Maturation induction of human peripheral blood mononuclear cell-derived dendritic cells

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Abstract. The aim of the present study was to explore an optimal method for maturation induction of dendritic cells (DCs). Human monocyte-derived DCs were induced in the presence of GM-CSF and IL-4. On Day 6, the maturation of DCs was induced with CD40L, LPS, TNF-α and cocktail of cytokines (TNF-α, IL-6, IL-1β and PGE_2), respectively, for 24 h. Then, DCs were harvested and subjected to flow cytometry (FCM) for the detection of CD80, CD83, CD86 and HLA-DR. FITC-dextran endocytic activity was measured by FCM, IL-12 production by ELISA and T lymphocyte proliferation following DC stimulation by MTT assay. CD40L, LPS, TNF-α and a cocktail of cytokines induced DC maturation. Induction with the cocktail of cytokines was the most efficient, and the expression rate of CD83 was 66.91% ($P<0.05$). The FITC-dextran endocytic activity of mature DCs was significantly reduced, and IL-12 production was dramatically increased in mature DCs, particularly in those following induction using the cocktail of cytokines. The mature DCs had potent ability to stimulate the proliferation of lymphocytes. The cocktail of cytokines is a favorable strategy for the induction of DC maturation.

Introduction

Dendritic cells (DCs) have been found to be the most potent professional antigen-presenting cells (APCs) to date and are initiators of the immune response. They play central roles in the induction, regulation and maintenance of anti-tumor immunity (1). Human peripheral blood mononuclear cells (PBMCs) can be induced into DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) and using this method a large amount of DCs with high quality can be obtained for clinical application (2). Biological therapy of tumors based on DCs has been a focus of research, and extensive progress has been made. Autogenous antigen-bearing DCs following *in vitro* culture have been re-transfused to induce specific immunity, which has been a routine method (3). Following culture for 5-7 days, PBMCs can be induced into immature DCs which may be further induced into mature DCs in the presence of inflammatory factors. The maturation of DCs is critical for the function of DCs. Only mature DCs can function to activate an immune response. Currently, the DC vaccine used in clinical practice consists of mature DCs, and the therapeutic efficacy is closely related to the degree of DC maturation. Thus, induction of DC maturation is a key step in the preparation of DC vaccine. Various cytokines including TNF-α, IL-1β and PGE_2, CD40L, LPS and CpG ODN have been applied to induce DC maturation. In the present study, human PBMCs were induced into immature DCs which were then induced to mature DCs using different stimulants. Our study aimed to compare the effectiveness of these various stimulants in inducing DC maturation and to identify an optimal method for clinical preparation of DC vaccine.

Materials and methods

Collection of human PBMCs. Peripheral blood (50 ml) was collected from volunteers aged 20-40 years and isolation of PBMCs was carried out within 2 h.

Main reagents. RPMI-1640 powder containing L-glutamine (Gibco), fetal bovine serum (FBS; Hangzhou Sijiqing Biotech Co., Ltd.), penicillin-streptomycin (P-S; Gibco), Ficoll lymphocyte separation solution (relative density, 1.077±1 g/l; Lymphoprep™), recombinant human GM-CSF (rhGM-CSF), recombinant human IL-4 (IL-4), tumor necrosis factor-α (TNF-α), recombinant human IL-6, recombinant human IL-1β, CD40L (Pepro Tech), prostaglandin E_2 (PGE_2; Cayman), LPS (Sigma), FITC-dextran (40,000 MW, Sigma), phycoerythrin (PE)-conjugated mouse anti-human CD83 and HLA-DR, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD86 and CD80 fluorescence monoclonal antibody (BD Pharmingen) and human IL-12(p40) ELISA kit (Dia Clone) were used in the present study.

Main instruments. FACS Calibur flow cytometer, CellQuest analysis system (B-D) and Wellscan Mk3 microplate reader (Labsystems Dragon) were used in the present study.

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Isolation of PBMCs. PBMCs were isolated using Ficoll density gradient centrifugation. In brief, heparin-treated fresh blood was diluted in normal saline of equal volume. Then, 9 ml of lymphocyte separation solution was added to a 50-ml centrifuge tube followed by addition of 15 ml of diluted blood. Centrifugation was carried out at 20˚C for 20 min at 250 x g. The mononuclear cells at the interface were collected and washed in PBS twice. These cells were used for DC induction.

