

## SHORT PAPER

# Efficiency and Impact of Positive and Negative Magnetic Separation on Monocyte Derived Dendritic Cell Generation

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### ABSTRACT

**Background:** The immunomagnetic separation technique is the basis of monocyte isolation and further generation of monocyte-derived dendritic cells. **Objective:** To compare the efficiency of monocyte positive and negative separation, concentration of beads, and their impact on generated dendritic cells. **Methods:** Monocytes were obtained using monoclonal antibody-coated magnetic beads followed the Ficoll-Paque gradient separation of mononuclear cell fraction from the peripheral blood of 6 healthy volunteers. CD14 expression was analyzed by flow cytometry. **Results:** The percentage of MDDCs generated from monocytes obtained by positive and negative selection was comparable ( $51.8 \pm 15.0$  and  $46.7 \pm 3.4$ , respectively;  $p=0.885$ ). The median values for the number of MDDCs obtained from monocytes after positive selection ( $3.9 \times 10^6$ ) and for MDDCs obtained from monocytes after negative selection ( $3.1 \times 10^6$ ) were comparable ( $p=0.194$ ). The use of the recommended or half of the amount of beads for both types of separation had no significant influence on the percentage of isolated cells. **Conclusions:** Both types of magnetic separation including recommended and reduced concentrations of beads did not affect the yield and the purity of monocytes and their surface CD14 expression. However, DCs originated from the “positively” separated monocytes had noticeable higher expression of CD80.

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## INTRODUCTION

The methods that allow maturing dendritic cells (DCs) from *in-vitro* treatment of other cell types are highly desirable as DCs constitute only 0.16-0.68% of leukocytes and 0.55-1.63% of peripheral blood mononuclear cells, and play a prominent role in the immune mechanisms (1). Among many available methods of monocyte isolation, the immunomagnetic separation (MACS microbead system) from the peripheral blood mononuclear cells (PBMCs) leads to acquiring monocytes with high purity. Positive immunomagnetic selection of CD14<sup>+</sup> cells is one of the varieties of MACS separation and results in acquiring “touched” monocytes. It means that the anti-CD14 monoclonal antibodies are attached to the cell surface. This fact raises some concerns about the possible adverse effects of bounded antibodies on monocyte activation. It is poorly investigated whether monocyte activation may have negative effects on monocyte-derived dendritic cells (MDDCs) generation. This issue seems to be crucial, especially in the context of the use of MDDCs in medical applications, such as cancer immunotherapy or vaccine preparation. In recent years a new type of monocyte separation by MACS system has been developed. The main difference is to deliver the fraction of “untouched” monocytes obtained by depletion of the magnetically labeled all non-CD14<sup>+</sup> cells (B cells, T cells, NK cells, DCs, early erythroid cells, platelets, and basophils) by an indirect labeling system using a cocktail of biotin conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A) (2-4). One of the remaining issues, however, is the relatively high cost of obtaining MDDCs by immunomagnetic separation. Taking into consideration the need to decrease the costs to make this technique more affordable, we checked the efficiency of the isolation using half of the recommended amount of beads stated in the manufacturer’s protocol. Furthermore, we compared the effectiveness of positive and negative separation and their influence on MDDCs generation.

## MATERIALS AND METHODS

**Subjects.** Blood was collected from 6 young healthy volunteers with a mean age of  $30 \pm 3$  years (range: 25-35 years). All studies were approved by the local Ethics Committee. Informed consent was obtained from each participant.

**Isolation of Peripheral Blood Mononuclear Cells.** Peripheral blood (40 ml) was drawn in vacutainer tubes containing spray-coated heparin (Becton Dickinson). After centrifugation ( $150 \times g$  for 15min, RT.) and plasma removal, blood was diluted (1:1) in RPMI 1640 (PAA, Linz, Austria) and layered on Ficoll-Paque PLUS (GE Healthcare) at a ratio of 4:3. After centrifugation at  $400 \times g$  for 30 minutes at 20°C without brakes, PBMCs were collected in the interphase ring. The cells were harvested and washed twice with RPMI 1640. After the final wash the supernatant was completely aspirated and the cells were suspended in 2 ml of the RPMI 1640 medium at RT. PBMCs were counted in the Bürker’s chamber. The mean cell yield after PBMCs isolation out of peripheral blood was  $5.6 \times 10^7 \pm 0.9$  (total number of cells from the donor). The cell viability was assessed using a trypan blue dye and ranged from 98 to 99%. After isolation procedure, PBMCs were immediately used for monocyte isolation with the use of MACS system.

