Comparative study of dendritic cells matured by using IL-1β, IL-6, TNF-α and prostaglandins E2 for different time span

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Abstract. Interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and prostaglandins E2 is considered as the standard cocktail for maturing dendritic cells (DCs). However, the appropriate time span for DC maturation with the standard cocktail remains unclear. The present study aimed to compare the differences between DCs matured with the standard cocktail for 24 and 48 h, respectively, and determine whether 24-h stimulation was sufficient for DC maturation. The findings demonstrated that, compared with DCs matured for 48 h, the levels of cluster of differentiation (CD)80, CD83, CD86 and programmed death-ligand 1 expression in DCs matured for 24 h were relatively lower. However, with the exception of CD80 whose mean fluorescence intensity (MFI) was higher in DCs matured for 48 h, the MFI values of other surface markers were comparable. Notably, the MFI of CD40 was higher in DCs matured for 24 h. In addition, the viability, T cell stimulatory capacity in allogeneic mixed lymphocyte reaction and cytokine production, including IL-12p40, IL-12p70 and IL-10, were all comparable between DCs matured for 24 and 48 h, respectively. These results indicated that 24-h stimulation may be sufficient for DC maturation when using the standard cocktail.

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Abbreviations: DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; PGE2, prostaglandin E2; PD-L1, programmed cell death ligand 1

Key words: dendritic cells, monocytes, cytokines, immunotherapy

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells and they have a critical role in innate and adaptive immunity (1,2). Notably, they have a unique ability to initiate naive T cells (3). Immature DCs (imDC), which are located in peripheral tissues (such as the skin, capture and process antigens), migrate to the draining lymphoid organs where they are able to prime cluster of differentiation (CD)4+ and CD8+ T cells (4). However, whether they induce T cell-mediated immune response or tolerance is determined by the status of DCs (5).

Over the past few years, great interest has been focused on the development of DC-based immunotherapy due to the unique capacity of DCs to initiate naive T cells. It is now straight-forward to generate monocyte-derived DCs (moDCs) *in vitro* using granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (6,7). A number of protocols have been tested for their capacity to induce DC maturation (8-11) due to the fact that fully mature DCs are more powerful than imDC at inducing immune responses (5). Among these protocols, IL-1β, IL-6, TNF-α and prostaglandins E2 (PGE2), which was developed by Jonuleit *et al* (8), has become the gold standard cocktail for DC maturation. To date, DCs matured with this standard cocktail have been applied in the treatment of patients with different malignant tumors and promising results have been demonstrated in several clinical studies (12-16).

Although this standard cocktail has been widely used, the appropriate time span for DC maturation has not been determined. It has been reported that DCs gradually lose their function over a few days after maturation (17). Therefore, shortening the time to mature DCs *in vitro* may be beneficial for the effectiveness of DC-based therapeutic vaccine *in vivo*. Therefore, the present study comprehensively compared DCs matured for 24 and 48 h using the standard cocktail to determine the appropriate time span for DC maturation.

Materials and methods

Isolation of PBMCs and positive selection of CD14+ monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare, Chicago, IL, USA) density gradient centrifugation from healthy human heparinized blood (Beijing 307 Hospital of Chinese People's Liberation Army,

Beijing, China). All subjects were recruited in August 2015 (n=3; male: Female, 1:2; age 27.33±1.53 years). Inclusion criteria for subjects in this study were i) no fever; ii) normal renal and hepatic function; iii) no drug use within one month. Exclusion criteria were i) infection with hepatitis B virus, hepatitis C virus, hepatitis D virus or human immunodeficiency virus; ii) liver cirrhosis or hepatocellular carcinoma, fatty liver or alcoholic hepatitis. PBMCs were washed by PBS twice before CD14+ monocytes isolation using human CD14+ microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated CD14+ monocytes was >90%. The Ethics Committee at the Second Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China) approved this study. Informed consent was obtained from all participants.

DCs generation. Monocyte-derived DCs were generated as previously described with minor modifications (9). CD14+ monocytes were re-suspended in serum-free AIM-V medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and placed in 24-well plates (Corning, Inc., Corning, NY, USA) for incubation at 37˚C in a humidified atmosphere containing 5% CO_2 for 2 h. Following complete aspiration of the supernatant, fresh AIM-V medium supplemented with GM-CSF (1,000 IU/ml) and IL-4 (500 IU/ml; both PeproTech, Inc., Rocky Hill, NJ, USA) was added to the cells. The cells were supplied every 2 days with fresh medium. On day 5, imDC were harvested and cultured in the presence of IL-1β, IL-6, TNF-α (1,000 IU/ml; PeproTech, Inc.) and PGE2 (1 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for a further 24 or 48 h, respectively, to obtain mature DCs (mDC). Supernatants were collected and retained for cytokine analysis.