Culture and maturation induction of DCs. The PBMC density was adjusted to $5x10^9$ cells/l with complete RPMI-1640 medium containing 10% inactivated FBS, 2 mmol/l L-glutamine, 0.05 mmol/l 2-mercaptoethanol and 100 U/ml P-S solution. The cell suspension was added to a 25-cm^2 flask followed by incubation at 37˚C in an atmosphere with 5% CO_2 for 2 h. The suspended cells were removed, and the adherent cells were maintained in complete RPMI-1640 medium containing 1000 U/ml rhGM-CSF and 500 U/ml IL-4. On Day 3, fresh medium containing rhGM-CSF and IL-4 was added. On Day 6, the immature DCs were collected and seeded into a 24-well plate at a density of $2x10^5$/ml (1 ml/well). These cells were further induced with rhGM-CSF and IL-4 and then divided into 5 groups: Group A, control group (cells were treated with rhGM-CSF and IL-4 alone); Group B, CD40L group (cells were treated with rhGM-CSF and IL-4 as well as 500 ng/ml CD40L); Group C, LPS group (cells were treated with rhGM-CSF and IL-4 as well as 1 μg/ml LPS); Group D, TNF-α group (cells were treated with rhGM-CSF and IL-4 as well as 1000 U/ml TNF-α); Group E, cocktail of cytokine group (cells were treated with rhGM-CSF and IL-4 as well as 1000 U/ml TNF-α, 10 ng/ml IL-6, 10 ng/ml IL-1β and 1 μg/ml PGE_2). DCs were harvested 24 h later and subjected to detection of phenotypes by flow cytometry. At the same time, the supernatant was also collected and stored at -70˚C for the detection of IL-12p40.

Detection of maturation markers of DCs by flow cytometry. The maturation markers (CD80, CD83, CD86 and HLA-DR) of DCs were determined with direct immunofluorescence method. The DC density was adjusted to $2\text{-}5x10^5$ cells/ml. Then, 50 μl of cell suspension was mixed in 5 μl of antibody working solution followed by incubation in the dark at 4˚C for 30 min. After washing in PBS twice, cells were fixed in 450 μl of 1% paraformaldehyde. Cells were re-suspended and then subjected to flow cytometery (FACS Calibur flow cytometer). CellQuest software was employed to calculate the positive expression rate of these markers in $1x10^4$ cells. Fluorescence-conjugated isotype IgG was used in the negative control group.

Detection of endocytic activity of DCs. The extent of FITC-dextran uptake reflects the antigen uptake ability of DCs. Detection of endocytic activity of DCs was performed according to the method developed by John *et al* (4) with modification. DCs were maintained for 7 days and then DCs were collected and cell density was adjusted to $4x10^8$ cells/l with RPMI-1640 medium containing 10% FCS. Then, $2x10^5$ cells were mixed in 0.5 mg/ml FITC-dextran solution followed by incubation at 4˚C and 37˚C for 2 h. After washing in cold PBS twice, cells were re-suspended in 1% paraformaldehyde and subjected to flow cytometry. The mean fluorescence density represents the uptake ability. In each group, cells were consistently incubated at 4˚C as a control and the fluorescence density was subtracted to avoid the non-active uptake.

Detection of IL-12 secretion by DCs with ELISA. A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was employed to measure the IL-12p40 content according to the manufacturer's instructions. Following visualization, absorbance (A) was measured at 450 nm in a microplate reader. The IL-12 content was calculated according to the standard curve. The lower limit in the detection of IL-12p40 was 20 pg/ml.

Mixed lymphocyte reaction. Allogeneic lymphocytes were used as effector cells. These cells were seeded into a 96-well plate at a density of $2x10^5$/well with the ratio of DCs to effector cells of 1:10. The control group included lymphocytes alone. Three wells were included in each group. Incubation was performed at 37˚C in an atmosphere with 5% CO_2 for 3 days. Then, 10 μl of 5 mg/ml MTT was added to each well followed by further incubation for 4 h. The supernatant was removed and 100 μl of DMSO was added to each well followed by gentle shaking. When the blue-violet formazan crystals dissolved, absorbance was measured at 595 nm (A595) 10 min later. The proliferation index (PI) was calculated as follows: $PI = A_{experiment}/A_{control}$.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD) and statistical analysis was carried out with SPSS version 10.0. Analysis of variance and correlation analysis were performed.

Results

DC morphology. After 2-h culture of PBMCs, the adherent cells were small and round mononuclear cells. On Day 1, after culture, the DCs had a large volume and were adherent and pleomorphic. The suspended cells increased over time. On Day 3, colonies were present. On Day 6, cells were still pleomorphic and the majority of cells were suspended DCs. Following maturation induction, the mature DCs were largely suspended and had obvious processes and large volume. These cells were irregular and presented evidence of dendritic processes.