**Positive and Negative Separation.** PBMCs suspension from each blood donor was split into two equal parts and separately processed either with a positive bead selection or with negative depletion (MACS CD14 MicroBeads #130-050-201, and MACS Monocyte Isolation Kit II #130-91-153, respectively, Miltenyi Biotech, Germany), according to the manufacturer's protocol. Briefly, during the positive separation PBMCs were incubated with magnetic beads conjugated with mouse monoclonal anti-human CD14 antibody at two different ratios of beads per  $10^7$  cells: 20  $\mu$ l (as recommended) or 10  $\mu$ l of CD14 Microbeads (half of the recommended amount). After washing with MACS buffer (2 ml of buffer per  $10^7$  cells), cells were centrifuged at  $300 \times g$  for 10 min at  $4^\circ\text{C}$ . The supernatant was removed and the cells were suspended in 500  $\mu$ l of MACS buffer and applied onto a LS column placed in the magnetic field of MACS separator. The magnetically labeled  $\text{CD14}^+$  cells were retained in the column while the unlabeled cells ( $\text{CD14}^-$ ) passed through the column. After removal from magnetic field, magnetically retained  $\text{CD14}^+$  cells (monocytes) were eluted as a positively selected cell fraction. In the case of negative separation, monocytes were obtained from PBMCs through the depletion of B cells, T cells, natural killer cells, DCs, early erythroid cells, platelets and basophils by an indirect magnetic labeling using a cocktail of biotin conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A, as primary labeling reagent, and anti-biotin mAb coated microbeads, as secondary labeling reagent. To this end, PBMCs ( $10^7$  cells) have been suspended consecutively in: 30  $\mu$ l of MACS buffer, 10  $\mu$ l FcR Blocking Reagent, and either in 10  $\mu$ l (as recommended) or in 5  $\mu$ l of Biotin-Antibody Cocktail, and incubated for 10 minutes at  $4-8^\circ\text{C}$ . Next, 30  $\mu$ l of MACS buffer and either 20  $\mu$ l (as stated in the manufacturer's protocol) or 10  $\mu$ l of Anti-Biotin magnetic beads were added. After incubation for an additional 15 minutes at  $4-8^\circ\text{C}$ , the cells were washed with MACS buffer and centrifuged. The cell pellet was suspended in 500  $\mu$ l of MACS buffer and applied onto a LS column placed in the magnetic field of MACS separator. The effluent of highly pure unlabeled monocytes was collected.

**Monocyte Differentiation into MDDCs and their Stimulation.** After MACS isolation, monocytes ( $2 \times 10^6$ /well/2ml) were cultured in RPMI 1640 medium supplemented with 10% FBS (Sigma, Germany) and 10 ng/ml IL-4 and 25 ng/ml GM-CSF (R&D System, USA) for 6 days ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) to differentiate into MDDCs. After that, the cells were incubated on ice for 30 min to detached them from the plastic surface of the well, centrifuged, and suspended in culture medium with 10% FBS (+100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine; Gibco, USA).  $1 \times 10^6$  cells/ml was seeded onto 12-well culture plate and infected with *M. tuberculosis* H37Rv (kindly provided by the Institute of Tuberculosis and Lung Diseases, Warsaw, Poland) at MOI of 1:1 for 24 h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ .

