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Original article

Differentiation plasticity of human monocytes in culture

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Summary

Introduction. Human monocytes are heterogeneous and plastic cell population with the ability to undergo phenotypic and functional changes as a response to a stimulus from a local microenvironment. Our aim was to determine the potential of human monocytes to differentiate into different cell populations depending on two different cytokines (IL-4 and IL-6) added to cultures as well as to compare their phenotypical and functional characteristics.

Methods. Peripheral blood mononuclear cells (PBMNC) were isolated from buffy coats of healthy donors. Monocytes, which were separated from PBMNC by plastic adherence, had been cultivated in Dendritic cell (DC), serum free medium for 5 days, either with granulocyte/macrophage colony-stimulating factor (GM-CSF) alone or with GM-CSF, with addition of interleukin 4 (IL-4) or interleukin 6 (IL-6), respectively. After cultivation, phenotypic characteristics of these cells were analyzed by flow cytometry, whereas the levels of produced cytokines in culture supernatants were quantified by ELISA. The potential of differentiated cells to modulate the proliferation of allogeneic T cells was examined by co-cultivation of these cells with PBMNC.

Results. GM-CSF differentiated monocytes into M0/M1 macrophages (MØ). The combination of GM-CSF and IL-4 favoured differentiation of immature DC, whereas GM-CSF and IL-6 transformed monocytes into monocytic myeloid derived suppressor cells (M-MDSC). All cell populations expressed typical monocyte/macrophage markers such as CD14, CD11b, CD16 and CD33, HLA-DR, CD209 and CD86, a co-stimulatory marker. DC and M-MDSC expressed CD1a and CD11c, in contrast to M0/M1 MØ. The expression of HLA-DR, CD1a, CD209 and CD86 was highest on DC. The expression of CD33 and CD16 was highest on M-MDSC, followed by lowest expression of HLA-DR. The potential of promoting T-cell proliferation was highest in cultures of PBMNC with DC, whereas M-MDSC had the opposite, suppressive, effect. These differences correlated with highest production of immunosuppressive cytokines such as IL-10, IL-27 and TGF-β by M-MDSC.

Conclusion. This study confirmed the differentiation plasticity of human monocytes, which are influenced by cytokines added in cultures. Phenotypic characteristics of these cells correlated with the production of cytokines involved in modulation of T-cell proliferation.

Key words: plasticity, differentiation, monocytes, culture, cytokines, T cell proliferation

Introduction

Human monocytes are heterogeneous and plastic cell population which has an important role in the immune response by sensing their local microenvironment, clearing pathogens and dead cells. In response to a stimulus from a local microenvironment, these cells have the competence to undergo phenotypic and functional changes [1]. It is well known that the addition of human recombinant granulocyte macrophages colony stimulating factor (GM-CSF) is necessary for *in vitro* differentiation of human monocytes into mature and functionally competent M0/M1 macrophages (M0/M1 MØ). The term M0 means that macrophages are undifferentiated, whereas M1 are a key subset of pro-inflammatory MØ [2]. However, for differentiation of monocytes into dendritic cells (DC) and myeloid derived suppressor cells (MDSC), except GM-CSF, the addition of interleukin-4 (IL-4) and interleukin-6 (IL-6), respectively, is required [3, 4].

Depending on the type of environmental stimuli, MØ have a wide array of functions and they have been primarily classified into: pro-inflammatory (M1), induced by GM-CSF with addition of interferon-γ and/or lypopolysaccharide (LPS) and anti-inflammatory (M2) MØ induced by macrophage colony stimulating factor (M-CSF) [5]. Macrophages are predominant cell population in the tumor and these cells are frequently named as tumor associated machrophages (TAM). Unlike M2, TAM, which predominantly promote the progression of tumor, M1 MØ have the opposite effect [6].

