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Phenotypic and Functional Comparison between Flask Adherent and Magnetic Activated Cell Sorted Monocytes Derived Dendritic Cells

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ABSTRACT

Background: Generation of an effective dendritic cell (DC) based cancer vaccine depends on appropriate differentiation of monocytes in vitro. **Objective:** To compare the effects of monocyte separation methods, flask adherence (Flask-DC) and magnetic activated cell sorting (MACS-DC), on phenotypic and functional characteristics of resultant DCs. **Methods:** DCs from healthy volunteers were generated from plastic adherence and MACS isolated monocytes in the presence of GM-CSF and IL-4 as well as TNF-α and monocyte conditioned medium (MCM) in 7 day cultures. Mature DCs were then subjected to phenotypic analysis using anti-CD14, anti-CD83 and HLA-DR monoclonal antibodies. Functional and cytokine release assays were carried out using [³H] thymidine uptake test and commercially available ELISA kits for the determination of IL-12, IL-10, IFN-γ and IL-4, respectively. **Results:** We found that MACS-DCs were more homogenous and the yield and viability were fairly higher than Flask-DCs. MACS-DCs expressed higher levels of CD83 and HLA-DR as well as CD14 compared to the Flask-DCs. Induction of T cell proliferative responses were higher in Flask-DCs and also they elicited higher levels of IL-12: IL-10 and IFN-γ: IL-4 ratios in cytokine generation assays. **Conclusion:** MACS method was superior for mass production of viable homogenous and fully mature DCs but their cytokine profile had the potential to polarize the immune system toward Th2 type immune response.

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INTRODUCTION

Successful usage of dendritic cell (DC) based immunotherapy for numerous types of tumors (1,2,3) as well as specific cytotoxic T cell priming for passive immunotherapy or adoptive transfer (4), generation of fully mature and functional DC and their successful in vitro loading by tumor antigens are the goals of many reported studies. Cancer patient's own immature immune cells are suppressed by tumor cells, and there are needs for mature and functional DCs as cell based tumor vaccines to trigger full immunologic responses against tumor cells (3).

Mature DCs express a full array of costimulatory molecules including CD40, CD80/86 as well as a maturation marker CD83, upregulated HLA-DR and secrete polarizing cytokines including IL-12, and IL-10. These maturation properties of DCs enable them to exert stimulating effect on T cells through multiple signals. Therefore, stimulated T cells proliferate and secrete IFN-γ or IL-4 in response to an effective action of mature and functional DCs. Higher IL-10 and IL-4 profile represents polarizing toward Th2 and the subsequent humoral immune response whereas the dominance of IL-12 and IFN-γ in the reaction of DCs and T cells, reveals a Th1 followed by cell mediated immune response (5).

Among the many reported methods on bulk DC preparation, such as isolation of DC from peripheral blood (6) and differentiation of DC from $CD34^+$ cells as well as peripheral blood monocytes in the presence of appropriate cytokines such as IL-4, GM-CSF and TNF-α (7). Recently, methods have established the clinical phase of DC based immunotherapy and are accepted as standard ways to produce DC in vitro (8).

There are multiple methods for obtaining monocytes as an abundant and simply available cells in the peripheral blood. Some of these methods include plastic/glass adherence (9), density gradient centrifugation (10), and specific marker based separations such as magnetic activated cell sorting (MACS) (Miltenyi Biotec, Germany), fluorescent activated cell sorting (FACS) and bipolar tetrameric antibody (Ab) based separation (11).

In this study we compared the effects of two monocyte separation methods including MACS and cell culture flask adherence methods on the production of DCs by several functional and phenotypic analyses. The MACS method is based upon a monocyte specific marker i.e. CD14 and the cell culture flask adherence method is based upon the fact that β_2 integrin expressing cells can adhere to plastic or glass.

The population of human monocytes are divided into two different subsets including $CD14^{low}CD16^+$ (5- 10%) and $CD14^+CD16^-$ (90-95%) (12,13). Therefore, the difference in CD14 expression in these subsets, which plays a central role in MACS method, and their reported difference in CD11c expression (12) involved in plastic adherence, may produce monocytes with variable composition.

