The Effect of Monophosphoryl Lipid A on Maturation of DCs from Patients with Acute Myeloid Leukaemia

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ABSTRACT

Background: Generation of monocyte-derived dendritic cells (MDDC) is induced in the presence of GM-CSF and IL-4, and a maturation stimulus is added to the monocyte culture to obtain mature Dendritic Cells (DCs) suitable for therapy. TNF-α is the most common cytokine used for activating DCs and generating mature MDDC either alone or in combination with other cytokines. Objective: To compare effects of traditional cytokine cocktail (TNF-α + IL-1β) versus TLR4-agonist monophosphoryl lipid A on the viability, phenotype, cytokine profile and functionality of MDDC. Methods: The study included 32 individuals; twenty Acute Myeloid Leukaemia (AML) cases in complete remission and 12 healthy volunteers. They were divided into 3 groups: Group 1: control group: 12 subjects to measure the baseline levels of all markers in the monocytic preparation; Group 2: cytokine cocktail (TNF-α) group, which included 10 AML subjects; Group 3: MPLA group which included 10 AML subjects. Results: TNF-α group showed higher expression of CD83 than MPLA group indicating higher capacity to induce DC maturation but both were similar in CD86, CCR7 and IL-10 expression. Preparation of dendritic cells from AML cases in remission and loading them with tumor peptides was successful. Conclusion: The effect of MPLA in DC maturation is comparable with traditional DC maturation cocktail.


Keywords: DCs Maturation, Monocyte-Derived DCs

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INTRODUCTION

Since the time when dendritic cells were identified as master cells in the modulation of the immune response, a lot of efforts have been made to bring their potential to the clinical setting. MDDC (monocyte-derived dendritic cells) established the starting point to the development of new potential therapeutic approaches for several diseases that are refractory to conventional treatments. Among those, cancer and infectious diseases were the first to be treated with MDDC, with partially satisfactory results, and also autoimmune disorders that were non-responding to current treatments (1).

Immature, antigen capturing ‘sentinel’ DCs such as Langerhans cells are matured into a T-cell activating state by Tumor Necrosis Factor (TNF-α), Interleukin-1b (IL-1b), lipopolysaccharide (LPS) or CD40 ligation (2). Induction of Ag-specific CD4+ and CD8+ T cells can be achieved by loading immature dendritic cells with the whole protein content of lysed tumor cells. Induction of necrosis and apoptosis in tumor cells can be achieved by mechanical/thermal lysis and UV irradiation (3).

Effective induction of antitumor cytotoxic T lymphocyte (CTL) responses requires fully mature DCs that express high levels of costimulatory molecules (3,4) and that can migrate in response to lymph-node-produced CCR7 ligands. In addition, high interleukin-12p70 (IL-12p70) secretion dramatically enhances the ability of DCs to induce tumor-specific Th1 cells and cytotoxic T Lymphocytes (CTLs), and promotes tumor rejection (4). For DC maturation, different combinations of pro-inflammatory mediators and Toll-like receptor ligands have been tested, obtaining DCs that differ in their properties and the type of immune response they promote. Therefore, it is necessary to find an optimal cytokine environment for DC maturation to obtain a cellular product suitable for DC-based immunotherapeutic protocols (5).

(TNF-α) is the most common cytokine used for activating DC and generating mature MDDC, (6-8) either used alone or in combination with other cytokines. Different preparations of cytokine cocktail have been tried using different combinations of cytokines with TNF α. The standard cytokine cocktail (CC) containing TNF-α, IL-1β, IL-6 and PGE-2, has largely proved to effectively mature DCs in-vitro (9).

A new cost-effective DC maturation cocktail consisting of MPLA and IFN-γ was tested in-vitro by Anja Ten Brinke et al.; by which matures DCs meet the criteria important for efficient immunotherapy, being the capacity to migrate and production of IL-12p70 upon CD40 triggering (1). A chemically modified LPS (Lipo-poly saccharide), monophosphoryl lipid A (MPLA), is one of the promising toll like receptor ligands, also called detoxified LPS. MPLA has been shown to exhibit potent adjuvant activity, while exhibiting essentially no toxicity. It has been safely used as an adjuvant in various vaccine trials in humans (10).

