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ORIGINAL ARTICLES

Differentiation of human dendritic cells from monocytes *in vitro* using granulocyte--macrophage colony stimulating factor and low concentration of interleukin-4

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Several laboratories have developed culture systems that allow the generation of large numbers of human dendritic cells (DC) from monocytes using granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-4 (IL-4). In this work we provided evidence that GM-CSF (100 ng/ml) in combination with a low concentration of IL-4 (5 ng/ml) was efficient in the generation of immature, non-adherent, monocyte-derived DC as the same concentration of GM-CSF, and ten times higher concentration of IL-4 (50 ng/ml). This conclusion was based on the similar phenotype profile of DC, such as the expression of CD1a, CD80, CD86, and HLA-DR, down-regulation of CD14, and the absence of CD83, as well as on their similar allostimulatory activity for T cells. A higher number of cells remained adherent in cultures with lower concentrations of IL-4 than in cultures with higher concentrations of the cytokine. However, most of these adherent cells down-regulated CD14 and stimulated the proliferation of alloreactive T cells. In contrast, adherent cells cultivated with GM-CSF alone were predominantly macrophages, as judged by the expression of CD14 and the inefficiency to stimulate alloreactive T cells. DC generated in the presence of lower concentrations of IL-4 had higher proapoptotic potential for the Jurkat cell line than DC differentiated with higher concentrations of IL-4, suggesting their stronger cytotoxic, anti-tumor effect.

Key words: **dendritic cells; monocytes; phenotype; granulocyte-macrophage colony-stimulating factor; interleukin-4; apoptosis; T-lymphocytes.**

Introduction

Dendritic cells (DC) are a family of bone marrow-derived professional antigen presenting cells (APC) with sparse, but wide tissue distribution (1, 2). Their capacity for migration, constitutive expression of major histocompatibility complex (MHC) class I and II molecules, costimulatory and adhesion molecules, make them ideal for the activation of naive T cells (3, 4). It has been shown that DC are heterogeneous by their origin, maturation stage, and function (5–8). DC reside in tissues in an immature form, where they are adapted for capturing and accumulating antigens. A variety of danger signals, including microorganisms, dying cells, or proinflammatory cytokines, induce terminal differentiation, also known as maturation of DC. Mature DC migrate to lymph nodes, acquire potent antigen-presenting capacity, and stimulate T cell responses vigorously. Moreover, the maturation of DC is strengthened during interactions with T cells by signals provided by T cells themselves (3, 9).

Culture conditions for the generation of myeloid DC from blood, bone marrow, or $CD34^+$ stem cells, have been developed by several laboratories (10–13). 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 was needed for the preparation of large numbers of functionally potent DC so they could be transferred *in vivo*. Immunotherapy with mature DC is required for the induction of anti-tumor immunity (14, 15). In contrast, immature DC possess natural killer (NK) activity against various tumor cell lines *in vitro* (16,17). On the other hand, tolerant DC could be used for the treatment of autoimmune diseases, and for the prevention of transplant rejection (7, 18–20).

DC can be easily generated from monocytes using RPMI medium containing fetal calf serum (FCS) with addition of granulocyte-macrophage colony stimulating factor (GM-SCF), (usually 1 000 U/ml or 80 ng/ml to 100 ng/ml), and interleukin-4 (IL-4) (1 000 U/ml or 50 ng/ml to 100 ng/ml) (10–13). Alternatively, FCS could be replaced by autologous serum or plasma (21–23). Some authors generated DC using lower concentrations of IL-4 (20 ng/ml or 250 U/ml), but phenotypic and functional characteristics of such prepared DC have not been yet thoroughly examined (23, 24).

Therefore, the aim of this study was to check whether a combination of GM-CSF (100 ng/ml) with lower concentrations of IL-4 (5 ng/ml) was as efficient in the generation of human monocyte-derived DC as GM-CSF together with a ten times higher concentration of IL-4 (50 ng/ml).

