Artigo / Article

Maturation of dendritic cells following exposure to different maturational stimuli
Matucação das células dendríticas após exposição a diversos estímulos

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Dendritic cells (DCs) are professional antigen-presenting cells that are highly effective to immunize against pathogens and tumor antigens. In order to obtain mature DCs several in vitro methods have been reported. Selecting the most efficient and effective method of generating morphologic and phenotypic DCs within 7 days of culture is an essential prerequisite for success in immunotherapy strategies. Herein, we report a method of obtaining an enriched monocyte population from blood donors and performed a comparison of DC maturation in response to four agents. Monocyte populations with 91% ± 5 of purity were obtained from 15 healthy donors. The resulting monocyte populations were cultured in the presence of GM-CSF and IL-4 during 5 days. At day 5 different maturation conditions were performed and morphological and phenotypical changes were analyzed. Our study demonstrates that TNF-α or PGE1 by themselves can induce the expression of CD1a 2.4 and 2.7 times respectively more than DC cultures in the absence of maturing agents. On the other hand, for other costimulatory or accessory molecules (CD80, CD86, CD83 and CD40) TNF-α was more potent in the induction of expression than PGE1, although in the presence of TNF-α plus PGE1, this effect is more pronounced compared to TNF-α alone. Under TNF-α plus PGE1 treatment the phenotypical maturation of immature DCs are comparable to LPS and therefore TNF-α+ PGE1 might be useful for generating ex-vivo DCs to use in protocols of cell vaccination. Further functional evaluation of these mature DCs is warranted. Rev. bras. hematol. hemoter. 2006;28(2):89-96.

Key words: Dendritic cells; monocytes; Percoll; maturation; immunotherapy.

Introduction

Dendritic cells (DCs), which were discovered more than 25 years ago, are the most potent members in the class of antigen-presenting cells (APCs) and are initiators and modulators of the immune response. They are mobile sentinels that bring antigens to T-cells and express costimulators for the induction of immunity.¹² The exceptional ability of DCs to stimulate T-cells in vivo is attributed to their dichotomous nature, as immature and mature DCs. Immature DCs (iDCs), exist in peripheral tissues, where they continuously capture and process antigens, but they are relatively poor stimulators of T-cell responses. Upon exposure to inflammatory cytokines, iDCs upregulate costimulatory molecules and chemokine receptors. The first are required for the activation of antigen-specific T-cells, which takes place in the T-cell region of draining lymph nodes and the second are required for their migration to the lymph nodes. The mature dendritic cells (mDC) express a unique repertoire of cell-surface molecules including high levels of: a) molecules involved in antigen presentation to T-cells, such as, MHC class I, MHC class II and CD1a; b) adhesion molecules, such as, ICAM-1 (CD54), LFA-3 (CD58), Mac-1a (CD11c), SIRP-1a (CD172), VLA-4a

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As maturation is an essential part of DC function increasing effort has been devoted to study other maturation stimuli. Some other DC maturation stimuli include microbial products (e.g., lipopolysaccharide LPS), prostaglandin E (PGE), CD40 ligand and several combinations of these molecules have been used for ex vivo generation of mature DCs.13-21-23

It is well-established in these studies that exogenous prostaglandins E₂ or E₃ can enhance the TNF-α-induced maturation in DCs as analysed by morphology, phenotype, ability to stimulate the allogeneic mixed leukocyte reaction, and induction of IL-12 production, but a systematic comparison of the phenotypical maturation of DCs generated from normal volunteers has not been performed yet.

In this study we performed a comparative study among four different DC maturation agents to investigate the influence of proinflammatory factors (TNF-α and PGE₁) in isolation or TNF-α in combination with PGE₁, as well as bacterial products (LPS) in the expression of each molecule characteristic of mature DCs, including CD1a, CD80, CD86, CD83 and CD40 evaluated by flow cytometry. Also, the characteristics of DCs were determined by evaluating their morphology by light microscopy. Our study demonstrates that TNF-α or PGE₁ by itself can increase the CD1a expression by 2.4 and 2.7 times, respectively, more than DC cultures in the absence of maturing agents. On the other hand, for other costimulatory or accessory molecules (CD80, CD86, CD83 and CD40) TNF-α was more potent to induce the expression compared to PGE₁, although in the presence of TNF-α plus PGE₁, this effect is more pronounced compared to TNF-α alone. Under TNF-α plus PGE₁ treatment the phenotypical maturation of immature DCs are comparable to LPS.