Flow cytometric analysis. Flow cytometry was performed using FACS Calibur (BD Biosciences). Cells were stained with the following monoclonal antibodies: Fluorescein isothiocyanate (FITC)-labeled antibodies against CD40 and CD86, phycoerythrin (PE)-labeled antibodies against CD80, CD83 and programmed death-ligand 1 (PD-L1), peridinin chlorophyll protein (PerCP)-labeled antibodies against human leukocyte antigen-D related (HLA-DR), allophycocyanin-labeled antibodies against CD14, CD11c, and isotype matched control antibodies (BD Biosciences). FACS data were analyzed using FlowJo software (version 5.7.2; Tree Star, Inc.).

Apoptosis assay. Freshly harvested mDC ($1x10^5$) were washed twice with cold PBS and incubated with Annexin-V-PE and 7-amino-actinomycin D (7-AAD) for 15 min before fluorescence-activated cell sorting (FACS) analysis. FACS data were analyzed using FlowJo software (version 5.7.2; Tree Star, Inc., Ashland, OR, USA).

Endocytic ability during the maturation of DCs. ImDC and mDC ($1x10^5$) cells were suspended in 100 μl of AIM-V and incubated with FITC-dextran (1 mg/ml) for 60 min either at 37˚C or 4˚C (negative control). Afterwards, cells were washed three times in cold PBS prior to FACS analysis. FACS data were analyzed using FlowJo software (version 5.7.2).

Allogeneic mixed lymphocyte reaction (MLR). mDC matured for 24 or 48 h were treated with 50 μg/ml mitomycin-C at 37˚C in a humidified 5% CO_2 atmosphere for 45 min. Afterwards, DCs were washed three times and added to allogeneic CD14+ monocytes depleted PBMCs (10^5 cells) at a ratio 1:10 (DCs:PBMCs) in 96-well plates (Corning, Inc.) for 4 days, then 20 μl CellTiter 96 Aqueous non-radioactive reagent (Promega Corp., Madison, WI, USA) was added to each well and cultures were continued for another 4 h. Following this, absorbance at 490 nm was recorded using an ELISA plate reader.

Cytokines secretion analysis. Production of IL-12p40, IL-12p70 and IL-10 was assayed by ELISA kit (BioLegend, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis. Comparisons between groups of quantitative variables were performed using the Mann-Whitney U test. The test was two-sided and differences were considered significant if $P<0.05$. Data handling and analysis were performed with SPSS software for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Purity of CD14+ monocytes and mature DCs. In order to improve the purity of monocyte-derived DCs, we first selected the CD14+ monocytes from PBMCs by using human CD14+ microbeads instead of the conventional cell adherent technique. Our data showed that the proportion of CD14+ monocytes in PBMCs was approximately 6.79% prior to selection. However, this number increased to 91.98% following selection (Fig. 1A). The purity of DCs matured for 24 and 48 h was 90.22 and 92.95%, respectively, of which the majority of mature DCs were CD11c+ DCs (Fig. 1B).

Phenotypic characteristics of mature DCs. In the present study, co-stimulatory and co-inhibitory surface markers, including CD40, CD80, CD83, CD86, HLA-DR and PD-L1, were compared between DCs matured via a standard cocktail for 24 and 48 h by flow cytometry. Compared to DCs matured for 24 h, DCs matured for 48 h expressed higher levels of CD80, both in frequency and mean fluorescence intensity (MFI). For CD83 and CD86 exhibited higher levels of frequency when matured for 48 h instead of 24 h, however no differences were found in MFI. Notably, similar expression levels of CD40 were found in frequency after 48 h, whereas MFI was higher in DCs matured for 24 h. No differences of HLA-DR were found in terms of frequency and MFI. However, for the inhibitory molecule, PD-L1, a higher frequency was also observed in DCs matured for 48 h. The frequencies of co-stimulatory molecules for both DCs all exceeded 90%, with the exception of CD83 (Table I).

Viability and endocytosis of mature DCs. High viability is important for the preparation of effective DC-based therapeutic vaccines. Therefore, we determined and compared the viability of DCs matured via the standard cocktail for different time spans. Our data showed that the viability of DCs matured for 24 and 48 h, respectively, were similarly

Table I. Phenotypic characteristics of mature DCs.