MATERIALS AND METHODS

Media and Reagents. Complete medium (CM) including RPMI-1640 (Gibco, Germany) supplemented with 10% human AB serum (Blood Transfusion Organization, Tehran, Iran), 2.5×10^{-5} M 2ME, 2mM L- glutamine (Sigma Chemical Co, Munich, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Chemical Co, Munich, Germany) were used

to culture cells from peripheral blood mononuclear cells (PBMCs). Recombinant human GM-CSF (Novartis-Basel, Switzerland), IL-4 (Peprotech, USA), TNF-α and monocyte conditioned medium (MCM) which is the supernatant solution from an overnight culture of adherent PBMCs (25% V/V) were used to obtain mature DCs (mDCs) from peripheral blood monocytes.

Preparation of Apoptotic Tumor Cell. 6×10^6 T47-D breast cancer cell line were cultured in t T25 flasks (2×10^6 cells in each flask) in DMEM medium supplemented with 10% human AB serum (Iranian Blood Transfusion Organization, Tehran, Iran). The optimum dose and post irradiation incubation period to induce maximum number of apoptotic tumor cells were as mentioned before (4). The cells were irradiated up to 8 Gy (optimum dose) by a gamma emitting ^{60}Co radioisotope source (Omid Hospital, Urmia, Iran). The irradiated cells were incubated for 48 hours (optimum incubation period) at 37°C and 5% CO_2. The resultant apoptotic tumor cells were frozen in liquid nitrogen until use.

Monocyte Isolation. Fresh peripheral blood was taken with informed consent from five healthy volunteers into sterile falcon tubes containing heparin (200 IU/ml) (Sigma Chemical Co, Munich, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by using 1.077 g/ml Ficoll/Hypaque (Sigma Chemical Co, Munich, Germany), as previously described (14). Monocytes were isolated from PBMC either by positive selection of CD14$^+$ cells using a MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol, or by cell culture flask adherence as plastic adherence method (Flask). For monocyte isolation by Flask, $10\text{-}15 \times 10^6$ PBMC per flask were distributed into T25 cell culture flasks, and allowed to adhere in a 5% CO_2 incubator at 37°C for 2 hrs in 5 ml of CM. Non-adherent cells were removed and the adherent cells were carefully washed, twice with CM.

DC Generation. For the generation of immature DCs (immDCs), monocytes isolated by either MACS or flask adherence methods were cultured in cell culture flasks containing 5 ml of CM per flask supplemented with 800 U/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sandoz Basel, Switzerland) and 400 U/ml human IL-4 (Peprotech, USA). Additions of these amounts of cytokines were repeated on day 3, and again on day 4. Apoptotic Ag of tumor were added to immature DC's at a ratio of 1:1 and incubated overnight. On day 5, maturation factors including MCM (25% V/V) and TNF-α (10 ng/ml) were added to all flasks and tumor antigen-pulsed mature DCs were harvested on day 7. At the same time, the supernatants of mature DCs were removed and kept frozen at -80°C for cytokine assay. Such, DC's generated from monocytes obtained with either MACS or Flask methods will be referred to as MACS-DCs and Flask-DCs.

Estimation of DC Yield and Viability from Plated PBMC. Separated DCs from cell culture flasks in day 7 were submitted to counting and assessment of their viability by trypan blue exclusion test. The percentage of yield was estimated by the following formula:

$$\%Yield = \frac{DC}{PBMC} \times 100$$

Microscopic Analysis. The bottom of the culturing flasks were observed by inverted microscope daily. Shape and size of cells as well as their composition were compared in both groups. Extended cells without projections were considered as macrophages (15) and

the round and unchanged cells were accounted as lymphocytes. Platelets were considered as the smallest cells with irregular surfaces.

Phenotypic Analysis. Immunophenotyping of monocyte-derived DCs was performed by direct immunofluorescence staining of cell surface antigens using FITC-conjugated mouse antibodies against CD14, CD83, HLA-DR and the appropriate isotype matched controls (DAKO, Denmark). Samples were analyzed on FACS DAKO (Partec, Germany) using FlowMax software.

Allogenic Mixed Leukocyte Reaction (MLR). Allogenic MLR was performed using mature DCs which irradiated with 3000 rad as a stimulator and the allogenic T cells as responder cells in the ratios of 1:5, 1:10 and 1:20. A 2.5% phytohemagglutenin stimulated T cells (Sigma Chemical Co Munich Germany) and the DC or T cells alone served as positive and negative controls respectively. Cultures were made in V bottom 96 well plates at a final volume of 200μl of CM supplemented with 10% AB serum for 5 days and [^{3}H] thymidine was added at a concentration of 1μCi/well 18 hours before the end of the culture. Proliferative responses were measured by a liquid scintillation counter (Wallac Inc Turku Finland) and expressed as mean count per minute (CPM) obtained for triplicate wells.