DC are the most potent antigen presenting cells orchestrating the adaptive immune response. In response to infection, DC are capable to differentiate into mature cells that can initiate immune responses, while in the absence of infection, most of them remain in an immature form and induce tolerance to self antigens [7]. The main function of mature DC is to process and present antigen peptides to $CD4^+$ and $CD8^+$ T cells. However, in the tumor microenvironment (TME), this maturation process is disturbed by increasing the accumulation of immature DC [8]. As a result, DC fail to activate tumor reactive T cells and become tolerogenic. Dysfunction of DC and reduction in their number in patients with cancer may be the result of cross-talk between MDSC and DC [9]. MDSC are critical in the regulation of immune responses by suppressing function of DC and T cells [10].

MDSC present immature cell population of myeloid origin that accumulate in patients with cancer, burns, sepsis, or chronic inflammation. Their main physiological function is to avoid and reduce tissue damage during inflammation [11]. Immature myeloid cells generated in bone marrow can differentiate into mature granulocytes, MØ or DC, without causing detectable immunosuppression in healthy individuals. In pathological conditions such as cancer, a partial block in differentiation of immature into mature myeloid cells results in induction of pathological MDSC. These cells are capable of blocking adaptive immunity by inhibiting the activation of $CD4^+$ and $CD8^+$ T cells [12]. Neoplastic cells in TME affect bone marrow by releasing soluble factors such as cyclooxygenase-2, prostaglandin E-2, IL-6 and GM-CSF that drive the accumulation, expansion and activation of local MDSC [13]. Currently, two MDSC populations have been characterized: monocytic MDSC (M-MDSC) and granulocytic MDSC (G-MDSC). M-MDSC are significantly immunosuppressive in comparison to G-MDSC. M-MDSC are phenotypically described as $CD14^+$ $CD11b^+$ $CD33^+$ $CD15^{-/low}$ $HLA\text{-}DR^{-/low}$ [14].

However, despite the increasing amount of data on the potential benefits of M-MDSC in different clinical situations, the protocols for differentiation of human M-MDSC *in vitro* have not been fully established. There are only few published studies describing methods for the *in vitro* generation of human M-MDSC [4, 15, 16]. We have recently shown that the combination of GM-CSF and IL-6 is capable of transforming monocytes into potent M-MDSC [17]. It is obvious that GM-CSF is a key factor for monocytes to differentiate into different effector cells. Therefore, the aim of the study was to check how addition of two different cytokines (IL-4 and IL-6, respectively), changes phenotypic and functional properties of MØ, generated in the presence of GM-CSF alone.

Methods

Most experiments were performed at the Center for Biomedical Sciences, Medical Faculty Foca, University of East Sarajevo. Cell proliferation assay was performed at the Institute for Medical Research, Military Medical Academy, Belgrade, Serbia.

Cells

Buffy coats were obtained from voluntary healthy blood donors. Peripheral blood mononuclear cells (PBMNC) were isolated on Lymphoprep gradient (PAA Laboratories) by density centrifugation (2200 rpm, 20 min, 20˚C). Subsequently, PBMNCs were used for the isolation of monocytes by plastic adherence, as described [18].

M0/M1 MØ were generated by cultivating adherent monocytes (1 x10^6/ml) in Cell Genix® GMP Dendritic cell Medium (CellGenix. Freiburg, Germany), supplemented with 100 ng/ml of GM-CSF (Leucomax Basel, Switzerland) within the period of 5 days. For generation of immature DC, except of GM-CSF, human recombinant interleukin (IL)-4 (Rosche diagnostics, Basel Switzerland) was added in concentration of 20 ng/ml. Human recombinant interleukin (IL)-6 (R&D Systems, Minneapolis, MN, USA) was added in concentration of 10 ng/ml, together with GM-CSF, for generation of M-MDSC. The cultures had been maintained for 5 days.

Flow cytometry

The analysis of markers expressed by M0/M1 MØ, DC and M-MDSC was performed on a flow cytometer (Attune Acoustic Focusing Cytometer, Applied Biosistems, by Life Technologies) after labelling the cells with primary antibodies (Abs). The following Abs and reagents were used: anti-CD11c-FITC, anti-CD11b-PE, anti-CD1a-PerCP, anti-CD209-APC, anti-CD14-FITC, anti-CD16-PE, anti-HLA-DR-PerCP, anti-CD33-APC and anti-CD86-FITC (all from Biolegend, San Diego, CA, USA).

