5 different donors. All the differences were statistically highly significant ($p < 0.0001$).

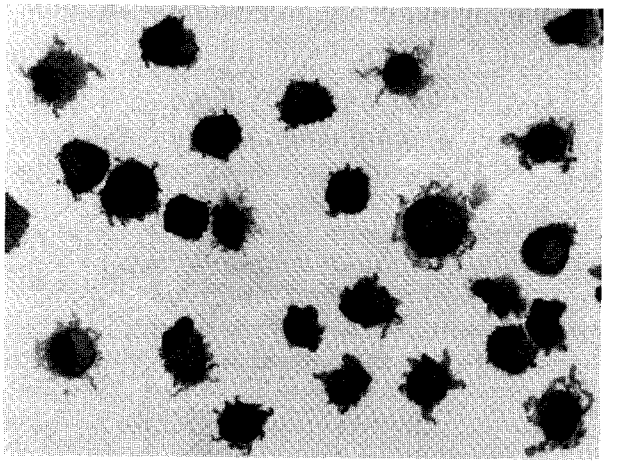

Fig. 2 – Morphological characteristics of MDDC in LPS-treated cultures.

Cytospins of non-adherent cells from LPS-DC cultures were stained with May-Grünwald Giemsa. Note typical dendritic morphology of the cells. PM-DC displayed very similar morphology. Magnification: × 520.

Phenotypic characteristics of DC

Flow cytometric analysis showed that most DC possessed the phenotype of mature DC, as judged by high expression of HLA-DR, costimulatory molecules (CD80, CD86, and CD40), adhesion molecules (CD54), and maturation markers (CD83). The results showed that, due to significant variations between donors, no statistically significant differences in the expression of HLA-DR, CD40, CD54, and CD80 were observed between LPS-and PM-treated DC (Table 1) (Fig. 3). However, PM-DC showed higher expression of CD86 and CD83 than LPS-DC.

Endocytic and allostimulatory activity of DC

Endocytic activity of DC matured under different conditions was also studied. Endocytosis of LPS-treated DC was higher than PM-treated DC (Fig. 4) suggesting that DC in cultures with the cocktail of PM were more mature than

Table 1

Comparison of phenotypic characteristics of human MDDC matured under different culture conditions

Markers	Phenotype			
			PM	
	%	mean FI	%	mean FI
HLA/DR	95.1 ± 3.4	202.4 ± 67.0	95.1 ± 5.9	223.4 ± 28.3
CD40	92.4 ± 7.8	89.8 ± 33.2	93.4 ± 8.7	100.9 ± 22.0
CD54	91.1 ± 4.2	126.7 ± 31.0	93.9 ± 2.0	179.7 ± 52.7
CD80	67.6 ± 35.9	51.7 ± 14.4	78.6 ± 29.7	75.5 ± 22.1
CD83	45.9 ± 20.7	10.4 ± 1.9	74.5 ± 15.2*	14.3 ± 1.7**
CD86	76.6 ± 28.6	62.5 ± 11.3	91.2 ± 7.8	81.8 ± 10.3*

MDDC were induced to mature with LPS or with a cocktail of PM for 2 days. Phenotypic characteristics of these cells were determined by flow cytometry as described in Methods. Values are given as mean percentages (%) of stained cells ± SD and mean fluorescence intensity (FI) ± SD ($n = 5$); * = $p < 0.05$; ** = $p < 0.01$ compared to the corresponding controls (LPS).