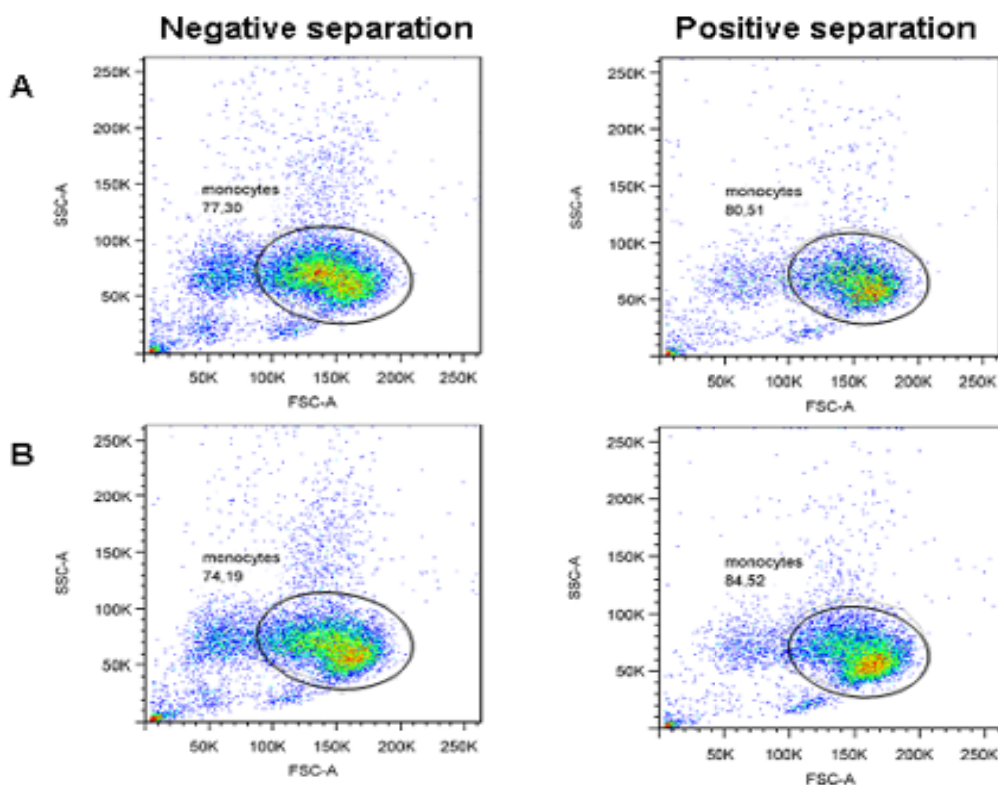
**Flow cytometry.** The monocytes obtained with positive and negative magnetic separation were adjusted to the density of  $3 \times 10^5$  cells/ml and incubated for 30 minutes at  $4^\circ\text{C}$  with the monoclonal mouse antibodies (mAb): FITC-conjugated anti-human CD14 or an irrelevant isotype-matched mAb as control. MDDCs, after bacterial stimulation, were treated with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free ice-cold PBS containing EDTA (2 mM) for 10 min, harvested and centrifuged. Finally, the pellet was suspended in 0.5 ml PBS (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and the cells were split up into equal samples and stained with the following mouse mAb: FITC-conjugated anti-human: CD86, CD40, HLA-DR, DC-SIGN, PE-conjugated anti-human CD80 for 30 minutes at  $4^\circ\text{C}$ . All mAbs were purchased from Becton Dickinson. After two washings with PBS and centrifugation, the cells were

acquired and analyzed using the FACS LSRII (Becton Dickinson) and FlowJo software, a minimum 10,000 events were collected. The calculated mean fluorescence intensity (MFI) represents the molecule density on the cell surface.

**Statistical Analysis.** Statistical analyses were performed with STATISTICA 10.0 PL software. Data are expressed as median  $\pm$  SD. Differences between samples were analyzed by Mann-Whitney U test (for unpaired data). P values of  $\leq 0.05$  were considered significant.

## RESULTS AND DISCUSSION

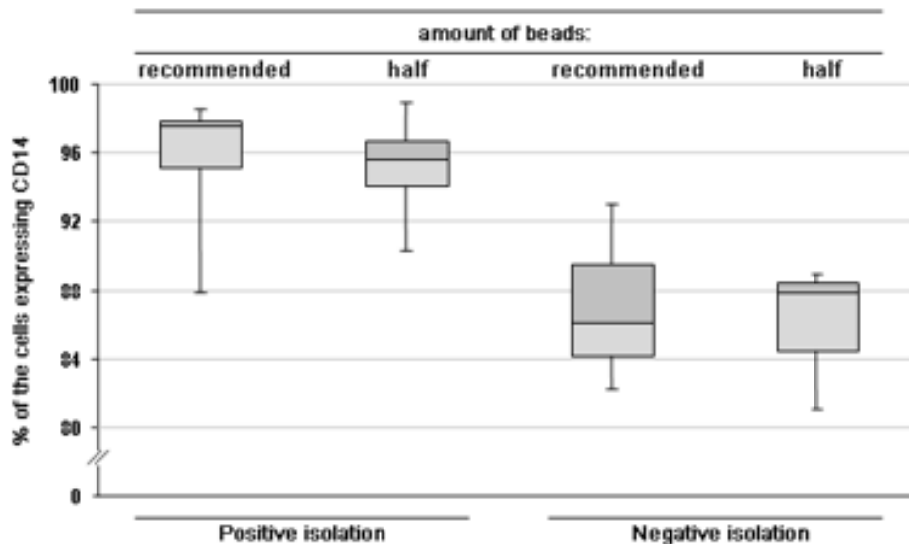
One of the methods for acquiring monocytes with high purity is the immunomagnetic separation from PBMCs. There are two varieties of this method: a positive and a negative technique, which lead to receiving “touched” or “untouched” monocytes, respectively. The separation using magnetic beads coated with the specific monoclonal antibodies (mAb) is quite common (5-11). Although both positive and negative separations are relatively uncomplicated to perform, their potential use is limited due to the high cost. To address the issue we tested whether the reduced number of magnetic beads will affect the performance of monocyte isolation. We showed that type of separation and the use of recommended or reduced to half the amount of microbeads did not influence the number of isolated monocytes.