Cytokine Release Assay. Concentration of IL-10 and IL-12 in the supernatants of 7-days cultured DC and IL-4 and IFN-γ in MLR supernatant were analyzed using commercially available ELISA kits according to the manufacturer's instructions (Peprotech, USA). Cytokine release was reported as ng/ml. The IL-12: IL-10 and IFN-γ: IL-4 ratios were also calculated as a polarizing parameter for the generated DCs. The sensitivity of ELISA kit was under 1ng/ml.

Statistical Analysis. The data shown in each figure corresponds to a representative experiment of at least three independently performed experiments. Data were expressed as mean ± standard deviation (S.D.). Statistical analyses were done by two-tailed Paired t-test and Bonferroni One-way ANOVA test.

RESULTS

Yield and Viability. Our results revealed that upon using MACS or Flask methods, either 6.56 ± 2.49 or 5.69 ± 1.75 percent of plated PBMCs were differentiated into DCs, respectively. The viability of MACS-DC and Flask-DC were 89.66 ± 10.4 and 88.66 ± 8.08 percent, respectively. The differences in either yield or the viability of resultant DCs by the two methods were not significant ($p<0.05$).

Microscopic Analysis. Daily observation of cells in both groups showed that in the Flask method, the cell culture contained more non DC cells including lymphocytes, macrophages and platelets, whereas, in the MACS method more homogenous cell culture dominated with dendritic cells was observed (Figure 1).

Immunophenotyping of Generated DCs. Flow cytometric analysis of DCs showed significantly increased CD83 expressing DCs among MACS-DCs compared with Flask-DCs (19.4 ± 3.9 vs. 52.3 ± 5.5) ($p<0.05$). Also a higher percentage of MACS-DCs expressed HLA-DR (70.6 ± 7 vs. 58.5 ± 1.01) and CD14 (54.4 ± 3.1 vs. 36.4 ± 2.3) compared with Flask-DCs, but their differences were not significant (Figure 2).

Allogenic Mixed Leukocyte Reaction. Stimulation of allogenic lymphocytes with Flask-DCs and MACS-DCs revealed that Flask-DCs produced a slightly higher, but non-significant, proliferative response from allogenic lymphocytes in comparison to MACS-DCs (Figure 3).
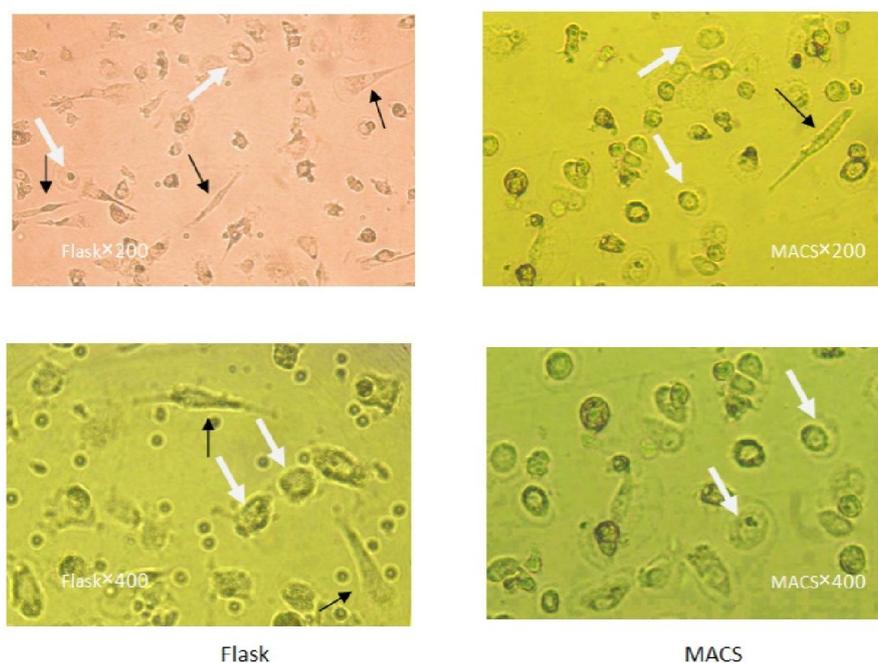

Figure 1. Morphologic appearance of Flask-DCs (left) and MACS-DCs (right). Analysis by inverted microscope revealed that in comparison to Flask method most of plated monocytes isolated by MACS method were differentiated to DC. Flask methods showed more non DC cells including lymphocytes, macrophages and platelets. White arrows present typical DCs and black arrows show macrophages.