Methods

DC cultures

Highly enriched monocytes (about 75–85% purity) were obtained and purified from buffy coats of seven healthy volunteers by Lymphoprep, gradient (Nycomed, Oslo, Norway), and plastic adherence, as described (11, 21, 25). Buffy coats were prepared at the Institute for Transfusiology, Military Medical Academy, Belgrade. Monocytes were cultivated for 7 days in 4-well tissue culture plates (Flow, Irvine, Scotland) in 4 ml of complete RPMI-1640, hepes/sodium bicarbonate buffered medium with the addition of 10% heat inactivated fetal calf serum (FCS) (ICN), streptomycin, gentamycin, penicillin, and 2-ME (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, Basle, Switzerland), and 5 ng/ml or 50 ng/ml of IL-4 (Roche Diagnostics GmbH, Mannheim, Germany). In some experiments monocytes were cultivated with GM-CSF alone. After day 3, cultures were fed with 3 ml of fresh medium with cytokines. At day 7, non-adherent cells were collected, washed twice with RPMI medium, and counted. Adherent cells were incubated with RPMI, serum-free medium and 5 mM EDTA at +4°C for 30 min, then collected, washed, and counted. For morphological and immunocytochemical analysis cytospins were prepared from cultivated DC and adherent cells using a cytocentrifuge (MPW-350 R, Warsaw, Poland), and stained with May-Grunwald-Giemsa or APAAP method, as described below.

Flow cytometry

Monocytes and non-adherent DC were collected, 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 (Becton-Dickinson), CD1a, CD80, CD83, and CD86 (Serotec), and CD14 (ICN), 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 and the cells were further incubated for 30 min at +4°C. Controls consisted of samples with an irrelevant mouse mAb. After washing, the cells were analyzed on EPICS XL-MCL flow cytometer, (Coulter, Krefeld, Germany).

Immunocytochemistry

Cytospins prepared from adherent cells were fixed with cold acetone, and then incubated with anti-CD1a or anti-CD14 mAbs for 60 min at room temperature. After washing in Tris-buffered saline (TBS), slides were incubated with anti-mouse Ig (Dako) for 30 min, and then with a mouse alkaline phosphatase-anti-alkaline pshosphatase (APAAP) complex (DAKO) for 30 min. Enzyme reaction was visualized using naphtol AS-MX phosphate dissolved in dimethyl formamide and fast red as a substrate. Levamisole was added to the enzyme complex to block endogenous alkaline phosphatase. Cytospins were analyzed by light microscopy. The percentage of positive cells was determined on the basis of 500 cells calculated per sample.

Allogeneic mixed leukocyte reaction (MLR)

Peripheral blood mononuclear (PBMN) cells were isolated from buffy coats using Lymphoprep gradient. T cells were purified from PBMN using immunomagnetic sorting with a pan-T cell isolation kit by MACS technology (Myltenyi Biotec, Bergish Gladbach, Germany), following instructions of the manufacturer. The purity of T cells recovered in the negative fraction was higher than 95%, as checked by anti-CD3 FITC mAb (Serotec) and flow cytometry.

T cells (2×10^5/well) were cultivated with different numbers of monocytes, allogeneic DC cultivated under different conditions, or allogeneic adherent cells from monocyte cultures in complete RPMI medium +10% FCS in 96-well, flat bottom cell culture plates, for 5 days. Cells were pulsed with $[^3H]$-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.

Apoptosis assay

A human T cell leukemic line, Jurkat, was previously obtained from the ATCC collection (Washington DC, USA). Jurkat cells were cultivated alone or with DC at a ratio 10:1 in complete RPMI medium for 24 or 48 hrs. After that, cells were stained with Türk reagent, as we originally described (26), and analyzed by light microscopy. Apoptosis was detected using classical morphological criteria (condensation of chromatin and/or fragmentation of nuclei). The percentage of apoptotic Jurkat cells, that were easily recognized from DC, was determined after the calculation of at least 500 cells per sample.