Materials and Methods

Cell culture media and recombinant cytokines

In all experiments, we used the complete RPMI-1640 (Sigma Chemical Company; St Louis, MO) medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone laboratories, Logan, UT), 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco-BRL). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant human interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), prostaglandin-E₁ and lipopolysaccharide (LPS) were all purchased from Sigma (Sigma Chemical Company; St Louis, MO).

Percoll gradient

The 50% Percoll gradient (density 1.064 g/ml) as described by Almeida was used with one modification. First the isosmotic Percoll solution was prepared with one volume of PBS 10X (137 mM NaCl, 2.6 mM KCl, 8.45 mM Na₂HPO₄ and 1.52 mM KH₂PO₄) and nine volumes of Percoll (Pharmacia, density 1.130 g/ml), afterwards the 50% Percoll solution was prepared by mixing 1:1 (v/v) isosmotic Percoll: PBS1X plus 0.6% ACDA.

Experimental design

DCs were generated from leukocyte-enriched Buffy coats. The Buffy coat, derived from 500 ml of whole blood drawn from a healthy volunteer, was supplied by the Blood Fractionation Laboratory of Regional Blood Center of Ribeirão Preto, in compliance with institutional guidelines and immediately processed. The leukocyte-enriched Buffy coats were diluted twice in PBS and the purification of high-enriched monocyte population was obtained as follows: first the mononuclear cells were purified by Ficoll-Hypaque gradient (Pharmacia, density 1.077 g/ml) and centrifuged at 700 g for 30 min at room temperature. Afterwards, the MNCs were washed twice with PBS (final volume 20 ml)
and centrifuged into 20 ml of 50% Percoll solution at 400 g for 35 min at room temperature. The cells from the interface were collected, washed twice with PBS and the number of cells counted. The purity of monocyte population was evaluated by flow cytometry using an anti-CD14 antibody. The monocyte (1 x 10⁷/ml) was subsequently cultured in 24-well plates (Greiner Bio-One GmbH, Frickenhausen) in complete RPMI-1640 medium. The following cytokines were used: 100 ng/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml of recombinant human IL-4 (both from Sigma) for 5 days. One-half of the culture volume was replaced with fresh medium and cytokines every 2 days. On day 5, fresh whole medium was added and four different maturation conditions were tested: 1) TNF-α (10 ng/ml); 2) PGE1 (10⁻⁶ M); 3) TNF-α (10 ng/ml) plus PGE1 (10⁻⁶ M) and 4) LPS from E. coli (100 ng/ml). Simultaneously, as a control one-step culture, some samples were also maintained without adding additional factors. Following 48 h DCs were harvested and analyzed by light microscopy and by flow cytometry

**Immuno-phenotype analysis**

DCs and monocytes were analyzed for expression of cell surface markers by single-color cytometry. Cells were harvested after Percoll gradient separation and after culturing for seven days. A total of 1x10⁶ cells was incubated for 30 minutes on ice in 2% BSA containing phosphate-buffered saline (PBS) with the fluorescein isothiocyanate (FITC) - or phycoerythrin (PE) - conjugated mAb, or with control irrelevant isotype-matched mAb. The monoclonal antibodies used were: CD1a, CD80, CD86, CD83, CD40, CD14 and HLA-DR. HLA class II were analyzed by fluorescent-labeled Abs against HLA-DR. The stained cells were washed twice with PBS and fixed with 500 µL of 2% paraformaldehyde in PBS. Then, cells were kept on ice, protected from light until flow cytometry analysis. Data acquisition of 10,000 events was performed using a FACSort™ flow cytometer (Becton Dickinson, San Jose, CA) and analysis was performed using Cell-Quest (Becton Dickinson) software. Dendritic cell regions were electronically gated according to light and forward scatter properties to exclude cell debris, dead cells and contaminating lymphocytes and granulocytes.