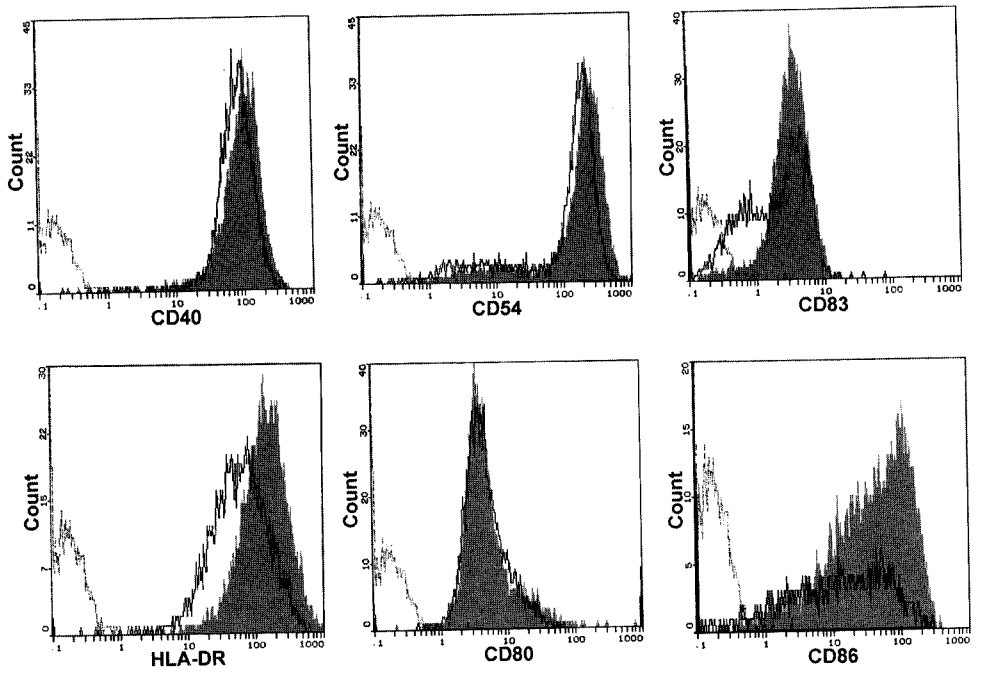

Fig. 3 – Phenotypic characteristics of human MDDC cultivated with two different protocols for the induction of their maturation.

MDDC induced to mature in the presence of LPS or PM, were stained with mAbs and analyzed by flow cytometry, as described in Methods. Representative histograms of one donor are presented. Grey histograms represent the fluorescence profile of PM-DC, whereas white histograms (thick lines) represent profiles of LPS-DC. White histograms (thin lines) represent non-specific staining using isotype controls.

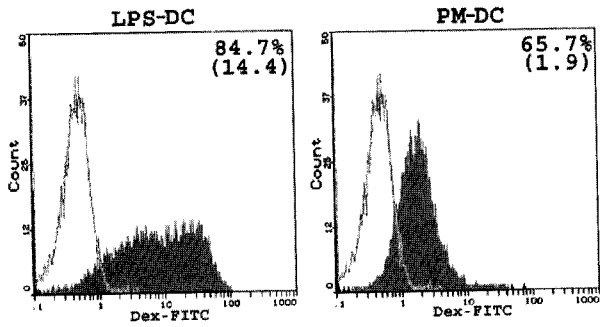

Fig. 4 – Endocytic activity of LPS-DC and PM-DC.

Cells were incubated with dextran-FITC at +4 °C (white histograms) or 37 °C (grey histograms) and then analyzed by flow cytometry. Values are given as the percentage of positive cells and mean fluorescence intensity (numbers in parentheses) of one representative experiment. Note the lower endocytic activity of PM-DC.

LPS-treated cells. These results were in accordance with the findings of their allostimulatory potential. PM-DC were more potent stimulators of proliferation of allogeneic lymphocytes in MLR than LPS-DC (Fig. 5).

Cytokine production by DC

Finally, we tested the production of two key cytokines (IL-12 and IL-10), mediating Th1 and Th2 immune responses, respectively, by mature DC. LPS was more potent inducer of IL-12 production by DC than PM, whereas PM was more potent stimulator of DC to secrete IL-10, compared to LPS (Fig. 6).

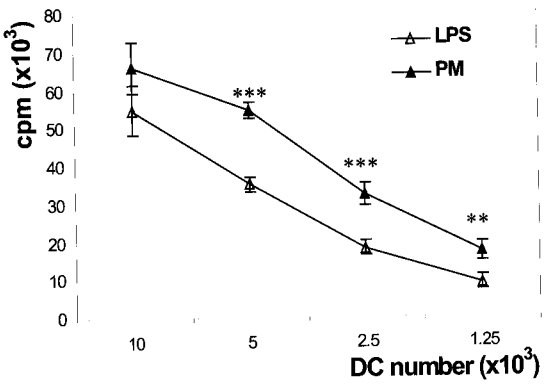

Fig. 5 – The effect of LPS-DC and PM-DC on proliferation of allogeneic lymphocytes.

Allogeneic lymphocytes (2×10^5/well) were cultivated with different numbers of DC, as described in Methods. Proliferation of lymphocytes was measured by ^{3}H-thymidine incorporation. Values (mean cpm ± SD of triplicates, from one representative experiment) are given. ** = $p < 0.01$; *** = $p < 0.001$ compared to the corresponding controls

Discussion

In the previous papers (15, 16) we published an alternative protocol for the generation of immature human MDDC using GM-CSF and low concentrations of IL-4. The

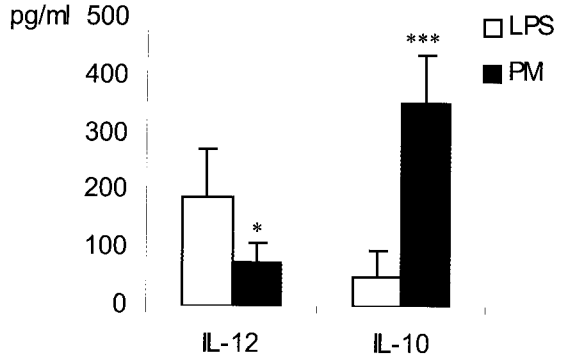

Fig. 6 – Production of IL-10 and IL-12 by LPS-DC and PM-DC in cultures.