Statistical analysis

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

Results

IL-4 is a necessary cytokine for the development of DC from monocytes

Human monocytes, mostly CD1a $^-$, CD14 $^+$ (Fig. 1), were cultivated with GM-CSF (100 ng/ml) alone, GM-CSF, and low concentrations of IL-4 (5 ng/ml), or GM-CSF, and high concentrations of IL-4 (50 ng/ml) for 7 days. Then, the numbers of non-adherent and adherent cells were calculated. As presented in Fig. 2 and Fig. 3, in cultures with GM-CSF and IL-4, the cells rapidly became non-adherent and displayed veiled and dendritic morphology to a different extent. The percentage of non-adherent DC was higher using GM-CSF and high concentrations of IL-4 than the percentage of non-adherent cells generated in the presence of lower concentrations of IL-4 (89.1 ± 6.2 vs 72.3 ± 13.0; $p<0.05$). In cultures without GM-CSF, only 21.6 ± 8.3% of

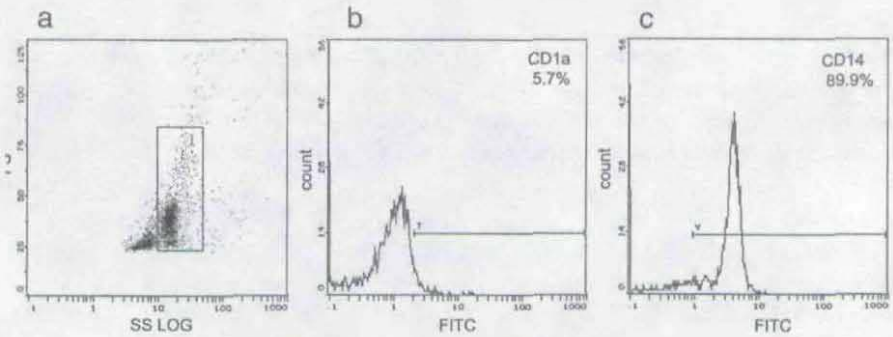

Fig. 1 – Expression of CD1a and CD14 by human monocytes.

Monocytes were isolated as adherent the cells from PBMN cells. After the detachement with cold RPMI-serum free medium containing 5 mM EDTA, the cells were stained with mAbs, as described in *Methods*, and analyzed on EPICS-XL MCL flow cytometer. Monocytes, representing about 85% of all adherent cells were identifed by selective gating (a). Horizontal bars on histograms (b, c), positioned according to the negative control, represent the levels of specific fluorescence. X-axis represents fluorescence intesity (log. the scale). Y-axis the represents the number of cells. Data are from one representative experiment out of four with similar results.

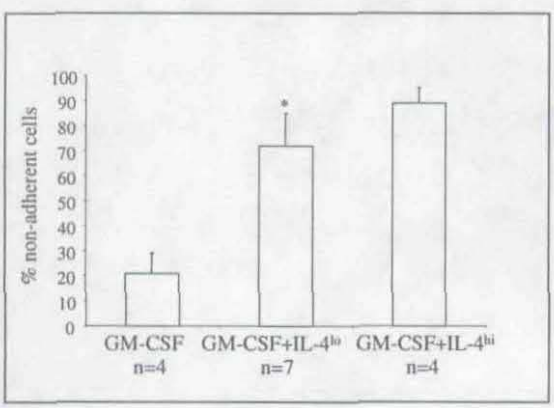

Fig. 2 – Yields of non-adherent DC, generated from human monocytes under different culture conditions.

Values are given as percentages of non-adherent cells (mean ± SD) calculated on the basis of the number of total cells (adherent + non-adherent cells) used as 100%. IL-4^{lo} - IL-4 (5 ng/ml); IL-4^{hi} - (50 ng/ml); * = $p < 0,05$ compared to the values in cultures with 50 ng/ml of IL-4.