Changes in the phenotype of DCs following maturation induction. The expression levels of CD80, CD86, CD83 and HLA-DR were determined by flow cytometry. Results showed the expression levels of these markers in Groups B, C, D and E were markedly increased when compared with levels in Group A. The expression of CD83, a known marker of DC maturation, 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. The mean positive expression rate of CD83 was 66.91% in Group E. These findings suggest that induction with a cocktail of cytokines is an optimal method with which to induce DC maturation (Fig. 1)

Mature DCs exhibit an obvious reduction in endocytic activity. DCs have a mannose receptor on their surface which

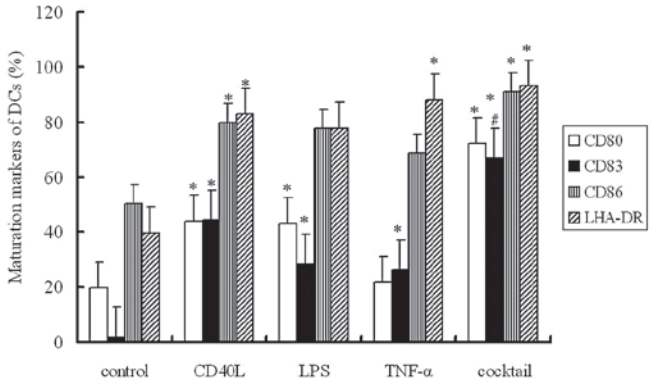

Figure 1. Expression of maturation markers on DCs following induction using different methods (n=5, *P<0.05 vs control, #P<0.05 vs. Groups B, C and D).

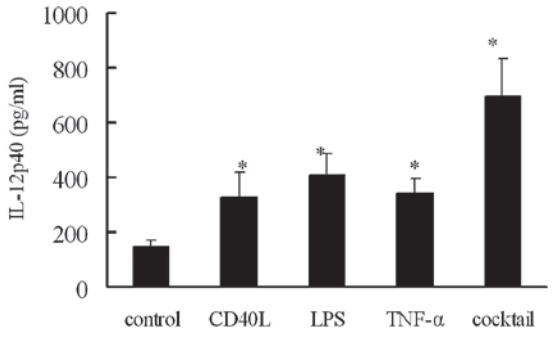

Figure 3. Level of IL-12 secreted by DCs following maturation induction using different methods (n=5, *P<0.05 vs. control).

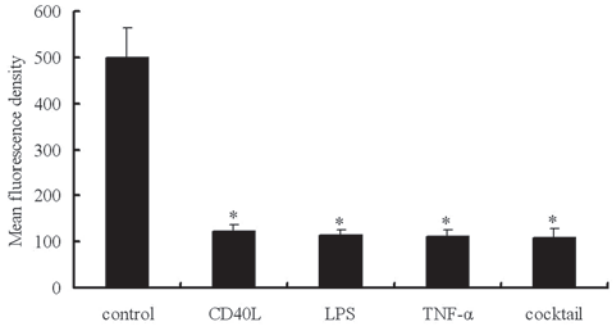

Figure 2. Endocytic activity of DCs following maturation induction using different methods (n=5, *P<0.05 vs. control).

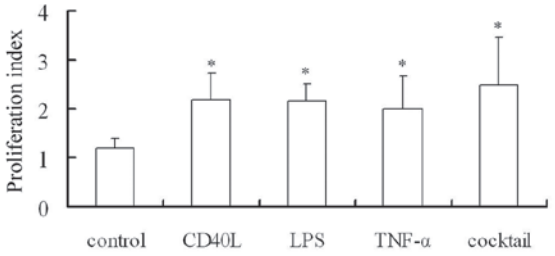

Figure 4. Proliferation index of T lymphocytes following co-culture with different DCs (n=5, *P<0.05 vs. control).

mediates dextran phagocytosis. The extent of active uptake of FITC-dextran reflects the antigen uptake ability of DCs. Flow cytometry and measurement of FITC-dextran uptake demonstrated the endocytic activity. In Groups A, B, C, D and E, the mean fluorescence density was 499.04±63.40, 123.86±14.21, 113.17±13.68, 112.11±14.66 and 108.73±18.41, respectively. Our results showed that the immature DCs exhibited potent endocytic activity, which, however, was markedly reduced in mature DCs (Fig. 2). The endocytic activity was negatively related to the expression levels of maturation markers (P<0.05).

IL-12 secretion by DCs following maturation induction using different methods. ELISA was employed to detect the IL-12p40 content in the supernatant of DCs following maturation induction for 24 h. The IL-12p40 content was calculated in $2x10^5$/ml cell suspension. In Groups A, B, C, D and E, the IL-12p40 content was 146.93±26.39, 330.76±87.23, 410.33±75.99, 342.45±52.60 and 693.65±138.52 pg/ml, respectively (Fig. 3). The IL-12p40 content in mature DCs in Groups B, C, D and E were markedly higher than that in Group A (P<0.05). The highest IL-12p40 content was found in Group E, while there was no marked difference among Groups B, C, D and E (P>0.05).