		Mature DCs	
Markers	Value	24 h	48 h
CD40	%	92.24	96.57
	MFI	67.21	59.22[a]
CD80	%	93.94	98.82[a]
	MFI	149.24	247.51[a]
CD83	%	83.55	94.98[a]
	MFI	81.90	89.17
CD86	%	94.47	99.52[a]
	MFI	198.56	216.83
PD-L1	%	95.03	98.01[a]
	MFI	105.18	135.52
HLA-DR	%	97.22	99.57
	MFI	629.59	675.37

Phenotypes of mature DCs were analyzed by flow cytometry. [a]P<0.05. MFI, mean fluorescence intensity; DCs, dendritic cells; CD, cluster of differentiation; PL-L1, programmed death-ligand 1; HLA-DR, human leukocyte antigen-D related.

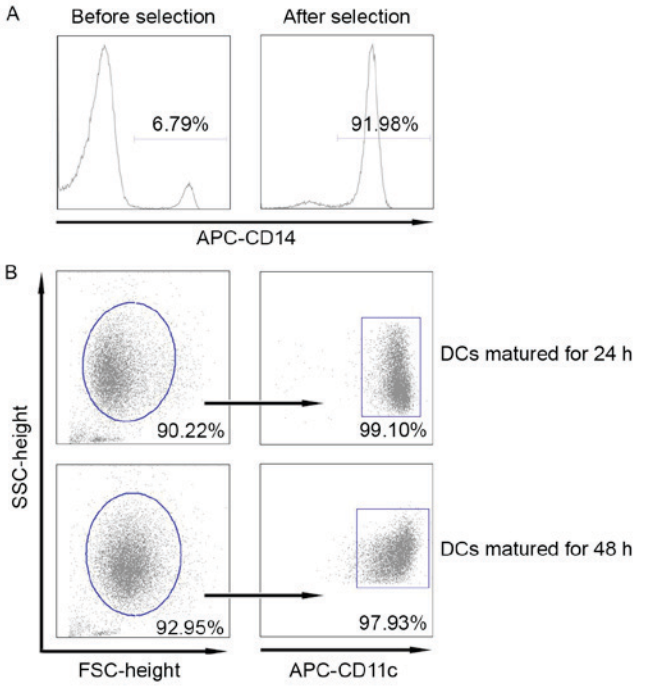


Figure 1. Purity of CD14+ monocytes after selection (A) and purity of DCs matured using a standard cocktail for 24 and 48 h, respectively. (B) Three experiments were performed; one is presented. CD, cluster of differentiation; DCs, dendritic cells; SSC, side scatter; FSC, forward scatter; APC, allophycocyanin.

high and exceeded 90% (Fig. 2A). It is known that the ability to take up antigens is one of the most important functions of immature DCs and this capacity decreases quickly upon maturation (18). Consistent with this, we found that imDCs showed high endocytosis while the endocytic capacity of DCs matured for 24 and 48 h both decreased rapidly to a similar extent (Fig. 2B).

T cell stimulatory capacity and cytokine productions of mature DCs. The T cell stimulatory capacity of DCs matured for different time spans was assessed via an allogeneic mixed lymphocyte reaction. Our data showed that the T cell stimulatory capacity of DCs matured for 24 and 48 h, respectively, was comparable (Fig. 3). We subsequently detected the cytokine production of mDCs from both time points and found that mDCs matured for 24 and 48 h, respectively, secreted comparable high levels of IL-12p40, which is a subunit of IL-12p70. Both groups of cells secreted comparable low levels of IL-10; however, mDCs matured for 24 h secreted relatively lower levels of bioactive IL-12p70 (Fig. 4).

Discussion

IL-1β, IL-6, TNF-α and PGE2 has been widely used as a standard cocktail for *in vitro* generation of mature DCs (8,12-16,19,20). However, the optimal time for DC maturation using this standard cocktail has not been established. In the present study, we found that DCs matured for 24 h were, phenotypically speaking, also fully mature compared to DCs matured for 48 h. DCs matured for 24 h expressed even higher levels of the CD40 co-stimulatory molecule in terms of MFI, whereas lower levels of the co-inhibitory molecule, PD-L1, were detected in terms of frequency. Notably, the viability, endocytosis, T-cell stimulatory capacity and cytokine production were all comparable between DCs matured for 24 and 48 h, respectively.

