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Technical note

Type of monocyte immunomagnetic separation affects the morphology of monocyte-derived dendritic cells, as investigated by scanning electron microscopy

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ABSTRACT

Dendritic cells (DCs) are increasingly being used for multiple applications and are useful tools for many immunotherapeutic strategies. The understanding of the possible impact of the DCs-generation methods on the biological capacities of these cells is therefore essential. Although the immunomagnetic separation is regarded as a fast and accurate method yielding cells with the high purity and efficiency, still little is known about its impact on the properties of the generated DCs. The aim of this study was to compare the morphology of the monocyte derived dendritic cells (MoDCs), generated from monocytes selected with anti-CD14 mAbs (positive separation) and treated with anti-CD3, -CD7, -CD16, -CD19, -CD56, -CD123, glycophorin A (negative separation), using laser scanning microscopy. We found that the type of the immunomagnetic separation method used strongly influences the shape and cell dimension of the MoDCs. We observed that the height of both immature and LPS-matured DCs generated from monocytes isolated by negative separation was significantly higher compared to the cells obtained by positive separation.

1. Introduction

Dendritic cells (DCs) are the major cells professionally presenting antigens (APC, antigen presenting cells) to T cells. The decision of DCs to mount an immunogenic or tolerogenic adaptive immune response against antigens has a strong impact on devising the most appropriate immunotherapeutic strategy. Immunomagnetic selection of monocytes is one of the methods widely used to generate DCs from peripheral blood cells. Adamson et al. (2009) described a method of enriching monocytes using the combination of leukapheresis and CliniMACS, which delivers highly purified monocytes and allows for the production of sufficient amounts of DC for vaccination purposes. However, there are still technical constraints in the methodologies used for the generation of DCs. It is known that different stimuli, such as bacterial or viral antigens, cytokines and ligands that attach to the cell-surface receptor induce DC maturation that results in functional and structural changes (Mildner et al., 2013). During *in vitro* DC generation from monocytes by using GM-CSF and IL-4, both the size and the shape of the cells change, going from rather spherical or round cells to more irregular cells with a large number of cytoplasmic projections (dendrites). In addition to the tracing of the expression of certain surface markers by flow cytometry, DCs can also be characterized by their morphology by using light microscopy or scanning electron microscopy (SEM) (Tan et al., 2010). However, there is still no comprehensive information on the impact of different variants of MACS separation on the formation of monocyte-derived dendritic cells (MoDCs). The aim of this study was therefore to compare the morphology of the MoDCs by SEM, generated from monocytes obtained either by positive or negative MACS separation.

2. Materials and methods

2.1. Blood samples and dendritic cell preparation

Peripheral blood mononuclear cells (PBMCs) were obtained from blood of 6 healthy volunteers with a mean age of 31 ± 4 years. All experiments were approved by the local Ethic Committee, and all the participants provided written informed consent. Briefly, 50 ml of peripheral blood was drawn in vacuum tubes containing spray-coated heparin (Vacutest Kima, Italy). After centrifugation (1000 rpm/min for 15 min, room temperature) and plasma removal, the cells were diluted in RPMI 1640 (1:1, Sigma-Aldrich, Germany) and layered onto Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Sweden) at a ratio of 4:3. After centrifugation at 400 ×*g* for 30 min, PBMCs were harvested,

Abbreviations: DCs, dendritic cells; BSA, bovine serum albumin; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MACS, magnetic-activated cell sorting; MoDCs, monocyte derived dendritic cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SEM, scanning electron microscopy.

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washed and suspended in PBS (Biowest, France) supplemented with 0.5% BSA (Sigma-Aldrich, Germany) and 2 mM EDTA (POCH, Poland), and immediately used for monocyte isolation either by positive or negative immunomagnetic selection, according to the protocol of manufacturer (MACS CD14 MicroBeads # 130-050-201, MACS Monocyte Isolation Kit II # 130-91-153, respectively, both from Miltenyi Biotech, Germany). In the first case, the cells were treated with anti-CD14 antibodies providing CD14+ tagged monocytes ("touched"). The negative selection was based on a cocktail of numerous antibodies: anti-CD3, -CD7, -CD16, -CD19, -CD56, -CD123, glycophorin A, and resulted in "untouched" monocytes. Positive and negative selection delivered comparable amounts of monocytes: 4.1 ± 0.9% and 3.6 ± 0.7% of PBMC, respectively. The variants of MACS isolation had no significant effect on the level of CD14 expression on the surface of freshly isolated monocytes, which was 4719 ± 1146 and 5168 ± 1318 (MFI values for positive and negative separation, respectively). The purity of the monocytes obtained by both methods of separation was determined to be 96% to 99% on the basis of forward and side scatter gating in conjunction with CD14 staining using standard flow cytometry (data now shown). The viability of the magnetically sorted cells was measured using a trypan blue dye. The monocytes were then suspended in RPMI-1640 supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine (Gibco, Grand Island, NY) and enriched with 10% (v/v) FCS (heat inactivated; Cambrex, Belgium). The density was adjusted to 1×10^6 cells/ml and the cells were seeded into 6-well flat-bottom plates with inserted plastic coverslips (Nunc, Denmark) and cultured for 6 days at 37 °C, 5% CO_2 in RPMI-1640 supplemented with 1% antibiotics and 10% FCS in the presence of 10 ng/ml IL-4 and 25 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) to allow the cells to differentiate into DCs. The efficiency of MoDC generation from positively- and negatively separated monocytes was: 50.9 ± 2.2% and 48.5 ± 2.9%, respectively.