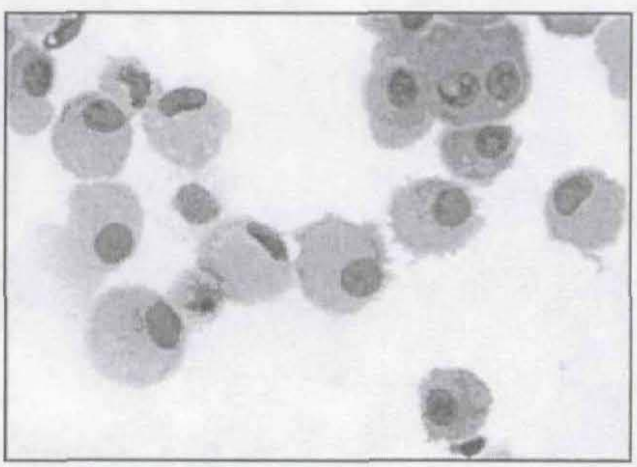

Fig. 3 – Morphology of non-adherent DC cultivated with GM-CSF and lower concentrations of IL-4.

Note that most cells displayed veiled and dendritic morphology. May Grünwald-Giemsa staining of a cytospin; magnification: × 840.

cells were non-adherent cells, whereas the rest of them were adherent, macrophage-like cells.

DC generated in the presence of GM-CSF and IL-4 are phenotypically immature

We next studied phenotypic characteristics of DC cultivated with GM-CSF and IL-4. The results given in Table 1, and Fig. 4 showed that most non-adherent cells had typical characteristics of immature DC, such as expression of CD1a, CD80, CD86 and HLA-DR, down-regulation of CD14, and the absence of CD83. No significant differences in the expression of any marker were detected between DC cultured with low and high concentrations of IL-4.

Table 1

Phenotypic characteristics of monocyte-derived DC cultivated with GM-CSF and different concentrations of IL-4

Markers	IL-4 (5 ng/ml)		IL-4 (50 ng/ml)		n
	%	expression	%	expression	
CD1a	52.4 ± 12.6	++	58.1 ± 15.0	++	5
CD14	38.0 ± 12.2	+	36.4 ± 11.8	+	5
CD80	36.1 ± 14.4	+	35.5 ± 8.2	+	6
CD86	51.3 ± 9.2	++	55.4 ± 7.6	++	6
CD83	6.0 ± 2.1	+	5.2 ± 2.0	+	4
HLA-DR	95.4 ± 2.0	+++	96.6 ± 1.3	+++	4

Phenotype of DC was determined by flow cytometry after 7 days of monocyte cultures, as described in Materials and methods. Differences in the percentages of positive cells between groups were not statistically significant ($p > 0.05$)

