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In vitro maturation of monocyte-derived dendritic cells results in two populations of cells with different surface marker expression, independently of applied concentration of interleukin-4

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ABSTRACT

Dendritic cells (DCs) play a crucial role in the development of adaptive immune response. Monocyte-derived dendritic cells (MDDCs) are generated in vitro to study DC biology and for use in immunotherapy. However, procedures to generate MDDCs vary and an impact this may have on their final phenotype is insufficiently studied.

Monocytes isolated from healthy blood donors were cultured for 7 days with granulocyte-macrophage colony stimulating factor (50 ng/mL) and low (500 IU/mL, L-IL4) or high (1000 IU/mL, H-IL4) interleukin 4 (IL4), to obtain immature DCs and for the following 2 days with addition of soluble CD40 ligand (500 ng/mL) and prostaglandin E_2 (1 μg/mL) to obtain mature DCs. We measured mean fluorescence activity and percentage of cells, positive for CD14, HLA-DR, CD80, CD83, CD86, CCR7, and CD1a or CD209 markers after 7 and 9 days of culture, in both IL4 concentrations.

Percentage of positively staining mature MDDCs was higher than among immature cells, for all studied markers. Interestingly, varying IL4 concentrations had negligible impact on staining of mature MDDCs. However, immature L-IL4 cultured MDDCs were less intensely stained for HLA-DR and CD209 than H-IL4 immature DCs. Flow cytometry revealed presence of 2 populations of cells (dominant P1 and less prevalent P2), when either L-IL4 or H-IL4 was used. Among mature MDDCs, population P1 had higher percentage of positively staining cells than P2, for all studied markers except CCR7.

In conclusion, both concentrations of IL4 produce in vitro heterogeneous populations of mature MDDCs with similar staining for cell surface markers.

1. Introduction

Dendritic cells (DCs) play a crucial role in initiation of an adaptive immune response. Immature dendritic cells with potent phagocytic properties scan peripheral tissues for antigens and phagocytosis of found foreign antigens initiates maturation of DCs. Mature cells lose capability to phagocytose and begin to express chemokine receptor CCR7, which directs them along the growing gradient of CCL19 and CCL21 homeostatic chemokines, towards the lymph nodes. Concomitantly, various co-activating molecules, e.g. CD80, CD83, CD86, and HLA-DR, are upregulated on the surface of mature DCs. These molecules enhance interaction between DCs and T lymphocytes. Depending on the surrounding milieu leads to tolerance, immunosuppression or immunity.

Central role of DCs in regulation and activation of T cells prompted the idea to use in vitro modulated DCs as therapeutic agents. The first drug based on in vitro matured and stimulated DCs (Sipuleucel T) was approved for the treatment of prostate cancer in 2010. There are many disorders, which can be potentially treated by modulation of DCs, e.g. in autoimmune [1,2], cardiovascular [3] and inflammatory diseases [4] as well as in cancer [5].

Therapeutic use of DCs requires that large number of cells be harvested from the patients and cultured for several days with exposition to antigens and growth factors to direct their maturation. The most popular sources of DCs are: circulating dendritic cells separated directly from blood, DCs differentiated from $CD34^+$ hematopoietic progenitor cells, and DCs differentiated in culture from monocytes. Cells to be used therapeutically need not only to be generated in sufficiently high number and purity but also need to be cultured with various growth factors to direct their differentiation and maturation towards the cells which are mobile and competent in interleukin production.

Several combinations of cytokines, growth factors and adjuvants

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were implemented to produce DCs. Differences between them exist not only in their composition but also in the concentration of the components as well as timing and duration of stimulation. These differences translate into cells with varying final immuno- and tolerogenic potential. Interleukin-4 (IL4) is a popular component of DC production cocktails, however, it was also claimed to inhibit DC maturation. Gross differences exist in the concentration of IL4 used for DC culture. The main goal of our study was to compare the effect of IL4 concentration on expression of cell surface markers of immature and mature DCs, accounting also for the possible heterogeneity among cultured cells.

2. Material and methods

2.1. Blood donors and monocyte isolation

Whole blood (9 mL) was obtained with EDTA anticoagulation from healthy blood donors, immediately before blood donation at the Regional Blood Center in Krakow. No specific information about the donors was sought. All procedures were approved by the Jagiellonian University Bioethics Committee.

Blood was diluted with 26 mL of phosphate buffered saline with 4 mM EDTA (PBS/EDTA) and mixed with 15 mL of Ficoll (Histopaque-1077, Sigma-Aldrich, St. Louis, USA), followed by 40 min of centrifugation at 400*g*. Resulting buffy coat of mononuclear cells was transferred to a new tube and washed with PBS/EDTA once for 10 min at 300*g* and twice for 15 min each at 200*g*. All centrifugation steps were performed at room temperature. Finally, mononuclear cell pellet was suspended at 4 °C in 4 mL of PBS/EDTA with addition of bovine serum albumin to a final concentration of 0.5%.

Immunomagnetic isolation of untouched monocytes was performed with Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Use of anti-CD3, -CD7, -CD16, -CD19, -CD56, -CD123, and anti-glycophorin A biotinylated antibodies, traps vast majority of non-monocyte blood cells on the column while monocytes are washed out untouched with the effluent.