Cytokine Profile of Matured DCs. We found that matured Flask-DCs and MACS-DCs produced 3.91 ± 1.27 and 4.39 ± 0.15 ng/ml IL-10 and 4.18 ± 0.39 and 4.18 ± 0.98 ng/ml IL-12 respectively. IL-12: IL-10 ratio was slightly, but insignificantly, higher in Flask-DCs (Figure 4).

Cytokines Released by Stimulated T cells. Concentration of IL-4 released by stimulated T cells was slightly higher in the MACS group, and there was a significant increase in the concentration of IFN-γ in the Flask group ($p<0.05$), therefore the ratio of IFN-γ: IL-4 had increased (Figure 5), this finding was in accordance to IL-12: IL-10 ratio which found in the supernatant of mature DCs (Figure 4).
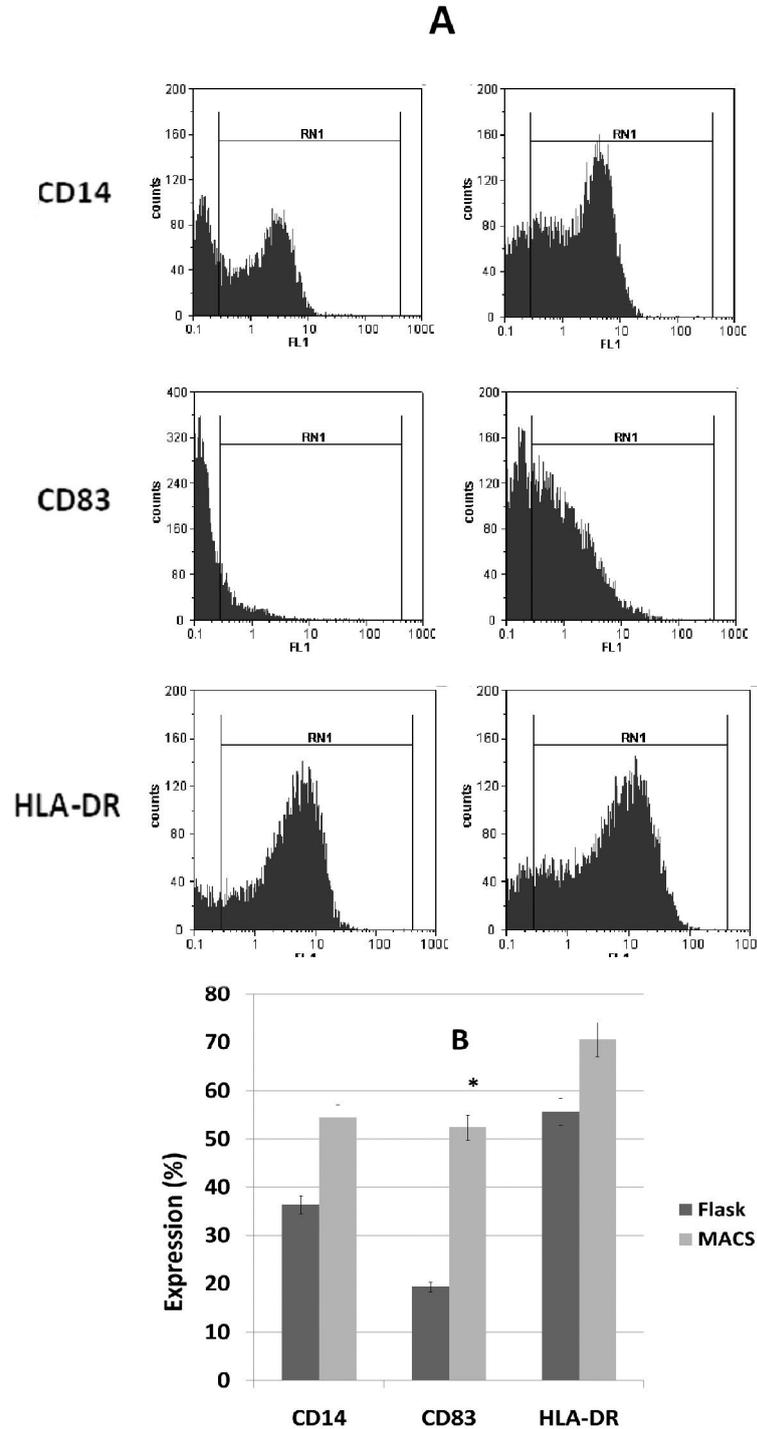
A