Our study aimed at comparing the effectiveness of MPLA vs. TNF-α and IL-1β in inducing DC maturation generated from peripheral blood monocytes of AML cases in remission, cultured in cellgro™ media and pulsed with tumor lysate.
MATERIALS AND METHODS

Study Design. This study was approved by Research Ethical Committee in Cairo University. We aimed to assess the best way to culture MDDCs loaded by tumor lysate of AML patients paving the way to in vivo stage of DCs vaccine. The study was conducted in two steps. First step to compare different culturing medias. Second step to compare TNF-α vs. MPLA as maturation cytokines.

Reagents. Cellgro™ DC serum-free medium was obtained from Cell Genix Freiburg, (Germany), Ficoll-hypaque density gradient (density 1.077, GIBCO, Axis-Shield PoCAs), 10% Heat inactivated Fetal calf serum (FCS, GIBCO) Phosphate buffer saline (PBS, Lonza), Gentamycin (Lonza) and Amphotericin B (Lonza), Trypan blue dye, Recombinant Human Interleukin-4 (IL-4) (R&D systems), Recombinant Granulocyte-Monocyte, Colony Stimulating Factor (GM-CSF) (R&D systems), Recombinant IL-1β (R&D systems), Recombinant TNF-α (R&D systems), MPLA from (Avanti Polar Lipids Inc., AL, USA), Monoclonal antibodies (mAb) used for flowcytometry were obtained from Becton Dickinson (BD, San Jose, USA), CY5-conjugated mouse anti-human CD83, &CD1a and ECD conjugated mouse anti-human CD14, Monoclonal antibody (mAb) phycoerythrin (PE)-conjugated mouse anti-human CCR7 used for the detection of CCR7 (Military Biotic Gm bH, Germany), Monoclonal antibody (mAb) fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD86 from R&D systems, UK), Quantitative human IL-12p70 ELISA kit (R&D systems), Quantitative human IL-10 ELISA kit (R&D systems) and Trypsin-Versene (EDTA) (Lonza).

Subjects. Twenty AML cases were recruited in remission after one month of last dose of chemotherapy and 12 normal volunteers. AML cases were patients treated in the clinical haematology unit of Kasr Al Aini hospital as well as the volunteers were staff ofhaematology clinic or relatives of the patients. Human peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparinized blood from subjects by Ficoll-hypaque density gradient centrifugation. Peripheral blood samples were collected with due approval from the Human Ethics Committee of Kasr al Aini hospital.

Cell Culture. Monocytes were purified by seeding PBMCs in bacteriological plastic dishes coated with human immunoglobulin G for a 2-hr adherence followed by removal of the non-adherent cells. The adherent cells were found to be 95% monocytes as assessed by CD14 staining by flowcytometry. The monocytes (0.2 \times 10^6/ml) were cultured. After the incubation period, the non-adherent cells were removed by washing the cell culture dishes with PBS. The adherent cells were cultured in cellgro™ medium (for 5-6 days) containing GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) to generate immature DCs. For the control group, after the non-adherent cells were removed by washing the cell culture dishes with PBS, the adherent cells were detached by incubating the cells at room temperature in 0.25% Trypsin (Gibco) in 0.02% EDTA for 5 min. In order to inactivate trypsin/EDTA, two parts of standard cell culture media [containing 10% FCS] per one part of used trypsin/EDTA was added to the cell suspension then harvested by gentle pipetting and resuspended in 1 ml of PBS. Cells were analyzed for the expression of surface markers CD14, CD1a, CD83, CD86 and CCR7.

Preparation of Tumor Lysate. Tumor lysate was prepared by the suspension of AML blasts in unsupplemented AIM-V medium at a concentration of 2\times10^7 cells/ml followed
by three successive freeze/thaw cycles at −80°C/37°C. Insoluble material was pelleted at 400×g for 10 min and the soluble antigenic fraction was placed at -80°C until needed for use. Protein concentration was determined by the bicinchoninic acid (BCA) assay according to the manufacturer’s instructions.