Levels of cytokines in supernatants of DC cultures were determined by ELISA. * = $p < 0.05$; = *** $p < 0.005$ compared to LPS-DC.

cytokine combination has been shown to be as potent in generating DC as ten times higher concentrations of IL-4. In this work, we studied the efficiency of two protocols (LPS versus PM) for the induction of maturation of immature MDDC. The results of direct comparison of these protocols have not been published so far.

Although the list of agents that can induce DC maturation both *in vivo* and *in vitro* is long and growing, the exact sequence of events and the composition of factors required for the efficiency of maturation processes of DC to become potent stimulators of T cells is not sufficiently elucidated. The difficulties have been underscored by recognition that, under certain conditions, *in vitro* generated DC can favor not only the desired protective immunity, but also induce Th2 $CD4^+$ T cell responses or tolerize the cognate T cells (2, 17–19). One of the most used agents for the induction of maturation of DC is LPS (1, 6, 20). LPS is a key component of bacterial cell walls that triggers host immune responses against the invading pathogen (21, 22). LPS can stimulate macrophages to produce proinflammatory cytokines that are crucial for the innate immune response (23). It can also induce the maturation of DC, which in turn produce more cytokines and express costimulatory molecules to activate T cells (24).

This study showed that LPS is a potent inducer of the maturation of MDDC, as judged by up-regulation of HLA-DR, costimulatory molecules (CD40, CD80, CD86), adhesion molecules (CD54), and a DC maturation marker, CD83 (1, 6, 10). LPS-treated DC had lower endocytic activity, but higher stimulatory capacity to induce proliferation of allogeneic lymphocytes in a MLR assay than immature DC (16). However, our results suggested that not all immature MDDC were converted to immunogenic mature DC, according to low levels of CD83 expression on a subset of these cells, and concerning the fact that up to 30% of MDDC remained firmly adherent to plastic. We observed that the proportion of adherent cells varied depending on the donor. Adherent cells could be macrophages or immature DC that are functionally different from conventional, non-adherent immature DC. According to our previous (15, 16) and unpublished results, adherent cells in LPS-treated DC cultures are probably immature DC. This hypothesis is based on down-regulation of CD14 and the expression of CD1a, a DC marker, in the majority of cells (6, 9, 25). The expression of low levels of CD83 in these cells (data not presented) indicated that certain adherent cells could be a specific transition stage from the immature to the mature stage of DC. Granucci et al, (26) published that LPS treatment of a growth factor-dependent immature, loosely adherent DC line, induced its firm adhesion to plastic in the early period of stimulation. It is not yet clear why a subset of MDDC failed to undergo final maturation with the influence of LPS. To our opinion, previous activation state of monocytes and/or isolation procedures during the preparation of monocytes could be relevant in explaining this phenomenon.

LPS signals are received and transmitted through Toll-like receptor (TLR) 4 (20, 27), a member of the TLR family proteins that are the receptors for various pathogen-associated molecular patterns (28, 29). The downstream signaling pathways triggered by LPS and TLR4 are complex and result in activation of the transcription factor NF-kB and various mitogen-activated protein kinases. These signals contribute critically to the induction of a variety of inflammatory cytokines, including TNFα, IL-1β, IL-6, and IL-12, as well as the expression of costimulatory molecules (CD80 and CD86) on the cell surface (20, 27–29). As mentioned above, costimulation is of crucial importance in generating signal two for the activation of T cells. In addition, IL-12 favors Th polarization of the immune response to Th1 (2, 8).

TNFα, IL-1, and IL-1β are identified as DC maturation stimuli, acting synergistically (2, 6, 8, 10, 25). Therefore, many protocols included these cytokines for the induction of maturation of DC *in vitro*. Better stimulation of DC was achieved when PGE_2 was added to the cocktail of proinflammatory cytokines. It was shown that PGE_2, although it did not induce final maturation of DC by itself, synergized with IL-1β and TNFα, allowing their effectiveness at 100-fold lower concentration (30).

Using such a protocol involving TNFα, IL-1β, IL-6, and PGE_2 (PM protocol), it was shown that this protocol was superior to the LPS-protocol in stimulating MDDC maturation. It was also demonstrated that PM-DC expressed higher levels of CD86 and CD83, had lower endocytic activity, and better stimulated T cell allogeneic response than LPS-DC. All these parameters, including the presence of predominantly non-adherent cells in DC cultures, suggested that PM-DC were more mature than LPS-DC (2, 6, 10, 16).