**Morphologic analysis**

Morphologic analysis was performed on an Axioskop plus, Zeiss microscope (Carl Zeiss from Brazil). A total of 2 x 10⁶ cells MNCs cultured for 2 days and DCs cultured for 7 days were cytocentrifuged using a Cytospin-3 centrifuge (Shandon Southern Products, Aalborg, UK) on microscope slides. Then, cytocenters were air dried and stained with May-Grünwald-Giemsa before examining by light microscopy. Digital pictures were taken with a Zeiss Axioskop 2 plus (Carl Zeiss) microscope using a x100 NA 1.25 Plan-Apochromat oil lens and a AxioCam camera (Carl Zeiss). Digital images were subsequently converted into JPG format and imported into Adobe Photoshop 4.0 for editing and printing.

**Statistical analysis**

Descriptive statistics (arithmetic mean, median, and standard deviation) was used to describe each group of data.

**Results**

**Enrichment of monocyte cells after Percoll gradient and morphological and phenotypical changes after culturing with GM-CSF plus IL-4**

We obtained an enriched-CD14+ cell population after Percoll gradient centrifugation (Figure 1B). Starting with approximately 2-8 x 10⁶ peripheral blood mononuclear cells,
we typically ended up with $5-8 \times 10^7$ monocytes. Analysis by flow cytometry showed that the frequency of CD14$^+$ cells was $91\% \pm 5$ and the lymphocyte contamination with CD3$^+$/CD19$^+$ cells was low (Figure 1C). Moreover, as expected, there is a strong decrease in the expression of CD14 after 7 days in the presence of GM-CSF plus IL-4 (Figure 1D) or other culture conditions (data not shown).

Morphologically, the CD14$^+$ cells appear with the typical monocyte morphology, which includes small cells with a plasma cell-like morphology and characterized by an eccentric nucleus and without dendritic projection (Figure 2A). After 7 days in the presence of GM-CSF plus IL-4 the cells became larger and few cytoplasmic projections were observed which is characteristic of immature DCs (Figure 2B). These results corroborated with the analysis by FACS where it was observed that, compared with the starting cell population, cells that were cultured with GM-CSF and IL-4 for 7 days increased in size and granularity (data not shown).

The expression of antigens including HLA DR and CD86 are up-regulated in the starting cell population (median value of 81% and 97%, respectively) while CD1a, CD40, CD80 and CD83 antigens are not detected (median value between 0.7 and 1.5%). In the presence of GM-CSF plus IL-4 for 7 days is observed the differentiation of CD14$^+$ cells into immature DCs as evaluated by FACS with a significantly higher expression of CD1a and CD80 (median of 34% and 9%, respectively). The expression of HLA DR is retained in cells without any maturating agent while CD86 expression is decreased. Other phenotypes, including CD40 and CD83 showed a slightly higher expression compared with the starting cell population (Figure 3).

**Effect of the addition of PGE$_i$ or TNF-α alone on phenotypical maturation of immature DCs**

In the next series of experiments, we investigated the effect of PGE$_i$ and TNF-α alone on the phenotypic maturation of human MoDCs. At day 5 immature MoDCs were treated with PGE$_i$ or TNF-α stimuli for 48 hours, and the expression of maturity-related surface markers was analyzed by flow cytometry. As shown by flow cytometric analysis, in the presence of TNF-α, or PGE$_i$ alone the expression of CD1a$^+$ is increased (median value of 82% and 94%, respectively, versus 34%). Related to the expression of the costimulatory CD80 molecule TNF-α - DCs had a higher level of expression (median of 40%) when compared to the levels of CD80 in immature DCs. On the contrary, CD80 expression could not be efficiently induced by DC exposed for 48 h to PGE$_i$ alone (median of 26%) when we compared with immature DCs (Figure 3).