+++ = strong expression; ++ = moderate expression; + = low expression

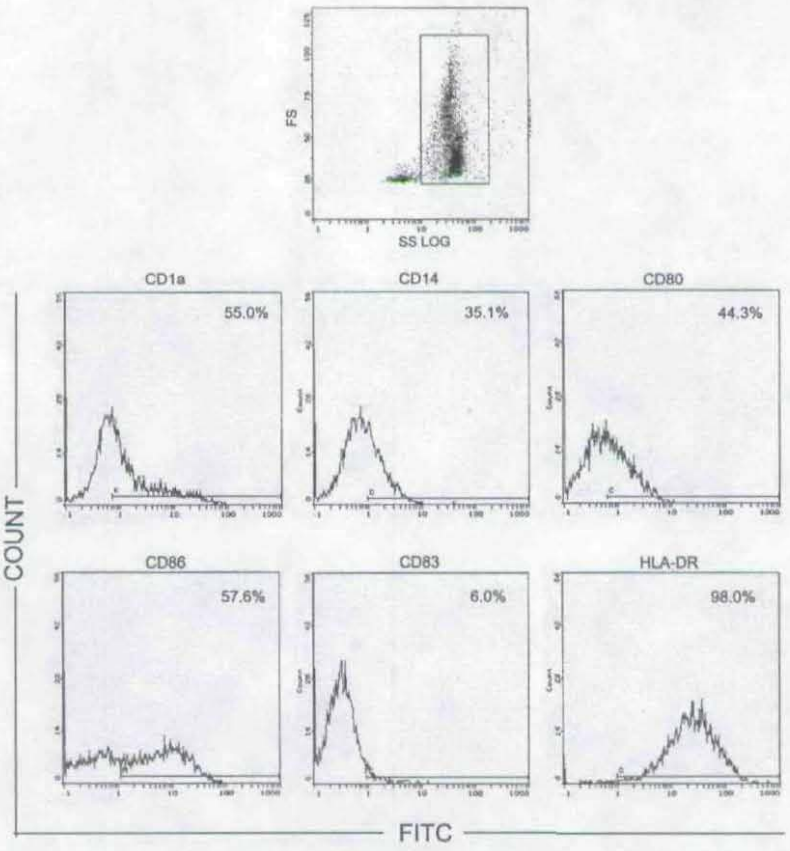

Fig. 4 – Phenotypic characteristics of DC generated from human monocytes in the presence of GM-CSF and lower concentrations of IL-4.

Cells were stained in suspension with mAbs and analyzed by flow cytometry, as described in *Methods*, and Fig. 1. Representative histograms from one culture are presented. X-axis represents fluorescence intesity (log. scale); Y-axis represents the number of cells.

Monocyte-derived DC better stimulate the proliferation of allogeneic T cells than the monocytes

Stimulation of allogeneic T cells is a typical characteristic of DC. Therefore, we compared the extent of proliferation of alloreactive T cells *in vitro,* using either monocytes or DC generated from the monocytes with GM-CSF and IL-4. The results presented in Fig. 5 showed that monocytes were much poorer stimulators of allogenic T cells, especially at higher monocyte/T cell ratio, than monocyte-derived DC. However, DC generated with lower concentrations of IL-4 were as efficient in allostimulatory activity as DC generated with higher concentrations of IL-4.

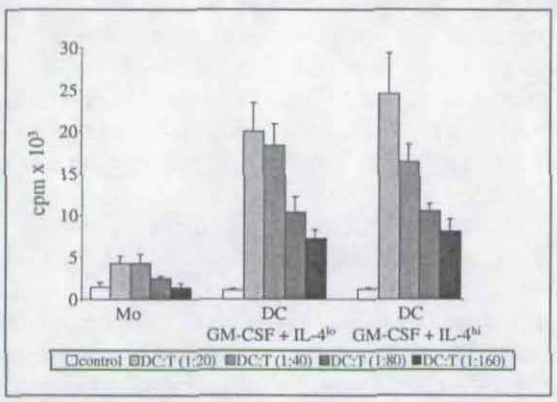

Fig. 5 – Effect of monocytes and monocyte-derived DC on proliferation of allogeneic T cells.

Purified allogeneic T cells (2×10^5/well) were cultivated with different numbers of monocytes (Mo), or DC differentiated from monocytes in the presence of GM-CSF, and low (^{lo}) or high concentrations (^{hi}) of IL-4, as described in *Methods*. Values (mean cpm ± SD of triplicates, from one representative experiment) are given. All the differences in the proliferation of T cells between monocytes and monocyte-derived DC, used as stimulators, were $p < 0{,}005$. No statistically significant differences were observed in T cell proliferation between cultures using DC as stimulators. Control = cpm (T cells, alone) + cpm (DC, alone)

Adherent cells generated in cultures with GM-CSF and IL-4 are phenotypically and functionally different from the adherent cells cultivated with GM-CSF alone

Based on previous results that in cultures with GM-CSF and lower concentrations of IL-4 a higher number of cells remained adherent than in the cultures with a higher concentration of IL-4, we framed a question whether these adherent cells differred from a predominant population of adherent cells developed from monocytes in cultures with GM-CSF alone. Results presented in Fig. 6 showed that both types of adherent cells were $CD1a^-$, but the percentage of $CD14^+$ cells was significantly higher in cultures with GM-CSF alone. In addition, while adherent cells from GM-CSF/IL-4 cultures showed a moderate allostimulatory activity, adherent cells from GM-CSF cultures were inefficient in the MLR assay.