Another important difference observed in our experiments was related to IL-10 and IL-12 production by MDDC. We found that PM-DC produced higher levels of IL-10 and lower levels of IL-12 than LPS-DC. These differ-

ences could be related to PGE_2. It was shown that DC matured with TNFα and IL-1β in the additional presence of PGE_2 showed impaired IL-12 production and bias naive Th cell development towards Th2 (30). IL-10 was shown to inhibit the production of IL-12 by human MDDC and suppress their maturation (31, 32). While IL-12 stimulated Th1 immune response, IL-10 favored the development of Th2 and tolerogenic T cells (11, 13, 32). Our results showed that PM-DC, which produced higher levels of IL-10 were phenotipically more mature than LPS-DC, which produced lower levels of the cytokine contradicted previous findings. These differences could be explained by the fact that the stimulation of Th1 immune responses depends on the balance between IL-12 and IL-10, as well as the presence of other components in culture media. In addition, it is believed that the final decision of Th polarization occurs in the close contact of DC with $CD4^+$ T cells through CD40-CD40L interactions. This signaling pathway upregulates the IL-12 production by DC (3, 33–35). In this context, it would be useful in future experiments to check the production of cytokines by Th cells stimulated with LPS-DC and PM-DC. Both human PM-DC and LPS-DC stimulate Th1 immune reactions (36, 37). We postulated that LPS-DC favored Th1-mediated immunity, whereas PM-DC were able to stimulate the development of both Th1 and Th2 cells. The immune polarization towards the Th1 response has been associated not only with the differentiation of $CD4^+$ Th1 cells but also with the development of cytotoxic T cells that were involved in protective anti-tumor immunity (38). The induction of Th2 response could confer humoral immunity for cytotoxicity mediated by antibodies (39). The mechanism may provide additional immune protection against the tumor growth. It was also shown that IL-10 produced by DC promoted the development of Th1 type of effector cells (32) and the maintenance of anti-tumor $CD8^+$ T cells (40).

In conclusion, our results suggested the advantage of using PM as stimulators of DC maturation before their potential use for anti-tumor immunotherapy *in vivo*. For that purpose, the analysis of cytokines produced by naive $CD4^+$ T cells in coculture with PM-DC is needed for each donor. These experiments are currently in progress at our Institute.

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Apstrakt

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POREĐENJE DVA RAZLIČITA PROTOKOLA ZA INDUKCIJU MATURACIJE HUMANIH DENDRITIČNIH ĆELIJA *IN VITRO*

Uvod. Dendritične ćelije (DC) se koriste u imunoterapiji malignih tumora, različitih vrsta infekcija i drugih oboljenja. U tom cilju neophodni su optimalni uslovi za dobijanje funkcionalno zrelih DC *in vitro*. U ovom radu smo poredili dva različita protokola za indukciju maturacije DC monocitnog porekla (MDDC). **Metode.** MDDC su dobijene *in vitro* kultivisanjem adherentnih monocita zdravih dobrovoljnih davalaca krvi pomoću faktora stimulacije granulocitno-makrofagnih kolonija (GM-CSF) i interleukina-4 (IL-4) u toku 6 dana. Tako pripremljene nezrele DC su indukovane na sazrevanje pomoću dva protokola. DC su stimulisane u toku 2 dana lipopolisaharidom (LPS) ili koktelom proinflamatornih medijatora (PM) koji je sadržavao IL-1β, IL-6, faktor nekroze tumora α (TNFα) i prostaglandin E_2 (PGE_2). Fenotipske karakteristike MDDC i njihova endocitozna aktivnost su ispitivani pomoću protočne citometrije. Alostimulatorna aktivnost ovih ćelija je ispitivana u testu mešane leukocitne reakcije (MLR), dok je stvaranje citokina određivano ELISA kompletima. **Rezultati.** MDDC koje su sazrevale u prisustvu PM (PM-DC) su bile predominantno neadherentne ćelije, dok su oko 30% DC koje su sazrevale pod uticajem LPS (LPS-DC) bile adherentne. U poređenju sa LPS-DC, PM-DC su ispoljavale više nivoe CD86 i CD83 molekula, imale slabiju endocitoznu aktivnost, produkovale više IL-10, a manje IL-12 i snažnije stimulisale proliferaciju alogenih limfocita. **Zaključak.** Protokol koji se bazira na primeni kombinacije proinflamatornih citokina i PGE_2 je bolji za indukciju maturacije humanih MDDC *in vitro* nego LPS protokol.

Ključne reči: **ćelije, dendritične; zapaljenje, medijatori; citokini; protokoli, klinički.**

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