Proliferation assay

PBMNC (3x10^5/well) were cultured in 96-well flat bottom plates (Sarstedt), alone, or co-cultured either with M0/M1 MØ, DC or M-MDSC, all at the concentration of 1x10^4/well. For stimulation, phytohemaglutin (PHA) was used at the suboptimal concentration of 1µg/ml. The cultures were set up in triplicates. After four days, ^{3}[H]-thymidine (1µ Ci/well) (Amersham, UK), had been added and the cultivation had been continued for the next 8 hours. After cell harvesting, the incorporated radioactivity was measured by using a scintillation counter (Ragbeta, Finland). The results are expressed in count per minutes (cpm). The proliferation index was calculated by dividing each cpm from PHA-stimulated cultures with mean cpm of control unstimulated PBMNC cultures (442 cpm).

Cytokines

The levels of cytokines from culture supernatants were determined by ELISA commercial kits (R&D, Minneapolis, USA), according to manufacturer's protocol. The concentrations of cytokines from M0/M1 MØ, DC and M-MDSC culture supernatants: transforming growth factor β (TGF-β), IL-10 and IL-27 were calculated from standard curves after the subtraction of blank controls.

Ethics statement

Approval for all experiments was obtained from the Ethics Committee of the University of East Sarajevo, Faculty of Medicine in Foca (permission date: September 12t^h 2018, Foca). Informed consent was obtained from participants in accordance with the Declaration of Helsinki.

Statistical analysis

The results are presented as mean ± SD values of at least three independent experiments carried out using cells of different healthy donors. To evaluate the differences between the experimental and corresponding control samples, the data were analyzed using One-way ANOVA. Re-

sults were analyzed in GraphPad Prism software (GraphPad, La Jolla, CA, USA) and as a level of statistical significance, p<0.05 was considered.

Results

The first aim of this study was to analyze phenotypic properties of cells differentiating from monocytes under different culture conditions: M0/M1 MØ, differentiated in the presence of GM-CSF; DC, differentiated with GM-CSF+IL-4; M-MDSC, differentiated with GM-CSF+IL-6. Figure 1 summarizes phenotypic properties of cultivated cells.

CD14 is a key monocyte marker which is modulated significantly upon differentiation of monocytes. The expression of CD14, which was highest on M0/M1 MØ, was significantly down-modulated on DC and M-MDSC (p<0.001). In addition, a decrease in CD14 expression on DC was higher compared to M-MDSC (p<0.05). Contrary to this, the expression of CD1a and CD11c which was hardly detectable on M0/M1 MØ was up-regulated on DC and M-MDSC (p<0.001). It is interesting, that the expression of CD11c on M-MDSC was higher in comparison to DC (p<0.05). The opposite was seen with CD1a, but the difference was not statistically significant. The expression of HLA-DR, CD209 and CD86 was significantly down-regulated on M-MDSC and M0/M1 MØ, when compared to DC (p<0.001). In contrast, the expression of CD16 and CD33 on M-MDSC was significantly higher in comparison to DC (p<0.05), while CD33 was up-regulated in comparison to M0/M1 MØ (p<0.05). The differences in the expression of CD11b were not found between these