Pioneering studies indicating the possibility of culturing murine DCs *ex vivo* from bone marrow precursors initiated DC vaccine development in the 1990s (21). Human applications followed soon thereafter and it was demonstrated that peripheral blood-derived monocytes and CD34+ hematopoietic progenitors are suitable for generating human DCs (22). In previous studies, DCs have been induced from adherent monocytes by washing out non-adherent cells, such as T and B cells. However, the purity of DCs obtained by this method is ~60%. In the present study, we induced DCs from CD14+ monocytes selected by using human CD14+ microbeads. The purity of DCs obtained from this method exceeds 90%, which is crucial for the improved effectiveness of DC-based immunotherapy (23).

The maturation state of DCs has been considered as a decisive factor in immune responses. Previous clinical studies have demonstrated that improved clinical outcomes were more frequently observed in trials using mature DCs in the therapeutic vaccination of patients with cancer, including prostate cancer, melanoma and glioblastoma (24-26), although moderate clinical benefit was also reported in trials using IL-4 immature DCs (27). Due to their low co-stimulatory and MHC class I and II molecule expression, immature and semi-mature DCs are prone to inducing suboptimal T-cell priming and causing T-cell tolerance. Fully mature DCs (for example, matured with proinflammatory cytokines or TLR agonists) are able to prime CD4+ T and CD8+ T cells (5,28). It is well-known that co-stimulatory molecules, such as CD80 and CD86, have a key role in the induction of effective T cell

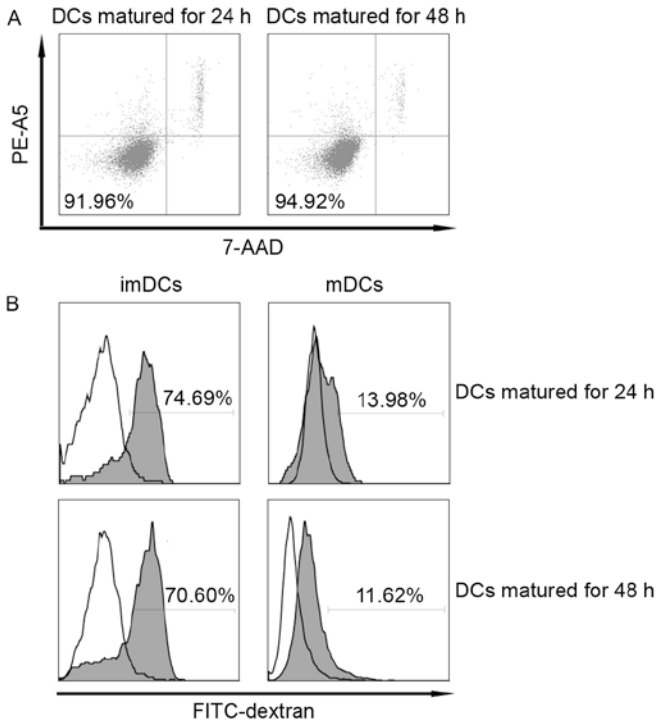


Figure 2. Comparison of (A) viability and (B) endocytosis of DCs matured for 24 and 48 h, respectively. Three experiments were performed; one is presented. DCs, dendritic cells; mDC, mature dendritic cells; imDCs, immature dendritic cells.

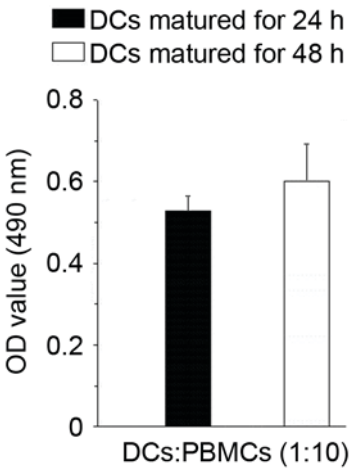


Figure 3. Comparison of T cell stimulatory capacity of DCs matured for 24 or 48 h. Data are presented as the mean + standard deviation. DCs, dendritic cells; OD, optical density.

responses (29). Our data showed that DCs matured for 24 and 48 h, respectively, expressed high levels of CD80 and CD86, which are important for the initiation of a robust immune response. Furthermore, CD40, which also has a critical role in T cell activation (30,31) expressed even higher levels in DCs matured for 24 h. Notably, PD-L1, which is well-known for its inhibitory role in T cell activation (32-34), expressed relatively lower levels on DCs matured for 24 h and this may be beneficial for T cell priming.