**Figure 1.** Forward (FSC) and side scatter (SSC) of the leucocyte population. The gated cells (monocytes) were isolated by negative and positive separation method with recommended amount of beads (A) or half of the amount (B).

After positive and negative separation with the use of recommended amount of beads, the cells constituted  $4.9 \pm 3.2\%$  and  $3.2 \pm 0.5\%$  of PBMCs, respectively, and  $5.5 \pm 2.8\%$  and  $4.1 \pm 0.8\%$  when half of the amount of beads was used. As shown in Figure 1, the use of the recommended or half of the amount of beads for both types of separation had no significant influence on the placement of monocytes on the FSC/SSC dot-plot.

The mean percentage of gated cells was approximately  $80.5\% \pm 3.3$  in case of the positive selection and  $77.3\% \pm 0.8$  in case of the negative selection. Following monocyte gate setting, the mean percentages of CD14<sup>+</sup> gated monocytes after positive selection using the recommended amount of beads and the half of the amount were  $95.4\% \pm 5.0$  and  $95.1\% \pm 3.6$ , respectively. Cognately, for the negative selection the results were  $87.1\% \pm 5.5$  and  $85.8\% \pm 4.1$ . The percentage of cells with the CD14 expression obtained by the separation with the use of recommended or half amount of magnetic beads was comparable (Figure 2) either in the case of positive or in the case of negative isolation.