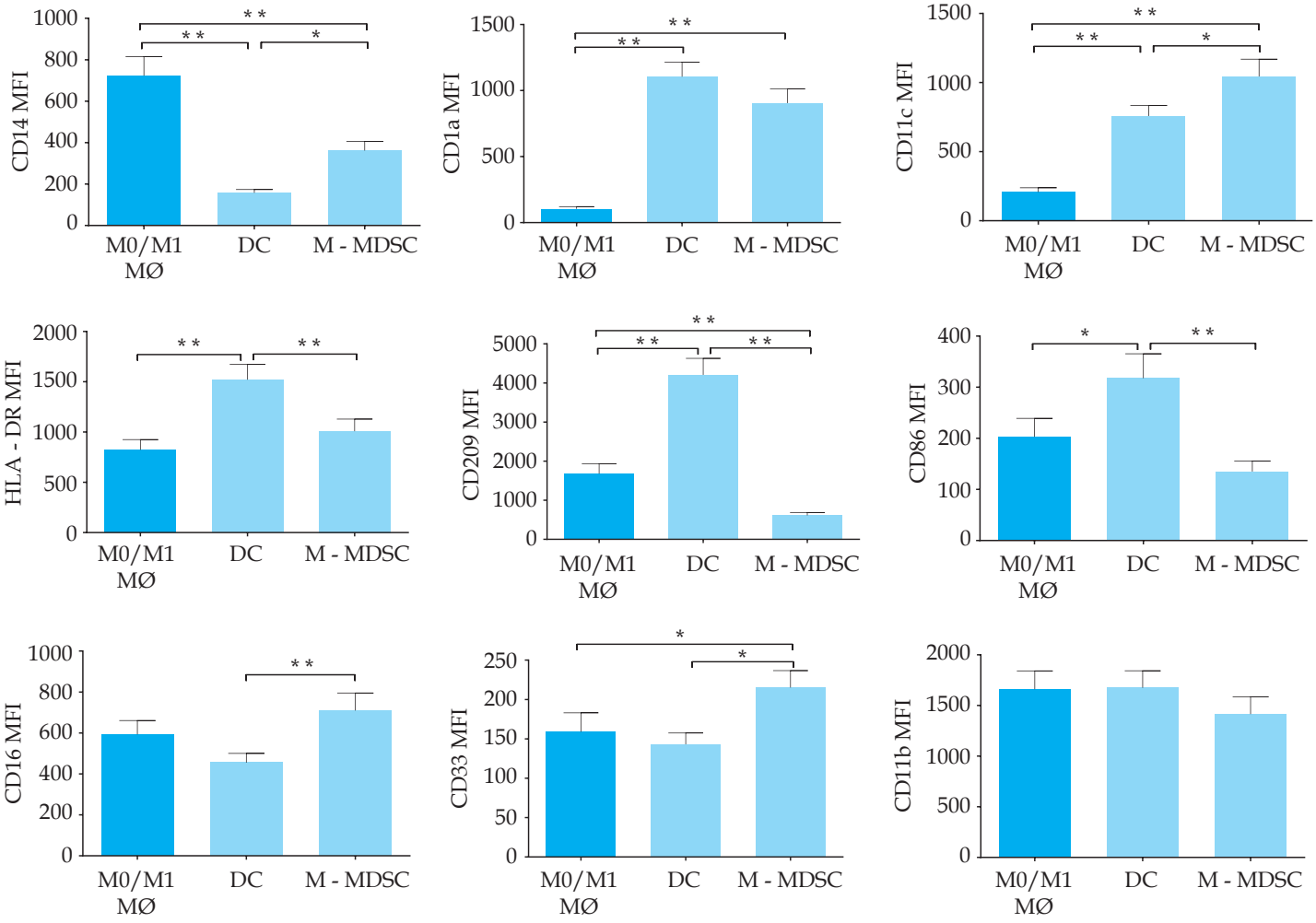

Figure 1. Expression of characteristic M0/M1 MØ, DC and MDSC markers
*p<0.05; **p<0.001, compared to corresponding cells indicated by bars (n=3)