**Antigen Loading and Induction of DC Maturation.** On day 6, autologous tumor lysate was loaded into immature DCs, as described below. DCs were incubated with lysate at a final concentration of 120 mg of protein/ml for 3 h. After being loaded with lysate, immature dendritic cells were cultured for three hours at 37°C. Maturation was induced at day 6 using either:

A proinflammatory cytokine cocktail (CC) consisting of TNF-α (100 ng/mL), IL-1β (10 ng/mL), for 48 h in group 2 or MP LA (10 µg/mL) in group 3. DCs were harvested 48 h later and targeted for detection of phenotypes by flow cytometry. At the same time, the supernatant was also collected and stored at -20°C for the detection of IL-10 and IL-12 by ELISA.

**MDDC Count, Morphology and Viability.** DCs were counted using the original cell counter (haemocytometer). DC morphology was assessed by light microscopy on Giemsa-stained cytospin specimens. Cells having a large size, copious gray cytoplasm, and long cytoplasmic processes were identified as DCs. DCs were checked for viability using trypan blue cell exclusion method. Percentage of viability was calculated by dividing the number of viable DCs by the total number of DCs.

**Flow cytometry.** Flow cytometry was performed to define the phenotypic characteristics of the cells cultured in the presence of the indicated cytokines and to quantify cytokines in the culture supernatants by Cytometric Bead Array™ Multiplex assays. Analysis was performed using a BD LSR™ flow cytometer (Becton Dickinson, San Jose, CA). Data on immunophenotyping were analyzed on Cell Quest™ software (Becton Dickinson).

**Statistics.** The results were analyzed using the SPSS computer software package, version 13 (Chicago, USA). Quantitative data were presented as mean ±SD for normally distributed data and as medians and percentiles for skewed data. Qualitative data were presented in the form of frequencies and percentiles.

Differences between two groups were compared by Student’s *t*-test and Mann-Whitney Rank test for normally distributed and skewed data, respectively. Pearson Chi-square test and/or Fisher’s exact test were used to detect associations between two variables. For comparison of means among the different groups, the Post Hoc of one-way ANOVA test (LSD) was used. All tests are considered statistically significant at p<0.05.

### RESULTS

Our study included 32 healthy donors, 20 females and 12 males aged from 20-40 years. They are divided into 3 groups:

- **Group 1**: control group consisted of 12 subjects (15-25 ml of blood). After removal of non-adherent layer, the adherent layer was harvested and the expression of all the markers was evaluated to measure the baseline level of these markers in the monocytic preparation.

- **Group 2**: cytokine cocktail (TNF) group which included 10 subjects.

- **Group 3**: MPLA group which included 10 subjects.
No statistical significant difference in age and sex was detected between the three groups.

**Count.** We obtained Dendritic cell rich population of about (2-5 x 10^5) cells from 10x 10^6 PMNCs.

**Viability.** Using trypan blue cell exclusion method, percentage of viability was ranging between (60-75%).

**Mean Fluorescence Intensity (MFI) of Flowcytometry.** Mean and standard deviation of CD14, CD1a, CD83, CD86 and CCR7 and IL-10 between the three groups (Table 1).

**Table 1.** Mean and standard deviation of CD14, CD1a, CD83, CD86 and CCR7 and IL-10 between expression (%) the three groups as determined by ANOVA.

<table>
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<th></th>
<th>N</th>
<th>Mean (%)</th>
<th>Std. Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P value</th>
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<tr>
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<td>20.00</td>
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</table>
Figure 1. Comparison of the mean expression of each marker among the three groups (CD14, CD1a, CD83, CD86, CCR7). Y axis shows % of each marker.

Application of Post-Hoc test for comparison of each two separate groups of the study groups shows the data in (Table 2).