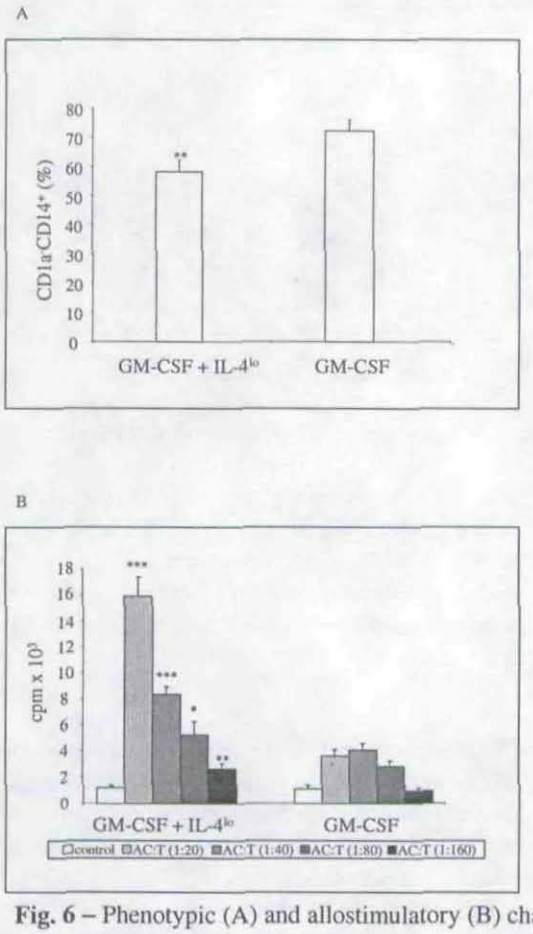

Fig. 6 – Phenotypic (A) and allostimulatory (B) characteristics of adherent cells generated from monocytes in the presence of GM-CSF or GM-CSF and low concentrations of IL-4.

A) Cytospins of adherent cells were stained by an APAP method, as described in *Methods*. Results (mean ± SD; n = 6) are expressed as percentages of $CD1a^-CD14^+$ cells. * = $p < 0{,}05$ compared to the corresponding control. B) Proliferation of T cells in MLR (mean cpm ± SD of triplicates) was measured using [^{3}H]-thymidine incorporation, as previously described. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$ compared to corresponding controls. Control = cpm (T cells, alone) + cpm (AC, alone), AC-adherent cells; T-T cells.

DC differentiated in the presence of lower concentrations of IL-4 had stronger proapoptotic activity for the Jurkat cell line

Finally, we examined the ability of monocyte-derived DC to induce apoptosis of the Jurkat cell line, based on recent results, showing that immature DC possessed NK-like activity (16, 17). As shown in Fig. 7 apoptosis of Jurkat cells was higher in cultures with DC than their spontaneous apoptosis. The percentage of apoptotic cells (especially after 48 hrs) was higher using DC generated with GM-CSF and low concentrations of IL-4, compared to the apoptosis induced with DC generated in the presence of higher concentrations of the cytokine.