Morphologically, under TNF-α (Figure 2C) or PGE$_i$...
(Figure 2D), the cells exhibited an irregular shape and multiple cytoplasmic processes in many directions that typify dendritic cells. These results corroborated with the analysis by FACS where it was observed that the forward – and side – scatter distribution where higher than those of immature DCs, suggesting an increase in their size and granularity properties (data not shown).

The influence of TNF-α or PGE₁ on the expression of other antigens was also observed. As shown in Figure 3 under TNF-α treatment the expressions of HLA DR and CD86 remained similar to untreated DCs (median of 79% versus 82%, and 69% versus 61%, respectively), while the expressions of CD40 and CD83 were higher (median of 27% versus 12%, and 22% versus 4%, respectively). Similarly, phenotypic analysis of DCs under PGE₁ treatment showed that the expression of HLA DR remained similar to untreated DCs (median of 78% versus 82%), and the expression of CD40 was slightly higher (median of 18% versus 12%). However, the expression of CD86 was downregulated (median of 31% versus 61%) and the expression of CD83 could not be stimulated (median of 4%) in both DC cultures, in the presence or absence of PGE₁.

Taken together, these data indicated that TNF-α or PGE₁ can increase the expression of CD1a 2.4 and 2.7 times, respectively, more than DC cultures in the absence of the maturing agent. However, for other costimulatory or accessory molecules (CD80, CD86, CD83 and CD40) TNF-α was more potent in the induction of the expression than PGE₁, suggesting that additional maturation factors are essential for obtaining efficiently matured DCs.
The presence of PGE$_1$ plus TNF-α has similar effect on phenotypical maturation of immature DCs compared to LPS

In the third series of experiments, we investigated the effect of TNF-α in combination with PGE$_1$, on the phenotypic maturation of human CD14$^+$ derived-DCs (Mo-DCs). At day 5, immature Mo-DCs were treated with TNF-α plus PGE$_1$, or PGE$_1$ alone. Moreover, CD1a showed an elevated expression. As shown by flow cytometry analysis in the presence of TNF-α plus PGE$_1$, the expression of CD1a and CD80 were elevated appreciably when compared with untreated-DCs (median of 90% and 79%, respectively, versus 34% and 9%). Also, as expected HLA DR and CD86 expression remained high and similar to untreated-DCs (median of 70% and 81%, respectively, versus 82% and 61%). Therefore, on the contrary to PGE$_1$, treatment where a decrease of CD86 expression (median of 14%) was observed the combination TNF-α and PGE$_1$ cooperate to activate the expression of CD86 (median of 70%). Surprisingly, TNF-α plus PGE$_1$, treated-DCs did not show significant increases of CD40 and CD83 expression compared to untreated or TNF-α-DCs and PGE$_1$-DCs (median of 28% and 19%, respectively, versus 12% and 4% for untreated-DCs; versus 27% and 22% for TNF-α-DCs and versus 12% and 4% for PGE$_1$-DCs). These results can be explained by DC-culture conditions (see comments on discussion) or by induction of a specific subset of dendritic cells.

Morphologically, under TNF-α plus PGE$_1$, treatment (Figure 2D) the cells exhibited a shape similar to TNF-α treated-DCs or PGE$_1$, treated-DCs with an irregular shape and multiple cytoplasmic processes in many directions that typify dendritic cells.

We next explored the relevance for the observed enhancement of DC maturation in the presence of TNF-α plus PGE$_1$ at day 5, immature CD14$^+$ -derived DCs were treated with LPS stimuli for 48 hours, and the expression of maturation-related surface markers was analyzed by flow cytometry. In LPS-DCs there were no significant differences in the levels of the expressions of CD1a, CD80, CD86 and CD83 compared to TNF-α plus PGE$_1$, DCs (median of 81%, 97%, 98% and 21% versus 90%, 79%, 81% and 19%, respectively, $p$=0.05). Sequential analysis of other antigens showed that, the expression of HLA DR and CD40 has a more pronounced effect in the presence of LPS compared to TNF-α plus PGE$_1$, treatment (median of 97% and 67% versus 70% and 28%, respectively).