Figure 2. Immunophenotyping of DCs. A: Representative flow cytomeric histograms, obtained from MACS-DCs and Flask-DCs, stained with FITC conjugated mAb against CD14, CD83, and HLA-DR. As shown in histograms the MACS-DCs (Right) produced a higher fluorescent intensity relative to Flask-DCs (Left) using the three stained markers. B: Flow cytometric analysis showing increased CD83, CD14 and HLA-DR expression among MACS-DCs compared with Flask-DCs. *represents a significant difference between two test groups (p<0.05).
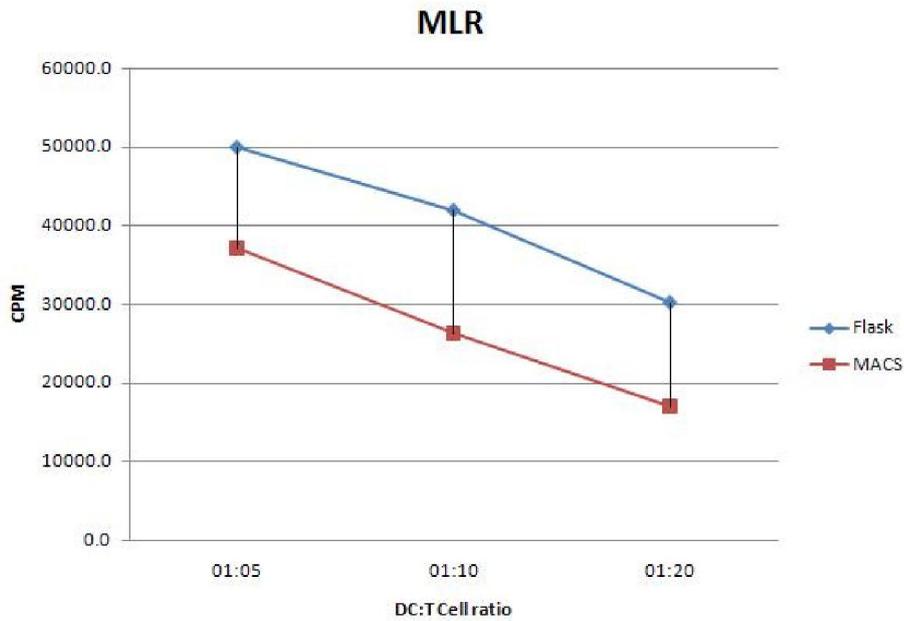
MLR

Figure 3. Allogenic MLR response induced by MACS-DCs and Flask-DCs. Allogenic T cells were incubated with MACS-DCs and Flask-DCs at a ratio of 5:1, 10:1 and 20:1 for 5 days. Uptake of [³H] thymidine during the last 18 hrs of incubation was then measured.
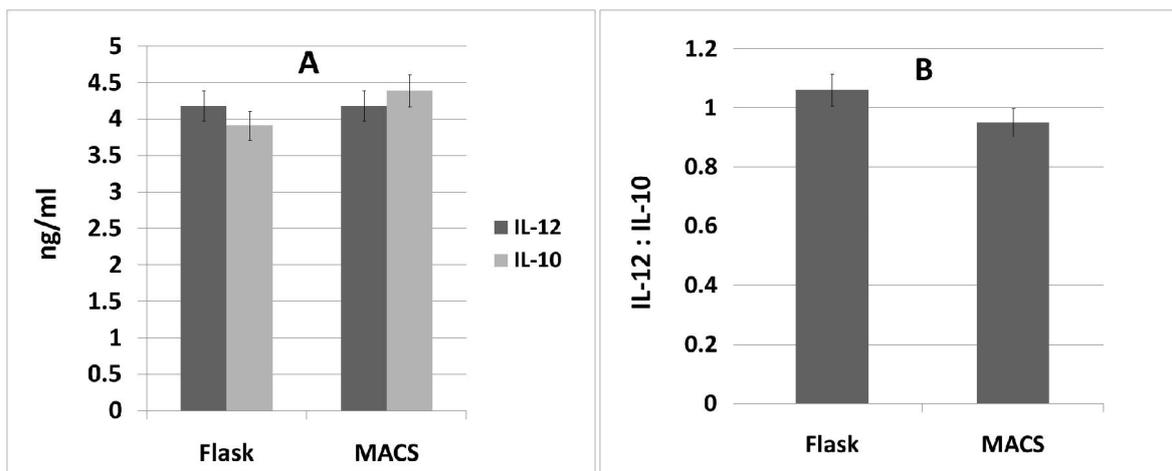