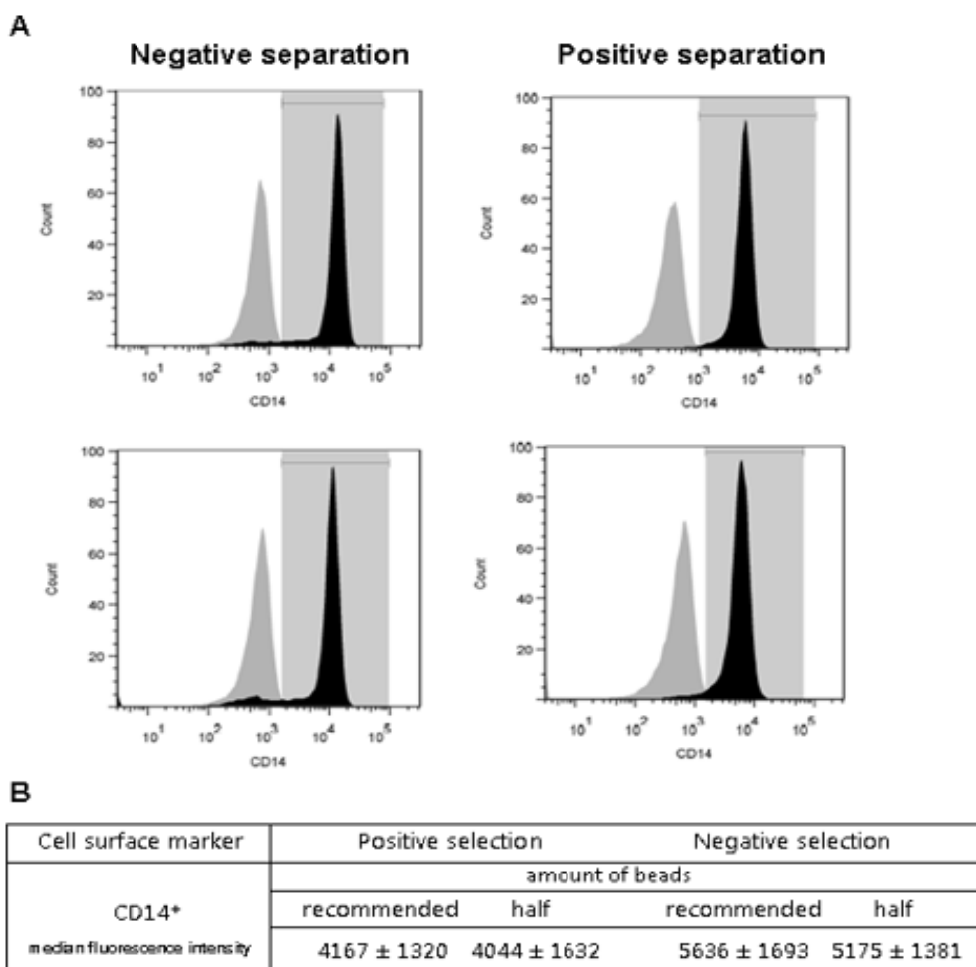


**Figure 2.** Percentage of positive cells with the CD14 expression obtained with positive and negative isolation methods using recommended or half amount of magnetic beads. A BoxPlot (box and whisker diagram) shows a measure of central location (the median) and two measures of dispersion (the range and inter quartile range).

Moreover, as it was shown in Figure 3, there were no significant differences ( $p > 0.05$ ) in the MFI values of the CD14 expression on the surface of monocytes obtained with positive and negative isolation method both when recommended or half of the amount of beads were used.

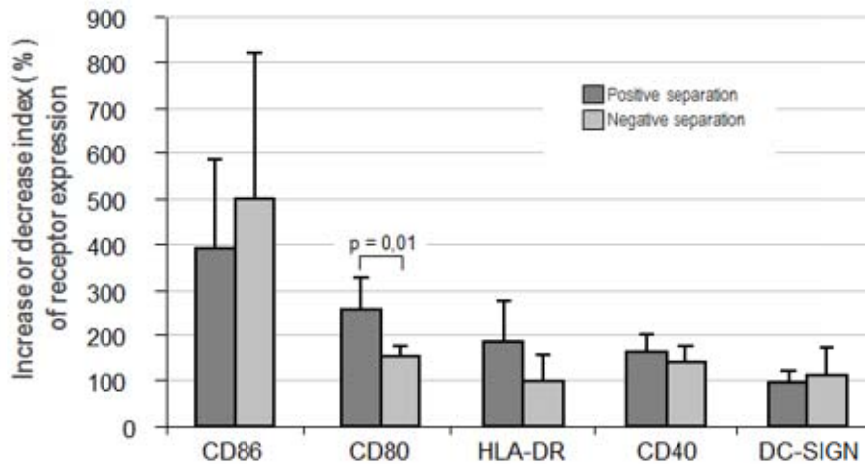
Since the efficiency of monocyte delivery by positive and negative separation was comparable using the recommended and half-reduced amount of beads, in the next step the effectiveness of dendritic cell generation was determined only for the recommended amount of beads-variant. The total number of monocytes obtained either by positive or by negative monocyte selection from the same donor was compared with the total

number of differentiated dendritic cells from these monocytes. The percentage of MDDCs generated from monocytes obtained by positive and negative selection was comparable ( $51.8 \pm 15.0$  and  $46.7 \pm 3.4$ , respectively;  $p=0.885$ ). The median values for the number of MDDCs obtained from monocytes after positive selection ( $3.9 \times 10^6$ ) and for MDDCs obtained from monocytes after negative selection ( $3.1 \times 10^6$ ) were comparable ( $p=0.194$ ). The method chosen to obtain monocytes may have an impact on the properties of monocyte-derived dendritic cells, particularly the expression of selected receptors. Therefore, the expressions of CD80, CD86, HLA-DR, CD40 (participating in antigen presentation and belonging to immune synapse) and DC-SIGN (indicating the dendritic cell maturity) were evaluated using MDDCs stimulated with *M. tuberculosis* H37Rv.



**Figure 3.** Panel A: CD14 surface expression of monocytes obtained with negative and positive isolation method. Upper row – recommended amount of beads, bottom row – half of the amount of beads. One representative experiment out of 6 independent ones is shown. White histograms represent the cell reactivity to fluorochrome-matched isotype control antibodies. Grey histograms represent the reactivity with the anti-CD14 antibody. Panel B: The median fluorescence intensity (MFI) values (median ± SD of 6 independent donors). Fluorescence intensity was calculated by the MFI of the CD14 expression from which the MFI obtained with a nonrelevant, isotype-matched antibody was subtracted. Statistical analyses were performed using the Mann-Whitney test.