2.2. Cell culture of monocytes to produce immature and mature dendritic cells

Viability of isolated monocytes was verified with 1% trypan blue staining. After each isolation, alive cells exceeded 98% of the total cell number. Mature dendritic cells (DCs) were produced during a two-step procedure, lasting nine days. Cells were cultured in RPMI medium 1640 (Thermo Fisher Scientific, Paisley, UK) with L-glutamine (2 mM), streptomycin (200 μg/mL) and amphotericin B (1 μg/mL). During initial seven days of culture with 10% fetal calf serum (single lot FCS, Sigma-Aldrich, St. Louis, USA), granulocyte-macrophage colony stimulating factor (GM-CSF, 50 ng/mL, R&D, Minneapolis, USA) and low (500 IU/mL, L-IL4) or high (1000 IU/mL, H-IL4) interleukin-4 (R&D, Minneapolis, USA), monocytes changed into adherent immature dendritic cells with characteristic protrusions.

After 7 days of culture, maturation of dendritic cells was stimulated for 2 days by addition of prostaglandin E_2 (PGE_2, 1 μg/mL; Sigma, St. Louis, USA) and of a soluble form of CD40 ligand (sCD40L, also known as CD154, 500 ng/mL, R&D, Minneapolis, USA), resulting in non-adherent DCs. Stages of DC maturation were verified using Corning Costar Transwell cell culture inserts (Corning, USA) with 8.0 μm pore size. Cells described as immature DCs (upon completion of 7 day culture, 10^5 cells per experiment) were generally unresponsive to CCL19 (CCR7 ligand, 300 ng/mL, R&D, Minneapolis, USA) added for 2.5 h to the lower chamber of the Transwell inserts (never > 2% of the cells undergoing chemotaxis in any experiment), in contrast to 48.7 ± 3.8% of mature DCs under the same experimental conditions, upon completion of a 9 day culture.

2.3. Cell surface markers of the monocyte derived dendritic cells

Markers on the surface of the monocyte derived dendritic cells were analyzed with FacsCantoII flow cytometer using FacsDiva software (Becton Dickinson, USA). To detect the markers, we used the following antibodies (Becton Dickinson, USA), conjugated with the respective fluorophores: anti-CD80 with FITC, anti-CD83 with APC, anti-CD86 with PE, anti-CD197 (anti-CCR7) with PE-Cy7, anti-CD209 with FITC, anti-CD1a with APC, anti-HLA-DR with PerCP, and anti-CD14 with PE-Cy7. All antibodies we used were mouse anti-human (except anti-CD197, which was rat anti-human), paired with the appropriate, isotype-matched control antibodies.

For each batch of cells, markers were measured in two parallel experiments, to allow use of the mentioned fluorophores: first using cells stained with antibodies against CD80, CD83, CD86, and CD197 and second using cells stained with antibodies against HLA-DR, CD14, and either CD209 or CD1a. In our initial experiments we aimed to detect CD1a, however, given high inter-individual variability of expression of this molecule, we replaced this marker with CD209 in subsequent experiments. Identical instrument parameters and staining procedures, suggested by the producers were used for each experiment. Before collection of the data, stained cells were washed with ice-cold washing buffer, centrifuged for 5 min at 400 *g* and suspended in 300 μL of washing buffer. At least 10,000 events were collected for each staining antibody. Cells were gated including information from forward and side scattered light detector. The results are presented as percentage and mean fluorescence intensity (MFI) of the cells stained with studied antibodies.

3. Results

3.1. Effect of IL4 concentrations in culture medium on cell surface markers of immature dendritic cells

Isolated monocytes were cultured for 7 days in low or high IL4 concentration. Mean fluorescence intensity and percentage of positively staining cells were compared between L-IL4 and H-IL4 groups ($n = 6$ in each group) for the following cell surface markers: CD14, CD209, HLA-DR, CD80, CD83, CD86, and CCR7. Percentages of the cells staining positively for HLA-DR were 78.4 ± 9.1% and 90.4 ± 3.1% for L-IL4 and H-IL4, respectively. For CD209, percentage of the cells staining positively were 81.0 ± 6.0% and 89.1 ± 1.1% while MFIs were 1031.3 ± 139.2 and 1634.7 ± 185.6 for L-IL4 and H-IL4, respectively ($p < 0.05$ for each of the three above comparisons). Results of all other comparisons of mean fluorescence intensity and percentage of positively staining cells between L-IL4 and H-IL4 groups of immature dendritic cells were not statistically significant. We analyzed the effect of the IL4 concentration on the total available during culture population of the immature MDDCs. However, immature MDDCs show some degree of heterogeneity, similarly to the mature MDDCs. This issue will be demonstrated below, when comparing immature and mature MDDCs.

3.2. Effect of IL4 concentrations in culture medium on cell surface markers, accounting for heterogeneity of mature dendritic cells

Plots from forward and side light scatter optical detectors (Fig. 1) reveal existence of two clearly demarcated populations of the mature MDDCs, which we called P1 and P2, respectively.

Altogether, these two populations, when gated, contain 92.6 ± 2.6% of all cells. The remaining 7.4 ± 2.6% of the cells are scattered randomly and are not subject of any further analyses. Mature MDDCs are divided into a numerically dominating population P1 (75.7 ± 11.5% of all cells) and less numerous P2 population (16.9 ± 9.9% of the cells). We were intrigued by the possibility that heterogeneity among mature MDDCs is affected by IL4 concentration used during cell culture. Percentage of the cells and mean fluorescence