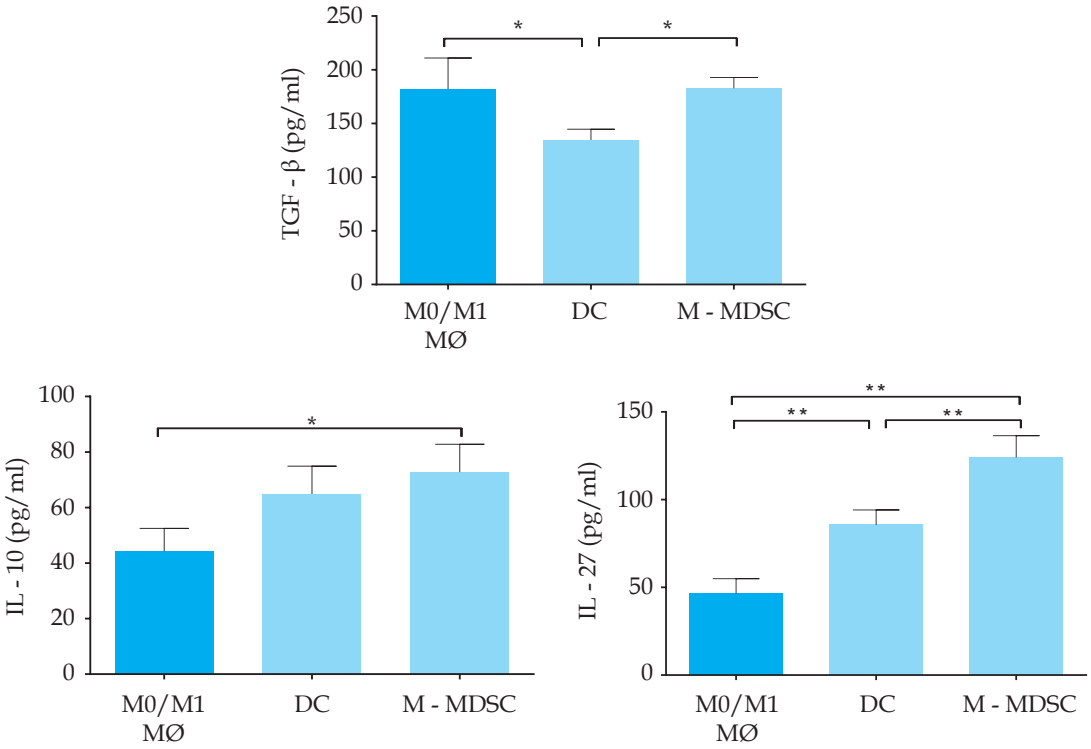

Figure 2. Production of IL-10, TGF-β and IL-27 by M0/M1 MØ, DC and MDSC in culture
*p<0.05; **p<0.001, compared to corresponding cultures indicated by bars (n=3)

cell populations.

After this, we tested the levels of three cytokines with predominant immunosuppressive functions in culture supernatants. M-MDSC and M0/M1 MØ produced significantly higher quantity of TGF-β (182.8±12.71 pg/ml and 182±29 pg/ml, respectively), compared to DC (134.6±11.20pg/ml) (p<0.05). M-MDSC also produced significantly more IL-10 (72.8±10.0 pg/ml) in comparison to M0/M1 MØ (44.3±8.2 pg/ml) (p<0.05). IL-27 was considerably more produced by M-MDSC (124.02±12.40 pg/ml) compared to DC (85.59±8.56 pg/ml) and M0/M1 MØ (46.30±8.60). Furthermore, the difference in mean concentrations of IL-27 was observed between DC and M0/M1 MØ (p<0.001) (Fig. 2).

PHA is a potent stimulator of human T-cell proliferation. Therefore, the final aim of this study was to examine how cells generated from monocytes in our culture model change T-cell proliferation using whole PBMNC. Fig.3. shows that DC significantly up-regulated PHA-induced proliferation of PBMNC (p<0.05). M-MDSC had the opposite effect (p<0.001), whereas M0/M1 MØ did not significantly modulate PBMNC proliferation.