Table 2. Application of Post-Hoc test for comparison of every two groups of the study are shown.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>(I) Activation</th>
<th>(J) Activation</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
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<td>MPLA</td>
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<td>30.83600*</td>
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<td>MPLA</td>
<td>-42.91917*</td>
<td>2.59057</td>
<td>0.000</td>
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<td>-3.59033</td>
<td>2.59057</td>
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<td>-3.59033</td>
<td>2.65907</td>
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<td>MPLA</td>
<td>-27.44417*</td>
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IL-10 expression shows no significant variations among the 2 study groups. Its mean expression in the TNF group (mean ± SD: 14.9 ± 11.8) and in the MPLA (12.3 ± 6.3) groups are compared in figure 2. There was no statistically significant differences (p = 0.608) between groups.

![Figure 2](image)

**Figure 2.** Comparison of the mean expression of IL-10 among the MPLA and TNF groups shows no statistically significant difference (p = 0.608). Y axis shows IL-10 concentration in pictogram.

Values of results of IL-12 were non-detectable.

**Morphology of mature DC.** shown in Figure 3 (Photo taken by Inverted phase-contrast microscope (Leitz) x 40).

![Figure 3](image)

**Figure 3.** Dendritic cell morphology in culture (x40).
Supplementary file contains study design, results of comparing different media on maturation of dendritic cells and the flow results of 2 cases of which, one treated by TNF and the other one by MPLA.

**DISCUSSION**

Our results on the comprise between TNF activation vs. MPLA revealed no significant difference between the two groups in CD14, CD1a, CCR7 and CD86 expression but statistical significant difference in the expression of CD83 was seen in favor of TNF.

In this study we used cellgro™ media which is characterized by no use of serum or albumin unlike other medias(supplementary file). Tkachenko et al., generated DC from adherent monocytes of human peripheral blood in the presence of GM-CSF, IL-4 and TNF-α. The following culture media were used in their study: RPMI 1640 supplemented with 2% human serum albumin and X-VIVO 15 and Panserin 501. Interestingly, the use of Panserin and RPMI with albumin preferentially gave rise to CD1a+ DC, whereas in X-VIVO they observed both CD1a+ and CD1a-. They found that all media led to the loss of monocyte surface marker CD14 (11).

Our results show some agreement with Fontes et al., who tested four different maturation conditions: [1] TNF-α; [2] PGE1; [3] TNF-α plus PGE1 and [4] LPS from *E. coli*. Data indicated that DCs exposed to TNF-α plus PGE1 have a higher expression of costimulatory and accessory molecules, CD80 and CD86 and CD1a than DCs stimulated with TNF-α or PGE1 alone. Surprisingly, TNF-α plus PGE1 treated-DCs did not show significant increases of CD40 and CD83 expression compared to untreated or TNF-α-DCs and PGE1-DCs. 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 PGE1-DCs (12).

In this protocol we omit IFN-γ due to the severe inflammatory reactions associated with its use. we used only MPLA unlike Brinke et al. who generated cytokine cocktail matured DCs (CC-DCs), and MPLA/IFN-γ DCs, both DC types were phenotypically mature as shown by expression of CD83 and the co-stimulatory molecules CD40, CD80 and CD86. The CD83 expression level was significantly lower for the MPLA/IFN-γ DCs compared to the CC-DCs (13).

A previous study used 5 maturation protocols including: Group A, control group (cells were treated with GM-CSF and IL-4 alone); Group B, CD40L group (cells were treated with GM-CSF and IL-4 as well as CD40L); Group C, LPS group (cells were treated with GM-CSF and IL-4 as well as LPS); Group D, TNF-α group (cells were treated with GM-CSF and IL-4 as well as TNF-α); Group E, cocktail of cytokine group (cells were treated with GM-CSF and IL-4 as well as TNF-α, IL-6, IL-1β and PGE2). The expression of CD83, demonstrated the most evident increase, and immature DCs almost had no CD83 expression. In Group E, the maturation induction was the most efficient and the expression of all markers was dramatically elevated. These findings suggest that induction with a cocktail of cytokines is an optimal method used to induce DCs maturation (8).