Figure 4. Concentration of IL-10 and IL-12 in the supernatant of 7-day cultured DCs. Cytokines released in the supernatant of mature DCs were measured using commercially available ELISA Kits. Concentration of cytokines (A), and IL-12: IL-10 ratio (B) in Flask-DCs and MACS-DCs.
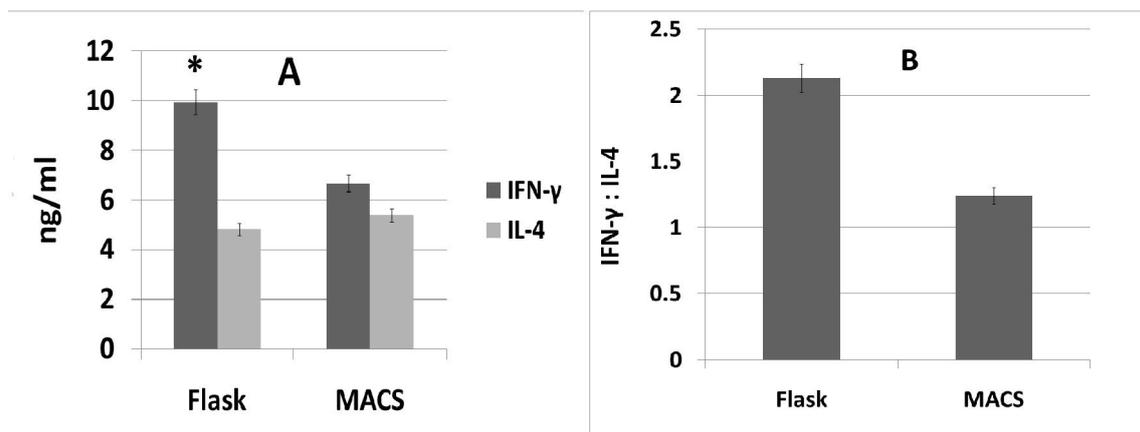
Figure 5. Concentration of released IL-4 and IFN-γ in the supernatant of stimulated T cells (A) and IFN-γ: IL-4 ratio (B) in Flask-DCs and MACS-DCs. Cytokines released in the supernatant of mature DCs were measured using commercially available ELISA Kits. *represents the significant difference between the two test groups ($p < 0.05$).

DISCUSSION

For more than a decade patients have been treated with dendritic cell (DC) immunotherapy against malignancies and infectious diseases. Results of principle studies demonstrated that immunogenicity and clinical responses were observed in a fraction of the patients. Overlooking more than 200 publications one realizes, however, that it is almost impossible to compare many of these trials even in a given clinical setting or in a given disease. This is primarily due to the fact that dendritic cell generation procedures are highly variable. There is a requirement for a the development of standardized DC generation protocol to provide 'reference dendritic cells' to which other dendritic cells could be compared (16). Generation of DCs for cancer immunotherapy should be usually accomplished by fully developing phenotypic and functional maturation resulting in the promotion of a Th1 cytokine profile and a subsequent induction of cell mediated immune responses (17). The most commonly used cell type for DC generation is the peripheral blood monocytes. Therefore, there is growing interest in the in vitro generation of dendritic cells from peripheral blood monocytes, but the effect of the method chosen to isolate CD14+ monocytes for subsequent DC generation is poorly documented. The method used to isolate monocytes may have an impact on the subsequent function of DCs by affecting their ability to express the maturation marker (CD83), allostimulatory reaction and/or to produce important immunomodulatory cytokines (18). Therefore, we have to choose a simple and comprehensive method for DC generation which provides maximum features of the required DCs.

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As mentioned above, when choosing a DC generation protocol for vaccination purposes, several critical parameters must be considered.