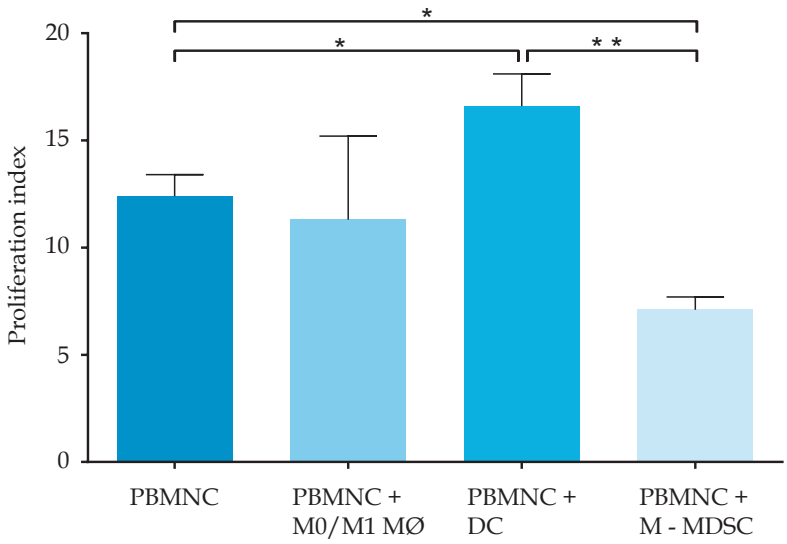

Figure 3. Proliferation of PBMNC in co-culture with M0/M1 MØ, DC or M-MDSC upon stimulation with PHA

Discussion

Two major characteristic features of monocytes are heterogeneity and plasticity. These cells have the ability to undergo phenotypic and functional changes in response to the stimuli from the local microenvironment [1]. It is known that monocytes can differentiate into different subsets of MØ, DC and M-MDSC, depending on the activation factors, but comparison of their phenotypic profiles and functions, which are changed during the differentiation process, is something which is rarely explored [2-4]. This was the reason why we studied these parameters during differentiation of human monocytes in culture.

In healthy individuals, monocytes are easily identifiable among the HLA-DR^{+}CD11b^{+} myeloid compartment. According to the Nomenclature Committee of the International Union of Immunologic Societies, population of monocytes has been divided into three subsets: classical (CD14^{high}CD16^{low} cells) constituting the majority of population (>90%), whereas CD14^{low}CD16^{high} cells, non-classical monocytes and intermediate subset (CD14^{high}CD16^{high} cells) account for only 5-10% of circulating monocytes. Classical and intermediate monocytes manifest inflammatory properties and are referred as inflammatory monocytes [1, 19]. The monocytes originate from monoblasts in the bone marrow that differentiate to promonocytes and then to monocytes. Monocytes play an important role in homeostasis and inflammation including the removal of apoptotic and necrotic cells [20]. Circulating monocytes get recruited to injury sites where they differentiate into macrophages [21]. Even though, the most common function of all macrophages is phagocytosis of microorganisms and apoptotic cells, they are very heterogeneous in terms of their functions and surface marker expression [1]. Macrophages are highly plastic cell population that respond quickly to a variety of environmental stimuli. This plasticity can be easily studied in culture experiments. Aiming to study macrophage induction *in vitro*, different stimuli are used to induce a particular macrophage phenotype [22, 23]. In general, GM-CSF is used as differentiation factor for their full maturation, including transformation of these cells into M0/M1 MØ. In contrast, LPS and IFN-γ are required as maturation factors for induction of M1 MØ. M-CSF together with IL-4 and/or IL-10 are used for M2 induction [5, 24]. M0/M1 MØ are known to produce pro-inflammatory factors and their main function is to phagocytose microorganisms and matrix debris [5]. After cultivation of monocytes with GM-CSF alone Chapuis et al. showed that HLA-DR was down-regulated, while CD14 was highly expressed compared to CD1a [25). Previous reports showed that M0/M1 MØ induced with GM-CSF alone had high expression of CD14 and CD11b [26). Both studies coincide with our results. In addition, we have showed that monocytes cultivated with GM-CSF also express CD11c, CD209, CD86, CD16 and CD33.

MDSC are potent immune suppressor cells. Their number is particularly increased in cancer patients [27]. Neoplastic cells release soluble cytokines that drive the accumulation of MDSC in TME, where they suppress the activation of T cells and stimulate FoxP3^{+} regulatory T cells (Tregs) by which they promote tumor progression [13]. Their detection is important because they have been recognized as a substantial limiting factor for the effectiveness of checkpoint inhibition therapy [28]. Immunotherapy by checkpoint inhibitors was very successful in treating metastatic melanoma, metastatic non-small cell lung cancer (NSCLC), head and neck squamous cell cancer, colorectal cancer, hepatocellular cancer and Hodgkin lymphoma [29]. However, these Abs which are designed to block signaling molecules that regulate T cell activation have impressive results in some, but not in all patients. This non-response to immunotherapy in some patients may be related at least in part to intensive immunosuppression which is mediated by MDSC [28]. Six ongoing clinical trials suggests that possible blockage of suppressive receptors on M-MDSC could improve checkpoint inhibition therapy for advanced renal cell cancer, melanoma, NSCLC and colorectal cancer [29] and that's why investigation of their phenotypical and functional characteristics is important. The selection of cytokines used to obtain M-MDSC from monocytes was based on our previous reports [17]. We showed that these cells expressed high levels of CD33, CD14, CD16 and low levels