It is increasingly recognized that abundant production of IL-12, particularly IL-12p70 during DC maturation has a

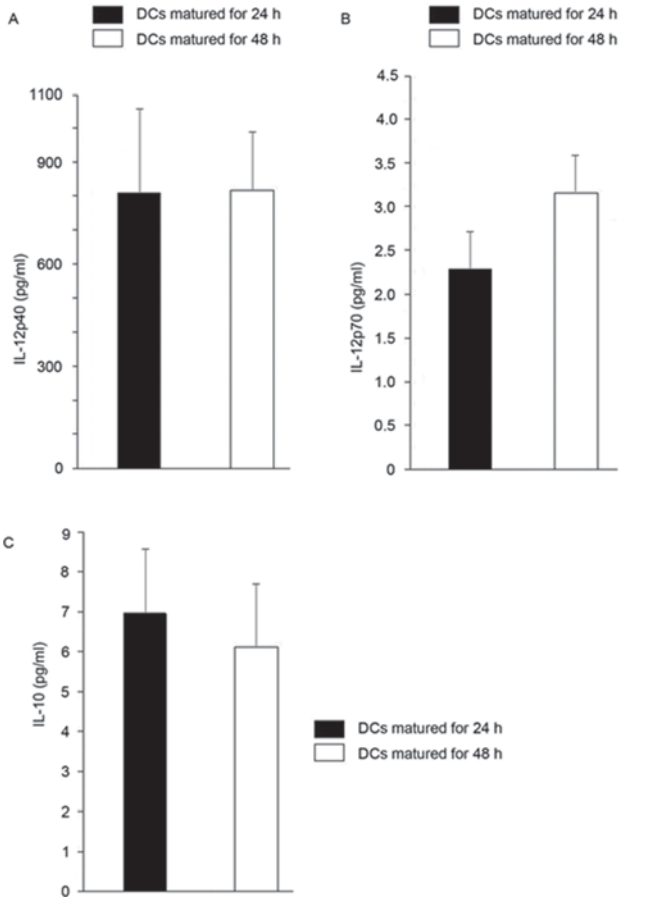


Figure 4. Comparison of the cytokine secretion of DCs matured for 24 or 48 h, respectively. Production of (A) IL-12p40, (B) IL-12p70 and (C) IL-10. Data are presented as the mean + standard deviation. DCs, dendritic cells; IL, interleukin.

crucial role in the differentiation and expansion of Th1 cell and Th1-polarized immunity (13,35,36). In clinical trials of melanoma (37) and glioblastoma (38), favorable outcomes were observed to be related to DC1-derived IL-12p70 production and Th1-polarized immunity. Similar to previous studies (39,40), the present study found that although DCs matured with a different time span secreted higher levels of IL-12p40, and secreted little bioactive IL-12p70. Therefore, studies to further improve the capacity of DCs to produce bioactive IL-12p70 are necessary. IL-10, known as an anti-inflammatory and immunosuppressive cytokine, was first described as a product of Th2 cells that inhibited cytokine synthesis in Th1 cells (41). It is now known that multiple immune cells, including macrophages, dendritic cells (DC), B cells, and various subsets of CD4+ and CD8+ T cells, are able to produce IL-10 (42). IL-10 inhibits the capacity of antigen-presenting cells, including DCs and macrophages, to present antigens to T cells in various ways to modulate immune responses (43). Recently, tumor cell-secreted IL-10 has been demonstrated to counteract the immunity of modified DCs in an established tumor model, which indicated that the high level of IL-10 within tumor microenvironment may impair DC vaccine functions (44). In the present study, DCs matured with the standard cocktail for different time spans (24 and 48 h) secreted minimal IL-10, which is a positive factor for DCs exerting immune responses.

High viability is another important factor in DC-based immunotherapy. In fact, DCs will gradually lose their function in a few days after maturation owing to apoptosis (17). In our present study, DCs matured for 24 and 48 h, respectively, exhibited high viability. The high viability may be due to the addition of PGE2 to the cocktail as previous studies have shown that PGE2 promotes apoptotic resistance and survival of DCs (45,46).

In conclusion, our preliminary results indicated that 24-h stimulation is sufficient for DC maturation when using IL-1β, IL-6, TNF-α and PGE2. Reducing the time to mature DCs *in vitro* from 48 to 28 h may be beneficial for the optimal preparation of tumor-pulsed DC therapeutic vaccine and improve its *in vivo* effectiveness.

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