This intracellular bacterial pathogen, besides its ability to stimulate the production of the wide panel of cytokines by DCs, is known to be an effective inducer of expression of DC molecules associated with DC competency in antigen presentation (12).



**Figure 4.** Level of the expression of selected receptors of monocyte-derived dendritic cells after stimulation with *M.tuberculosis* H37Rv. First bar in each pair represents data obtained after positive separation, the second bar — after negative separation. Median values (expressed in units of MFI) received for each type of receptor of unstimulated MDDCs were adopted as 100%. Alterations of receptors' expression after stimulation with bacteria were expressed as % index. Percentage index was calculated as follows:

$$\% \text{ index} = \frac{\text{MFI value for receptor on stimulated cells}}{\text{MFI value for receptor on unstimulated cells}} \times 100$$

The values exceeding 100% show the enhancement of the expression and values below 100% show decline in the expression.

After bacterial stimulation, the highest increase for CD86 marker was observed and was characteristic for both positive and negative isolation. The expressions of the other immune synapse receptors: CD80, CD40, HLA-DR were also elevated but not as dynamic as for CD86. That trend was a bit less intensive in relation to CD80 and CD40 receptors on DCs obtained from negative isolation (“untouched” cells). After stimulation with bacteria, the CD80 expression was significantly increased on the surface of positively separated dendritic cells compared with those negatively separated ( $p=0.01$ ). No significant changes were observed in the case of DC-SIGN. Reuter *et al.* (2) also reported that, regardless of the variants of monocyte magnetic separation method, the level of basal CD86 expression did not reveal any significant difference. However, they assumed that after cell stimulation the differences could appear. It is difficult to pinpoint the precise mechanism underlying the observed enhancement of the CD80 expression on DCs derived from monocytes obtained by positive selection in comparison to the CD80 expression on the cells generated by negative selection after *M. tuberculosis* stimulation. The way of monocyte isolation can implicate certain capacities of generated cells. For example, it was reported by Elkord *et al.* (10) that the monocyte

adhesion to the plastic surface resulted in high levels of TNF- $\alpha$ , IL-12p70 and IL-10 secretion after LPS stimulation in contrast to the monocytes isolated by positive MACS selection, however, the expression of CD80, CD86 and CD83 remained at the similar levels. The possibility of blocking mCD14 molecules by anti-CD14 microbeads followed by the positive selection is suggested. It cannot be excluded that the binding of the microbeads with the membrane CD14 may change the status of the cells (they become the “touched” cells) which later could have an impact on MDSCs phenotypic characteristics. The “touched” status can implicate various and numerous transcriptional changes which makes it extremely difficult to identify the exact single mechanism responsible for the distinct expression of CD80 on the cells after positive and negative selection. Our observation indicates that within the studied receptors, even after using *M. tuberculosis* H37Rv as the stimulator, the alterations in expression of investigated receptors are very limited. The lack of differences in the expression of HLA-DR, as well as CD11c, CD83 and CD1a in dendritic cells derived from monocytes obtained either by MACS-positive magnetic separation or by plastic adherence was also shown in the study done by El-Sahrigy *et al.* (13). Moreover, the authors suggested that to make the comparison of separation methods more accurate, the monocytes should be taken from the same donor. It is worth mentioning that in our work we used the cells originated from the same volunteers. Type of monocyte isolation procedure can modulate the intensity of cytokine production, which was reported by Delirez and Shojaeefar (14). They observed lower IFN- $\gamma$ : IL-4 and IL-12: IL-10 ratios in the case of dendritic cells obtained by MACS-positive selection compared to the cells received through adherence of monocytes to plastic surface. The modulation of cytokine release generated by the technique of monocyte selection can determine the area of DCs application. Moreover, the authors raise the issue of the high cost associated with a magnetic selection of cells. Our results indicate that the use of reduced quantities of mAb-microbeads allows to establish a cost efficient method, cutting the amount of beads in half does not affect the yield and purity of obtained cells as well as the surface CD14 expression on monocytes, and the presented procedure is applicable either for the positive or negative monocyte separation.

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