of HLA-DR, which is in agreement with the general phenotype of these cells [14, 17]. In addition, M-MDSC expressed CD1a, CD11c and CD11b and had the ability to suppress PHA induced proliferation of T lymphocytes.

When stimulated with GM-CSF and IL-4 in *in vitro* conditions, monocytes yield immature DC. Upon encountering microbial antigens, these cells are transformed into mature DC which are the most potent antigen-presenting cells [7]. Our results have showed high expression of HLA-DR, low expression of CD14, and high expression of CD16, CD11c, CD209 and CD1a on immature DC. These results are generally in agreement with other studies [25, 30]. Some differences depend on donor and medium used for generation of DC. The high expression of HLA-DR, CD1a and CD86 on DC, in comparison with other two cell populations, is associated with the antigen-presenting function of DC. In contrast, higher expression of CD16 and CD33 on M-MDSC, together with low expression of HLA-DR, in comparison to the other cells, supports the opinion that these molecules are of considerable importance for the immune suppressive function of M-MDSC. This is confirmed by strong stimulation of T-cell proliferation by DC in our culture system.

Even though CD1a is a cell surface molecule that is mostly studied in the context of antigen presentation by DC [31], previous reports also suggested up regulation of CD1a marker on human monocytes by GM-CSF [32]. In a study from Beyer at al., monocytes treated with GM-CSF showed significant up regulation of CD1a protein [26]. In our study CD1a has been significantly down regulated in M0/M1 MØ, while it has been up regulated in DC and M-MDSC population. Furthermore, CD11c surface marker being commonly up regulated in MØ [33] has been significantly down regulated when compared to DC and M-MDSC population. The possible explanation for this is that all these cells have been cultured in DC medium which is optimized for the differentiation of CD14+ monocytes into mature DC. Such a type of culture medium have not been used so far for differentiation of macrophage subsets.

To better characterize the functions of cell populations generated from monocytes in our cultures, we analyzed the production of three cytokines (TGF-β, IL-10 and IL-27). TGF-β is a pleiotropic cytokine with a dual function role in cancer and has a capacity to block early stages of tumor progression while promoting progression at late stages of tumor [34]. Within TME, TGF-β is considered as one of the main factors of inflammation by changing the activity of the innate and adaptive response [35-40]. It is considered to be the most immune-suppressive cytokine produced by cancer cells allowing them to escape from immune surveillance [40, 41], which contributes to tumor progression and metastatic potential [42]. Production of TGF-β by TME contributes to the increase of M-MDSC number and function [34] and *in vitro* is capable to induce functional M-MDSC from human monocytes [11]. In our research, we have showed that M-MDSC and M0/M1 MØ produced equal concentrations of TGF-β in comparison with DC which secretes lower levels of TGF-β. Since M-MDSC and M0/M1 MØ have opposite functions (inhibition versus stimulation of the immune response), it can be hypothesized that TGF-β is not a crucial immunosuppressive cytokine for M-MDSC. Another cytokine with immunosuppressive properties is IL-10 [43]. Our results, showing higher production of IL-10 by M-MDSC in comparison with M0/M1 MØ cells, are in line with these findings. However, in contrast to expected results, the difference in production of IL-10 between M-MDSC and DC have not been statistically significant, suggesting that the function of this cytokine can be considered in the context with other biomolecules involved in the adjustment of the immune response. IL-27 is a member of the IL-12 cytokine family. When IL-27 binds to the IL-27 receptor, there are two possible types of responses, pro-inflammatory and anti-inflammatory and the response is mostly dependent on the external surrounding of IL-27 [44]. IL-27 is involved in inducing or suppressing different T cell subsets. Th1 cells are activated, but Th2, Th17 and Tregs are inhibited by IL-27 [45, 46]. Our results show that M-MDSC produced significantly more IL-27 when compared to M0/M1 MØ and DC population suggesting that immunosuppressive function of the M-MDSC population involves IL-27, TGF-β and IL-10, which may act synergistically.