Our results also goes with Kaka et al., who found that TNF is essential in maturing DCs and that DCs matured with “TNF-α and PGE2” gave rise to the minimal DC maturation
and those matured with “IL-1, IL-6, TNF-α, and PGE2” gave rise to the standard DC maturation, and “IL-1, IL-6, TNF-α, IFN-α, and CD40L” cocktails gave rise to the optimal DC maturation (14).

Moreover, Abediankenari et al., generated DCs with GM-CSF and IL-4 cytokines and maturation was done by adding either TNF-α, LPS, Poly I:C (polyinosinic: polycytidyllic acid) or CpG (dinucleotide containing cytosine (C) and guanine (G)) in their culture media. Our results indicated that all of the studied maturation inducing factors can be used in DC maturation but TNF-α and CpG were the preferred in-vitro maturation factors. It is concluded that maturation of dendritic cells by CpG motif and TNF-α can be used to regulate immune responses LPS: 41%, poly I:C, 39%, TNF: 42% and CPG, 45% (7).

Contrary to our results, Raïch-Reguéa et al., used MPLA, compared to the traditional cytokine cocktail (CC; clinical grade TNF-α, IL-1β, PGE2) and a combination of both showed that (CC + MPLA)- immunogenic DCs expressed higher levels of CD83, CD86 and HLA-DR compared to CC or MPLA alone (1).

Brinke et al., compared a DC maturation cocktail consisting of MPLA and IFN-γ with two clinically available maturation cocktails, the ‘gold standard’ (TNFα, IL-1β, IL-6 and PGE2) and the ‘α type 1 polarizing’ (TNFα, IL-1β, IFNγ and p I:C) cocktail. All DCs had a mature phenotype and expressed the maturation marker CD83 to the same extent, while it was not expressed by imDCs. Furthermore, all types of DCs upregulated the expression of the co-stimulatory molecules, CD40, CD80 and CD86, and the chemokine receptor CCR7. Over all, no significant differences in the expression levels of CD40, CD83, CD86, HLA-DR and CCR7 were observed between the three different types of mature DCs (15).

Our results showed undetectable levels of IL-12 in the culture supernatant. These results are in agreement with Raïch-Reguéa et al., 2012 who used CC + MPLA on immunogenic DCs and found a higher production of IL-12p70 compared to CC or MPLA stimuli (1).

Brinke et al., generated cytokine cocktail matured DCs (CC-DCs), and MPLA/IFNγ DCs. The MPLA/IFN-γ DCs produced high levels of IL-12p70 during culture as well as upon restimulation by CD40L triggering, while no production was detected for the CC-DCs (10). Chiang et al., evaluated Day-2, Day- 4, and Day-7 cultured monocyte-derived DCs loaded with whole lysate and matured with LPS and IFN-γ. Day-4 DCs pulsed with lysate produced higher amount of IL-12p70 than did lysate-pulsed Day-2 or Day-7 DCs after LPS and IFN-γ stimulation. Day-7 iDCs that were not stimulated with LPS and IFN-γ did not produce IL-12p70, and they only did so after full activation from LPS and IFN-γ (16). Li et al., showed that the IL-12p40 content in mature DCs in Groups B, C, D and E were markedly higher than that in Group A. The highest IL-12p40 content was found in Group E, while there was no marked difference among Groups B, C, D and E (8). Dauer et al., studied to mature DC with additional activation with CD40 ligand and IFN-α. DC matured with proinflammatory mediators secreted low amounts of IL-12p70, even after additional activation with CD40L and IFN-α. Priming of DC with IFN-α reduced secretion of total IL-12 compared with unprimed DC (17). In another study IL-12 was notably undetected in all tolDC cultures. Similar differences were found when cytokine production by tolDCs and matDCs was measured directly after maturation with MPLA, before activation through CD40. IL-10 production by
tolDCs was non-significantly reduced as compared with their equivalent matDC counterparts (18).