Thus, CD14$^+$-derived DCs treated with TNF-α + PGE$_1$ display phenotypic mature characteristics for CD1a, CD80 and CD86 molecules similar to LPS-treated DCs and higher than their TNF-α treated-DC or PGE$_1$, treated-DC or untreated-DC counterparts.

Discussion

A variety of strategies to generate DCs are currently under investigation and there has been an increasing need to define the most promising candidate therapies for clinical studies. At present, most of the preclinical studies use DCs generated from monocytes isolated after adherence in culture flasks or from monocytes isolated after depleting lymphocyte cells with an immune magnetic column. Thus, the enriched-CD14$^+$ monocyte cell population is differentiated into DCs in a two-step culture. In the first step GM-CSF and IL-4 promote the differentiation of immature monocyte derived DCs (iDCs) and in the second step pro-inflammatory factors induce the terminal maturation of DCs.12-13,21,24,26-28

The studies described here have two proposes, first to establish a low cost and highly efficient technique to obtain an enriched-CD14$^+$ monocyte cell population and second to perform a systematic comparison of the phenotypical maturation of DCs generated from different maturing agents that have been used in clinical assays.

The adherence of PBMCs in culture plates for two hours at 37°C could be considered the "gold standard" to isolate monocyte cell populations from progenitor PBMCs. However, the contamination with lymphocytes may be high. On the other hand, the second approach, which consists of using an immune magnetic column, is too expensive for daily routine. In this study we obtained a CD14$^+$ enriched cell population by performing Percoll gradient from PBMCs. The results of FACS analysis of the recovered cells indicate that 91% ± 5% were CD14$^+$ cells and the content of cells expressing T and B markers were low. Additionally, these cells strongly express HLA DR and CD86 and lack expression of CD1a, CD40, CD80 and CD83.

We further observed that when these cells are stimulated in vitro with GM-CSF and IL-4 the cells can survive and enter cell cycles for in vitro DC development generating a typical iDC phenotype as they acquired CD1a and CD80 (median of 34% and 9%, respectively) while losing CD14 expression. Moreover, the expression of HLA DR is retained, while CD86 expression decreases.

The question about which is the most efficient cocktail of cytokines to produce mature DCs has been addressed previously by several laboratories. In these studies, the presence of exogenous prostaglandins E$_2$ or E$_1$ enhanced the TNF-α induced maturation of DCs as analyzed by morphology, phenotype, ability to stimulate the allogeneic mixed leukocyte reaction and induction of IL-12 production.13,21,29 Also, the bacterial lipopolysaccharide (LPS) is known to be an inflammatory stimulus and has been shown to activate DCs, as well as, whole pathogens or other
components of microorganisms (e.g. dsRNA, CpG DNA and toxins). However, our study is uniquely extensive in that we performed a systematic comparison with the yield of all spectra of DC markers that have been used to address the DC maturation before and after being exposed to four different maturing agents, including TNF-α, PGE₁, TNF-α plus PGE₁, and LPS.

Consistent with other studies, we show that TNF-α or PGE₁ alone are capable of inducing the maturation of DCs. Our TNF-α-treated DCs or PGE₁-treated DCs displayed higher expressions of CD1a compared with untreated DCs (median value of 82% and 94%, respectively, versus 34%) and some differences were found between both treatments. Analysis of the costimulatory and accessory molecules CD80, CD86, CD40 and CD83 showed a higher level after TNF-α treatment compared with PGE₁ treatment (median value of 40%, 78%, 27% and 22% versus and 26%, 13%, 4% and 4%, respectively). However, studies by Steinbrink et al. demonstrated that PGE₁-treated DCs are CD1a+ and display higher expressions of CD80, CD86, CD40 and CD83 (81%, 80%, 50% and 55%, respectively). One possible explanation for this discrepancy is the differences in the culture conditions chosen. Steinbrink et al. favored X-VIVO 15 + 1% autologous plasma + GM-CSF + IL-4 for 7 days followed by the maturation with PGE₁, for 2 days. As reported by Jonuleit et al., conceivably X-VIVO-cultured cell lines reveal lower expressions of CD83 in PGE₁-treated cells than in RPMI-cultured cells, which may need additional signals, or a longer time in cultures. These authors also detected lower expressions of CD83 in PGE₁-treated DCs or TNF-α-treated DCs under RPMI conditions, while Lee et al. obtained matured DCs with a higher expression of this CD marker on day 9 in cultured cells with RPMI conditions after TNF-α treatment. On the other hand, our TNF-α-treated DCs are CD1a+, and in agreement with a previous work this proinflammatory cytokine up-regulates the MHC class I expression as well as immune proteasome and TAP molecules at the cell surface of several Melanoma cell lines suggesting that TNF-α treatment is critical for an efficient immune response.