The first parameter which is important for the generation of DC vaccines is the number of DCs that needs to be generated. Relying on the results obtained in the present study, higher but not significant yield and viability were obtained using MACS method.

The second factor that affects resultant product is contamination of the DC preparation, which affects the efficacy of the DC vaccination. Since the type of contaminating cells present in the DC preparation can vary among protocols and in order to minimize the effect of the contaminants, it is necessary to aim for the highest possible DC purity. Based on the data from the literature, we propose to aim for DC purities of >75% (10,19). In the present study we obtained 65% and 56% DCs from the seeded monocytes by MACS and Flask methods, respectively. However, very homogenous DCs were obtained by the MACS method in comparison to the Flask method (Figure 1). Indeed more monocytes were driven to form DCs in the MACS method indicating that the differentiation capacity of this method is higher (20). Expression of β_2 integrins on the surface of granulocytes and natural killer cells, and that of CD11b on lymphocytes and CD18 on all leukocytes (21) make these cells to remain close to monocytes in the flask adherence method and make the resultant culture heterogenous (22,23,24).

An important feature of mature DCs is the upregulation of CD83 as a specific marker (25); however, the role of this surface molecule is not fully understood. It is believed that these markers may be involved in the regulation of cell mediated immune response (26). We found that in contrast to the Flask-DCs, the MACS-DCs significantly increased the expression of CD83, an indication of a more maturated status (Figure 2). On the other hand, CD14 expression in mature DCs is down regulated upon adding IL-4 which suppresses the transcription of the CD14 gene (27). The flow cytometric immunophenotype of generated DCs, however, showed a decreased CD14 expression in both groups in contrast to the estimated quantities of this marker in the original monocytes, but its expression was slightly higher in MACS-DCs. This difference is most probably the result of a higher positive selection of $CD14^+CD16^-$ subset of monocytes in the MACS method (12,13,28) and should not be interpreted as more maturation of the Flask-DCs, because the expression of other maturation markers such as HLA-DR and CD83 were higher in MACS-DCs. MHC II (HLA-DR) expression is another maturation marker in DCs which is upregulated during maturation process from intra cellular sources. Our results showed that expression of HLA-DR was slightly higher in MACS-DCs.

Higher expression of maturation markers, in particular HLA-DR, is well correlated to allostimulatory capacity of generated DCs. We have subjected Flask-DCs and MACS-DC to allogenic MLR. Our results revealed that Flask-DCs caused a more but insignificant proliferation response in allogenic lymphocyte reaction. This response probably arose from a more co-stimulation by flask adherence derived cells which may contain other HLA-DR expressing PBMCs such as B cells in addition to DCs.

It is reported that the positive selection of monocytes by anti-CD14-coated microbeads inhibits the lipopolysaccharide (LPS)-induced production of interleukin (IL)-12, IL-10 and tumour necrosis factor-α (TNF-α) from human DCs. However, when DC was grown from monocytes isolated by plastic adherence, LPS induced the production of much higher levels of these cytokines. DCs derived from adherence-isolated monocytes induced the

development of potent cytotoxic T lymphocytes and a Th1 cytokine profile as confirmed by interferon-γ production (10,18). In accordance to these findings, our results showed a significantly higher IFN-γ production which resulted in a higher IFN-γ: IL-4 ratio and also in a slightly higher IL-12 and a subsequent IL-12: IL-10 ratio in the Flask group. It seems that Flask-DCs polarized the immune response towards a Th1 cytokine profile and a cell mediated immune response which is a favorite feature in cancer immunotherapy.

The easiest and most cost-effective way for DC generation is through adherence of monocytes to plastic which has also been developed for use in closed systems. However, the variability in DC purity in this method remains as an important shortcoming. However, highly purified monocytes can also be obtained by positive immunomagnetic selection of CD14+ cells, but the reagents required are expensive, and limits their clinical use. Furthermore, positive selection of monocytes raises concerns about the use of xenogeneic antibodies and possible activation/alteration of the monocytes (29,30). However, it has never been investigated if monocyte activation has a negative effect on DC generation (23,24). In conclusion, MACS-DCs are preferred in terms of mass production, purity and maturity in particular, but their lower IFN-γ: IL-4 and IL-12: IL-10 ratios may decrease their chance of selection for cancer immunotherapy. However, they may be suitable for the treatment of autoimmune diseases due to Th2 the polarization of the immune response.

ACKNOWLEDGMENTS

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