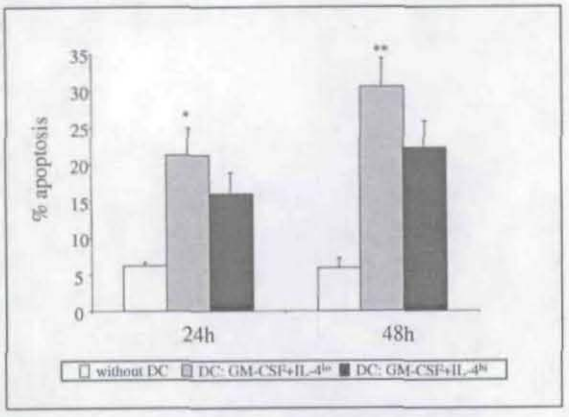

Fig. 7 – Apoptosis of Jurkat cells induced by monocyte-derived DC.

Apoptosis was determined using morphological criteria after staining of cells with Türck reagent, as described in *Methods*. Values (mean ± SD; n= 6) are given as percentages of apoptotic cells. * = $p < 0.05$; ** = $p < 0.01$ compared to values in cultures using DC generated in the presence of higher concentrations of IL-4.

Discussion

DC are recognized as the most efficient professional APC for the induction of primary immune responses (1, 2, 27). Several previous studies showed that human DC might have developed from $CD14^+$ blood monocytes cultivated with relatively high concentrations of GM-CSF (80 ng/ml – 100 ng/ml), and IL-4 (20 ng/ml – 100 ng/ml) (10–14, 23). We showed in this work that IL-4 was a necessary cytokine for the development of DC, since about 20% of non-adherent cells were generated from monocytes when culture medium was supplemented with GM-CSF only. In addition, we provided evidence that a much lower concentration of IL-4 (5 ng/ml) was sufficient for the differentiation of non-adherent DC that are phenotypically and functionally similar to DC generated in the presence of higher concentrations of IL-4 (50 ng/ml). Decreasing concentrations of GM-CSF bellow 80 ng/ml significantly lowered DC yield (data not presented).

In our culture system, non-adherent DC, generated from monocytes, had typical dendritic morphology, expressed high levels of MHC class II molecules, low levels of CD80, moderate levels of CD86, and down-regulated CD14. More than a half of them expressed CD1a, whereas CD83 – a typical marker for mature DC, was not detected. Such phenotypic characteristics were previously published for immature DC (2, 3, 6, 10), but some differences that were not related to IL-4 concentrations were observed. In our cultures CD1a was not expressed by all monocyte-derived DC, nor did CD14 completely disappear, as previously published for DC generated in the medium with FCS (10, 12). CD1a was not detected on monocyte-derived DC differentiated in medium with autologous serum, whereas the replacement of serum with heparinized plasma led to the expression of the molecule by about 60% of DC (23). These facts and our findings that the expression of CD1a and down-regulation of CD14 varied between donors, suggested that preactivation state of monocytes and/or quality of FCS could be responsible for the observed phenomenon.

We showed that immature DC were efficient activators of alloreactive T cells, in spite of relatively low expression of CD80 and CD86. These costimulatory molecules were of crucial importance in generating signal 2 by binding to CD28 on T cells, and together with signal 1 transmitted through TCR and coreceptor molecules, enabled the proliferation of T cells (9, 18, 27). These contradictory findings could be explained by the maturation of DC co-cultured with T cells. Among numerous signals provided by T cells, the interaction between CD40-ligand expressed by activated T cells and CD40, expressed by DC, was most important for the final maturation of DC (2, 12). Mature DC up-regulated costimulatory molecules and produce IL-12, a key cytokine that induced polarization of the T cell response towards Th1 (2, 3, 9, 12).

Adherent cells developed in monocyte cultures are mostly considered being macrophages by their phenotype ($CD1a^-$ $CD14^+$), expression of the M-CSF receptor and inability to stimulate naive T cells (11, 22). We demonstrated that adherent cells in cultures with GM-CSF, in the absence of IL-4, met these criteria, but unexpectedly, adherent cells in cultures with GM-CSF and IL-4, although phenotypicaly different from non-adherent immature DC, stimulated the proliferation of allogeneic T cells in MLR. By the characteristics, these cells probably represented a subset of the immature DC. The plasticity of monocyte differentiation pathway *in vitro* was well documented. Monocytes cultivated in the presence of GM-CSF differentiated into adherent macrophages. In contrast, the withdrawal of M-CSF and the addition of GM-CSF and IL-4 led to the differentiation of most macrophages into non-adherent, immature DC. However, under such conditions a proportion of cells still remained adherent (11, 28). Although the dynamics of phenotypic and functional changes of such cultivated macrophages had not been fully characterized, our results supported the concept that monocyte – or macrophage-derived DC were adherent cells at the early stage of their differentiation.