Conclusion

This study have showed that human monocytes undergo phenotypic and functional changes in culture, which are dependent on the cytokine cocktail used. M0/M1 MØ were differentiated in the presence of GM-CSF, DC were differentiated with GM-CSF+IL-4 and M-MDSC were differentiated with GM-CSF+IL-6. Some specific phenotypic changes of MØ subsets are different to those already published. These differences can be due to medium used which is designed for differentiation of DC. Phenotypic characteristics of these cells correlated with the production of cytokines involved in modulation of T-cell proliferation. Given the significant role of these cells in the tumor microenvironment, future research could address the effect of checkpoint blockage on these cells, especially M-MDSC as possible therapy of cancer.

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Ethical approval. The Ethics Committee of the Faculty of Medicine in Foca approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Diferencijalna plastičnost humanih monocita u kulturi ćelija

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Uvod. Humani monociti su heterogena i plastična populacija ćelija sa sposobnošću da se fenotipski i funkcionalno izmijene kao odgovor na stimuluse iz lokalne mikrosredine. Cilj našeg istraživanja bio je da se ispita potencijal humanih monocita za diferencijaciju u druge ćelijske populacije u zavisnosti od vrste citokina (IL-4 i IL-6) dodate u kulturi i da se uporede fenotipske i funkcionalne karakteristike dobijenih ćelija.

Metode. Mononuklearne ćelije periferne krvi (PBMNC) su izolovane iz leukocitnog koncentrata zdravih donora. Monociti, koji su izdvojeni iz PBMNC adherencijom na plastici, su kultivisani u medijumu za dendritske ćelije (DC), tokom 5 dana, u prisustvu faktora rasta granulocitno-makrofagnih kolonija (GM-CSF), ili sa GM-CSF uz dodatak interleukina-4 (IL-4) ili IL-6. Nakon kultivacije, analizirane su fenotipske karakteristike ovih ćelija protočnom citometrijom, dok su nivoi produkovanih citokina analizirani ELISA metodom. Potencijal diferentovanih ćelija u modulaciji proliferacije alogenih T ćelija su ispitivani ko-kultivacijom ovih ćelija sa PBMNC.

Rezultati. GM-CSF je diferentovao monocite u M0/M1 makrofage (MØ). Kombinovana primjena GM-CSF i IL-4 dovela je do diferencijacije monocita u nezrele DC, dok je primjena GM-CSF i IL-6 transformisala monocite u monocitne mijeloidne supresorske ćelije (M-MDSC). Sve ćelijske populacije su ispoljile tipične monocitno/makrofagne markere kao što su CD14, CD11b, CD16 i CD33, HLA-DR, CD209 i CD86, ko-stimulatorni marker. DC i M-MDSC su ispoljile CD1a i CD11c, nasuprot M0/M1 MØ. Eskpresija HLA-DR, CD1a, CD209 i CD86 bila je najviša na DC populaciji. Ekspresija CD33 i CD16 je bila najviša na M-MDSC, što je praćeno najnižom ekspresijom HLA-DR. Potencijal za proliferaciju T ćelija bio je najviši u kulturama PBMNC sa DC, dok su M-MDSC imale suprotan, supresivni, efekat. Ove razlike su bile povezane sa visokom produkcijom imunosupresivnih citokina kao što su IL-10, IL-27 i TGF-β od strane M-MDSC.

Zaključak. Ova studija je potvrdila plastičnost humanih monocita u pogledu njihove diferencijacije u zavisnosti od dodatih citokina u kulturi. Fenotipske karakteristike ovih ćelija su korelisale sa produkcijom citokina koji su uključeni u modulaciju proliferacije T ćelija.

Ključne riječi: plastičnost, diferencijacija, monociti, kultura, citokini, proliferacija T ćelija