2.2. DC stimulation and preparation for SEM analysis

Immature DCs were then pulsed with 1 μg/ml LPS (Sigma-Aldrich, Germany) as DC maturation inducer for 24 h at 37 °C, 5% CO_2. To see the effect of LPS, the phenotype of unstimulated and stimulated DCs was analyzed by flow cytometry (Fig. 1.). The DCs from both positively and negatively separated monocytes responded to LPS with an increase in CD86 and CD80 expression.

Subsequently, the cells were prepared for SEM as described (Echlin, 2009; Maser and Trimble, 1977). Briefly, DCs grown in monolayer culture were washed with PBS followed by fixation in 2.5% glutaraldehyde and 0.1 M cacodylate (POCH, Poland) for 30 min at room temperature. After three washes for 30 min with distilled water at room temperature, the cells were gradually dehydrated in ethanol (30%, 70%, 96%, 3 × 100%, 15 min for each step), critical point dried and sputter-coated with gold (6 nm). Finally, all specimens were examined in a PHENOM PRO X SEM (Phenom-World B.V., The Netherlands).

2.3. Statistical analysis

Statistical analyses were performed with the STATISTICA 10.0 PL program. Data are expressed as median ± SEM. Differences between samples were analyzed by the Mann-Whitney *U* test (for impaired data). p values of ≤0.05 were considered significant.

3. Results and discussion

The appearance of DCs can provide information about their maturation and activation status, which has important influences on the effective immune response to antigens. It is known that the transition from immature DCs to mature DCs is accompanied by numerous changes in cell morphology (Granucci et al., 1999; Verdijk et al., 2004; Li et al., 2012) including the increase in size and granularity (Fontes et al., 2006). Pereira et al. (2005) suggested that the DC protein profile depends on the stimuli used for differentiation and maturation. DCs become activated and mature into antigen-presenting cells that can prime naïve T cells for a proper immune response. We have previously shown that GM-CSF and IL-4-induced DCs generated from the monocytes (unstimulated DCs) obtained by positive or negative MACS separation expressed high levels of major histocompatibility complex class II molecules (HLA-DR), and a moderate-to high density of costimulatory molecules (CD86, CD40 and CD80) (Fol et al., 2016). In response to LPS (lipopolysaccharide), a DCs maturation stimulator, the positively separated MoDCs increased significantly the surface density of CD86, CD80 and CD40 molecules. In the case of negatively separated MoDCs, LPS induced the significant increase in CD86 and less marked increase in CD80 and CD40 receptors (Fol et al., 2016). Interestingly, the method of separation had no significant impact on the release of pro-Th1 and pro-Th2 cytokines (IL-12, IL-23, TNF-alpha, IL-10), by both unstimulated and LPS-stimulated DCs, as shown in our previous study. Furthermore, observing LPS-stimulated MoDC cultures under the light microscope, some cells, especially those that have been negatively separated, had more elongated projections, which might increase the surface area for antigen presentation. With this in mind, in this study we used SEM to compare the morphology of the MoDCs generated from monocytes

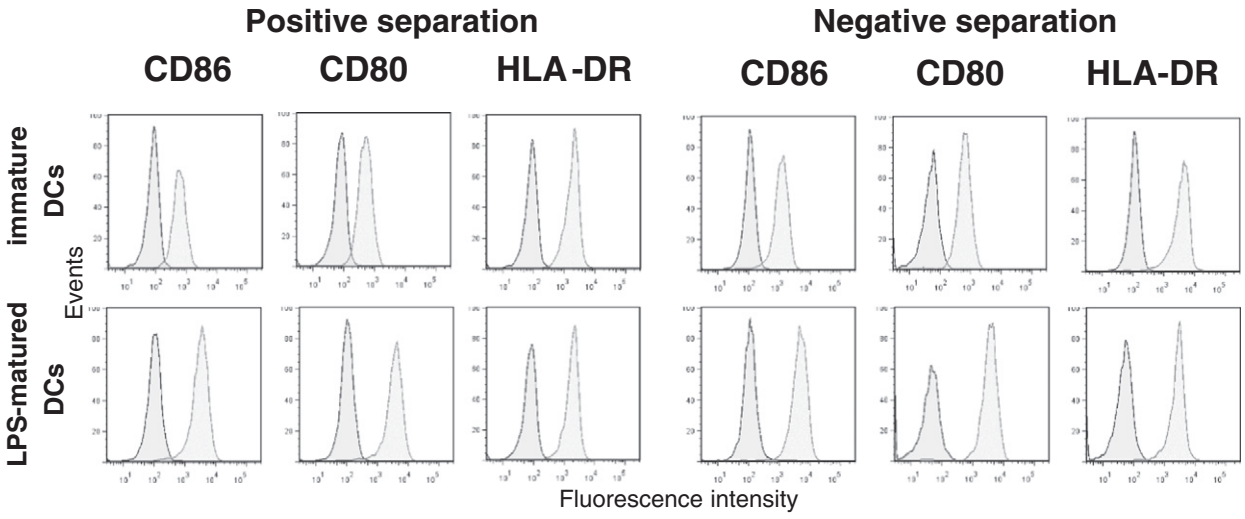

Fig. 1. Cell phenotype of unstimulated and LPS-stimulated MoDCs by flow cytometry. The expression of selected surface markers was determined with specific (light-grey histograms) or isotype-matched (dark-grey histograms) antibody conjugated with FITC (for CD86, HLA-DR) or PE (for CD80). One representative experiment out of 6 independent ones is shown.