Moreover, considering that our TNF-α plus PGE₁-treated DCs show a three to five fold higher expression of CD80, CD40 and CD83 (median of 79%, 28% and 19%, respectively) as well as CD1a remaining highly expressed (median of 90%) together with the fact that these data are comparable to DCs generated using LPS in vitro we therefore suggest that the combination TNF-α + PGE₁ might be useful for generating ex-vivo DCs for efficient processing and presentation of antigens and therefore could be used as a cellular vaccines. LPS is known to be a potent stimulator of DC maturation however it has not been approved for clinical use because it can induce substantial toxicity. Further functional evaluation of these mature DCs is warranted.

Also, these three maturing agents, PGE₁, TNF-α and LPS, mediate their effects by distinct mechanisms: a) PGE₁, a member of the same PGE family, mediates its effect on monocytes via 4 G protein-coupled receptors (EP1, EP2, EP3 and EP4) two of which mediate their signal through elevated cyclic adenosine monophosphate (cAMP, 33-34); b) TNF-α mediates its effects via CD134 and CD137 receptors; the latter has been shown to be expressed on follicular dendritic cells and c) LPS mediates its effect on DC activation via TLR4 specific receptors on the surface of the DC. The exact downstream signaling responses are not clearly understood, but these distinct receptors suggest distinct targeted genes and may also explain the differences observed among DCs generated ex-vivo.

Further studies to elucidate the molecular mechanism involved in the maturation process of DCs is essential not only to understand the biology of DCs but also to optimize dendritic cell immunotherapy in humans.

Resumo
Células dendríticas (CDs) são células apresentadoras de antígenos altamente eficientes para a imunização contra patógenos e antígenos tumorais. A obtenção de CDs maturis tem sido descrita por diferentes métodos. Portanto, a escolha do método mais apropriado para gerar CDs em cultura de sete dias é pré-requisito essencial para as estratégias imunoterápicas. Aqui relatamos um método de obtenção de uma população enriquecida em monócitos de doadores de sangue e compomos a maturação das CDs sob o estímulo de quatro agentes. Uma população de monócitos, com pureza de 91% ± 5, foi obtida de 15 doadores. A população monociotária foi mantida em cultura por cinco dias com GM-CSF e IL-4. No 5º dia, após diferentes condições de maturação, foram analisadas as modificações morfológicas e fenotípicas. Nosso estudos demonstram que o TNF-α ou o PGE₁, por si só podem induzir a expressão de CD1a de 2.4 a 2.7 vezes, respectivamente, mais do que culturas de CDs com ausência dos agentes de maturação. Alternativamente, para com outras moléculas coestimuladoras ou acessórias (CD80, CD86, CD83 e CD40) o TNF-α foi mais potente na expressão do que o PGE₁, embora na presença de ambos o efeito seja mais pronunciado. A maturação fenotípica sob TNF-α + PGE₁, pode ser comparável ao LPS, concluindo que o TNF-α + PGE₁, pode ser útil para geração ex-vivo de CDs e útil para protocolos de vacinação celular. Avaliação funcional das CDs é recomendável. Rev. bras. hematol. hemoter. 2006;28(2):89-96.

Palavras-chave: Células dendríticas; monócitos; Percoll; maturação; imunoterapia.

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