One important finding presented in this work is the ability of immature DC to induce apoptosis of the Jurkat leukemic T cell line. The property of immature, but not mature, human monocyte-derived DC to induce apoptosis of different tumor cell lines *in vitro* (16, 17) made these cells functionally similar to NK cells. These characteristics of DC should be considered, when DC are used for anti-tumor therapy. However, we are at the beginning of understanding the mechanisms involved in cell apoptosis induced by DC, and defining optimal conditions for the generation of DC with strong cytotoxic potential. Our finding that the pro-apoptotic effect of DC was higher when these cells were generated using lower concentrations of IL-4, a phenome-

non that has not been described yet, was a good starting point for further studies.

In conclusion, we described phenotypic and functional properties of immature DC generated from human monocytes using GM-CSF (100 ng/ml) and lower concentrations of IL-4 (5 ng/ml) that have not been published so far. Allowing cost reduction experiments, this method has some advantages in the generation of more cytotoxic DC against tumor cells over the protocol using ten times more IL-4. We are currently testing factors that stimulate maturation of DC *in vitro* and functional potential of such prepared mature DC.

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Apstrakt

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DIFERENCIJACIJA HUMANIH DENDRITIČNIH ĆELIJA OD MONOCITA *IN VITRO* KORIŠĆENJEM FAKTORA STIMULACIJE GRANULOCITNO-MAKROFAGNIH KOLONIJA I NISKE KONCENTRACIJE INTERLEUKINA-4

U više laboratorija su uspostavljeni sistemi za kultivaciju velikog broja humanih dendritičnih ćelija (DĆ) od monocita korišćenjem faktora stimulacije granulocitno-makrofagnih kolonija (GM-CSF) i interleukina-4 (IL-4). U ovom radu je pokazano da je kombinacija GM-CSF (100 ng/ml) i mala koncentracija IL-4 (5 ng/ml) podjednako efikasna za dobijanje nezrelih, neadherentnih, DĆ monocitnog porekla kao i kombinacija GM-CSF sa deset puta većom koncentracijom IL-4 (50 ng/ml). Ovaj zaključak izveden je na osnovu sličnog fenotipskog profila DĆ (ispoljavanje CD1a, CD80, CD86 i HLA-DR, smanjenje ekspresije CD14 i odsustva CD83), kao i slične alostimulatorne aktivnosti ovih ćelija za limfocite T. U kulturama sa nižim koncentracijama IL-4 prisutan je bio veći broj adherentnih ćelija nego u kulturama sa većim koncentracijama IL-4. Međutim, većina ovih ćelija je smanjivala ekspresiju CD14 i stimulisala proliferaciju aloreaktivnih limfocita T. Nasuprot njima, adherentne ćelije, diferentovane samo u prisustvu GM-CSF, koje su ispoljavale CD14 i nisu imale sposobnost stimulacije aloreakativnih limfocita T, pokazivale su karakteristike makrofaga. DĆ obrazovane u prisustvu manjih koncentracija IL-4 imale su veći potencijal za indukciju apoptoze Jurkat ćelijske linije, a time i snažniji citotoksični, antitumorski efekat nego DĆ diferentovane u prisustvu većih koncentracija IL-4.

Ključne reči: ćelije, dendritične; monociti; fenotip; faktor stimulacije granulocitno-makrofagne kolonije; interleukin-4; apoptoza; limfociti T.

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