Mature DCs activate and promote lymphocyte proliferation. Mature DCs demonstrate high expression of costimulatory molecules and are potent to activate and stimulate the proliferation of T lymphocytes. In the present study, MTT assay was employed to measure proliferation (proliferation index of T lymphocytes which were co-cultured with DCs). The proliferation index in Groups B, C, D and E was markedly different from that in Group A (P<0.05, Fig. 4). The DCs in Group E (after treatment with the cocktail of cytokines) exhibited the most potent ability to stimulate the proliferation of DCs, which may be attributed to the favorable maturation of DCs and high expression levels of costimulatory molecules on DCs.

Discussion

In 1973, Steinman and Cohn identified DCs in mouse spleen which induced immune responses (5). In early 1990s, Chapuis *et al* (2) first found that PBMCs could be induced into DCs in the presence of IL-4 and GM-CSF, in which PBMCs did not undergo proliferation and differentiation. The differentiation and development of DCs have two distinct stages: immature DCs and mature DCs. The immature DCs have weak ability to stimulate the proliferation of naïve T cells and may induce the disability of T cells. However, immature DCs can effectively capture and process antigens. After antigen uptake or following stimulation, immature DCs may become mature. Their antigen uptake ability is compromised but the antigen-presenting ability is enhanced. Mature DCs have high expression of MHC-I/II molecules, costimulatory molecules (CD80 and CD86), adhesion molecules (such as CD54) and

other maturation markers (such as CD83) and can secret multiple cytokines related to immunoregulation. Thus, these cells acquire antigen-presenting ability and can stimulate the immune response. Mature DCs have distinct characteristics when compared to immature DCs. Only mature DCs can stimulate the proliferation of lymphocytes and activate immune responses (6). Thus, induction of DC maturation has been a key step in the preparation of DC vaccine.

Numerous methods have been developed to induce DC maturation. Studies have reported that LPS, TNF-α, CD40L, monocyte-conditioned medium (MCM) and a cocktail of cytokines (TNF-α, IL-6, IL-1β and PGE_2) can be applied to induce DC maturation. LPS is a component of the cell wall of gram-negative bacteria. LPS can induce DC maturation via the Toll-like receptor. However, this is a bacterial reagent and infeasible for clinical application. CD40L is a type II membrane glycoprotein and belongs to the TNF superfamily. The CD40L can interact with CD40 on DCs to induce DC maturation, reduce the antigen uptake ability of DCs, promote the secretion of IL-12 by DCs and enhance the ability of DCs to activate the proliferation of lymphocytes (7). MCM is a type of medium containing several cytokines secreted by mononuclear cells including TNF-α, IL-6 and IL-1β, and has reliable effectiveness in inducing DC maturation (8). However, MCM should be tailored to the individual patient in clinical use. In addition, the preparation of MCM requires complex procedures and needs the isolation of autogenous mononuclear cells. These significantly limit the wide application of MCM in DC maturation induction. TNF-α is a pro-inflammatory cytokine and is usually applied to induce DC maturation. In the cocktail method, the combination of cytokines aims to mimic the MCM in which TNF-α, IL-6, IL-1β and PGE_2 are used. This method was first reported by Jonuleit *et al* in 1997 (9). The cocktail method can stably and efficiently induce DC maturation. To date, CD40L, TNF-α and a cocktail of cytokines have been applied in clinical practice in several trials (10,11). In the present study, the effectiveness of CD40L, TNF-α, LPS and the cocktail of cytokines in inducing DC maturation was compared. Our results showed these methods induced DC maturation in which the cocktail of cytokines had the most potent ability to induce DC maturation. The positive expression rate of CD83 was >60% in DCs following treatment with the cocktail of cytokines, and the expression levels of CD80, CD83 and HLA-DR were also higher than those in the remaining groups. Our findings also demonstrated that the DCs following induction with the cocktail of cytokines had the highest IL-12 content and these cells had the most potent ability to stimulate the proliferation of lymphocytes.

Taken together, in the present study, PBMCs were employed to induce immature DCs in the presence of GM-CSF and IL-4, and these immature DCs underwent maturation induction using different methods. Our findings revealed that a cocktail of cytokines stably and efficiently induced DC maturation, induced the secretion of IL-12 by DCs and potently induced the DCs to stimulate lymphocyte proliferation. The cytokines in the cocktail method meets the criteria of the Good Manufacturing Practice (2012, Ministry of Health, China) (12) and the cocktail of cytokines is feasible for application in clinical practice.

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