Kaka et al., measured secretion of the active form of IL-12, IL-12p70, in supernatants of differentially matured DCs. IL-12, produced by DCs matured using the minimal cocktail, was significantly less than standard DCs which in turn was significantly less than optimal DCs (14). Brinke et al. noticed no IL-12p70 was produced when DCs were matured with ‘gold standard’ cocktail. When matured with ‘α type 1 polarizing’ DC cocktail, a significant level of IL-12p70 was produced. However, DCs matured with MPLA in combination with IFN-γ produced even higher levels of IL-12p70. DCs matured with MPLA alone induced very low levels of IL-12p70, as previously described (5).

The difference in the results may be explained as:
- The concentration of IL-12 was highly donor-dependent, perhaps owing to IL-12 promoter polymorphisms in the population.
- Lack of IFN-γ in our culture: IFN-γ has a powerful effect in enhancing the ability of DCs to produce IL-12, and IL-12 also enhances the production of IFN-γ, thus creating a positive reinforcement loop.
- Very low concentrations which were not detectable by our assay as the MDD (Minimal detectable dose, that is < 5 pg/ml).
- Lack of stimulation by pansorbine (as described in the IL-12 ELISA kit).

Our results show that the mean expressions of IL-10 among the three study groups were not statistically different (p=0.608). In contrast to the results obtained by Brinke et al. 2007 who found IL-10 levels were somewhat higher upon CD40 ligation than during culture, although these levels were still negligible compared to the amounts of IL-12 produced. Raïch-Regüéa et al., found that IL-10 was detected in some samples when maturated in the presence of MPLA and CC +MPLA, but less with CC alone. Interestingly, IL-10 was preferentially produced by (MPLA)- or (CC + MPLA)-TolDC, when compared with (CC)-TolDC secretion. However, when comparing the IL-10 secretion by TolDC with their immunogenic DCs counterparts, (CC)-TolDC showed 1.4 times more of IL-10 production, in contrast to (MPLA)-TolDC and (CC- MPLA)-TolDC that produced about 50% less IL-10 than their MatDC counterparts. Brinke et al., showed that the CC-DCs produced higher levels of IL-10 during culture, while upon CD40L restimulation the MPLA/IFN-γ DCs produced more IL-10.

From the above discussion we conclude the following:

The preparation of DCs from AML cases in remission is feasible and with similar results to healthy volunteers.
- The expression of the monocytic marker CD14 was higher in the control group and showed marked down regulation in the TNF and MPLA groups due to the capacity of GM-CSF to skew monocyte differentiation toward the DC pathway.
- The expression of DC marker, CD1a, was low in the control group and showed marked up regulation in the TNF and MPLA groups, again due to GM-CSF effect which is perse sufficient to upmodulate the expression of CD1a.
- CD83 is the surrogate marker for DCs maturation. CD83 expression was low in the control group and showed marked up regulation in the TNF and MPLA groups.
- TNF induces higher expression of CD83 than MPLA indicating higher capacity to induce DC maturation.
MPLA is known to be a potent stimulator of DC maturation; however, it has not been approved to induce the same DC maturation effect as TNF.

-CD86 is one of the costimulatory molecules expressed by mature DCs. Its expression was low in the control group and showed marked up regulation in the TNF and MPLA groups. This change reflects the decreased antigen uptake, but enhanced presentation and T cell stimulation functions of mature DCs.

-Maturation of DC may induce acquisition of chemokine receptors such as CCR7 on the DC surface, which enables trafficking to lymph nodes. The mean expression of CCR7 among the three study groups shows no statistically significant difference.

-DCs provide the appropriate signals to T lymphocytes (Signal 1 and Signal 2) to allow generation of antigen-specific immune responses, in addition to Signal 3 which is secretion of either Th1 or Th2 cytokines.

-One well-studied third signal, IL-12p70, may be secreted by mature DCs for the induction of Th1 and CTL.

-DCs may secrete IL-10 which is the most important anti-inflammatory cytokine. It is a potent inhibitor of Th1 cytokines in addition to its activity as a Th2 lymphocyte cytokine. Our results showed no statistically significant difference in the mean expression of IL-10 among the three study groups.

-The most efficient method to induce DC maturation is the cocktail of cytokines that has the most potent ability to induce DC maturation evidenced by the expression level of CD83.

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