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Phenotypic and functional characteristics of dendritic cells derived from human peripheral blood monocytes*

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Abstract: Objective: This study is aimed at developing a simple and easy way to generate dendritic cells (DCs) from human peripheral blood monocytes (PBMCs) in vitro. Methods: PBMCs were isolated directly from white blood cell rather than whole blood and purified by patching methods (collecting the attached cell and removing the suspension cell). DCs were then generated by culturing PBMCs for six days with 30 ng/ml recombinant human granulocyte-macrophage stimulating factor (rhGM-CSF) and 20 ng/ml recombinant human interleukin-4 (rhIL-4) in vitro. On the sixth day, TNF-alpha (TNFα) 30 ng/ml was added into some DC cultures, which were then incubated for two additional days. The morphology was monitored by light microscopy and transmission electronic microscopy, and the phenotypes were determined by flow cytometry. Autologous mixed leukocyte reactions (MLR) were used to characterize DC function after TNFα or lipopolysaccharide (LPS) stimulations for 24 h. Results: After six days of culture, the monocytes developed significant dendritic morphology and a portion of cells expressed CD1a, CD80 and CD86, features of DCs. TNFα treatment induced DCs maturation and up-regulation of CD80, CD86 and CD83. Autologous MLR demonstrated that these DCs possess potent T-cell stimulatory capacity. Conclusion: This study developed a simple and easy way to generate DCs from PBMCs exposed to rhGM-CSF and rhIL-4. The DCs produced by this method acquired morphologic and antigenic characteristics of DCs.

Key words: Dendritic cell, Monocyte, Phenotype, Flow cytometry
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INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APC) that are responsible for the activation of undifferentiated T cells and the generation of primary T-cell responses (Cella *et al*., 1997). The specific role of DCs is to capture, process and present antigens to T cells. Immunogenic and inflammatory signals are responsible for the migration of DCs from tissues to lymphoid organs where they initiate an immune response. These processes induce the maturation of DCs, causing a shift from a processing to a presenting stage.

Recently, DCs have shown promise for gene therapy treatments of tumor or immune diseases. However, its potential is limited because of the low number of DCs in the human body (Jenkins *et al*., 1998). Therefore, to meet the demands of clinical use of DCs, it is critical to find a way to proliferate DCs in vitro. Peripheral blood monocytes (PBMCs) are the precursor cells of DCs and can be differentiated into DCs by exposure to some pathogens or inflammatory factors (Zhou and Tedder, 1996). Monocytes are directly obtained from peripheral blood and seem to represent cell populations with high developmental flexibility towards DCs.

Although some studies have already established methods to generate DCs in vitro (Duperrier *et al*., 2000; Hashimoto *et al*., 1999; Matteo Rigolin *et al*., 1999), the purpose of this study was to modify and

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simplify these methods, and then characterize their phenotype and the functionality. Recombinant human granulocyte-macrophage stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) were used to stimulate the differentiation of monocytes in this study.

MATERIALS AND METHODS

Samples

Fifty milliliters of white blood cell was obtained from healthy donors from the local central blood bank (*n*=10, male 5, female 5), aged 10~45 year-old (Average age, 32.6).

Reagents

RPMI 1640 was purchased from Gibcol Company. Ficoll-Hypaque sedimentation gradients were purchased from Nycomed Pharma, AS; 10% (*V*/*V*) fetal bovine serum (FBS) was purchased from Hyclone Company. rhGM-CSF, rhIL-4 and rhTNFα were purchased from PerRo Tech. EC. Ltd., UK, lipopolysaccharide (LPS) from *E. coli*. 0111:B4, Sigma, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (anti-CD1a-FITC, anti-CD83-FITC, anti-CD86-PE, anti-CD80-FITC, anti-CD3-PE and anti-CD14-PE) from Catalag, Laboratories, USA, and mitomycin (40 μg/ml) was purchased from Zhengjiang Haimen Pharmacies.

Isolation of PBMCs and generation of DCs

In the present study, PBMCs were prepared following protocol that was simplified and modified from that previously reported (Bhardwaj *et al*., 1994; Romani *et al*., 1996). Briefly, PBMCs were isolated directly from white blood cell rather whole blood by sedimentation over Ficoll-Hypaque (1.077 g/ml) density gradient centrifugation. After the cell concentration was adjusted to 2×10^7 ml^{-1}, they were cultured in RMPI 1640 for 2 h, and then the suspension cells were gently washed away. The lymphocytes should be washed completely to increase the purity of DCs. The cells attached to the flask bottom were considered as PMBCs. After collection, the concentration of PMBCs was determined. DCs were prepared from PBMCs by culturing cells in 1 ml of RPMI 1640 supplemented with 10% FBS, antibiotics, L-glutamine, and in the presence of 30 ng/ml rhGM-CSF and 20 ng/ml rhIL-4 in an incubator with 5% (*V*/*V*) CO_2 and 37 °C for six days. Cultures were maintained at 37 °C in 5% CO_2 by replacing culture medium and cytokines on day three. To determine the influence of TNFα on DCs, cultures were stimulated on Day six with 30 ng/ml TNFα for 48 h (Rieser *et al*., 1997). The culture was monitored by light microscopy throughout the duration of incubation.

Electron microscopy

Cultured cells (sampled after six days of culture) were fixed in 2% (*w*/*V*) glutaraldehyde, postfixed in 1% (*w*/*V*) osmium tetroxide (Euromedex, Souffelweyersheim, France) and embedded in Epon 812 (Sigma). Ultra-thin sections were then cut, stained with uranyl acetate and lead citrate, and examined using an electron microscope (Philips CM10; Lyon 1 Microscopy Center, Lyon, France).

Flow cytometry

DCs on Day six or DCs treated with TNFα for two additional days were subjected to flow cytometry assays as previously described (Kunitani *et al*., 2002; Summerfield *et al*., 2003). In indirect assays, reactivity was detected using either FITC- or PE-conjugated antibodies. Intracellular staining was performed using the Cytofix/Cytoperm plus kit (PharMingen). Cells were analyzed using fluorescence-activated flow cytometry (EPICS XL, Coulter, USA) calibrated with Calibrate beads (Becton-Dickinson) for FITC and PE. The distribution of debris, dead cells and any contaminating red blood cells, was assessed on the basis of forward and right-angle scatter before proceeding with the analysis. A total of 10000 events were examined using 488-nm wavelength excitation.

Autologous mixed lymphocyte reaction (MLR)

To determine the functional activity of DCs, autologous MLR of DCs was assayed as previously described (Johansson *et al*., 2003; Paillot *et al*., 2001). Briefly, after DCs were isolated by centrifugation over a 14.5% metrizamide gradient, 1×10^6 ml^{-1} DCs were cultured with the medium containing TNFα 30 ng/ml or LPS 100 ng/ml respectively for 24 h before autologous MLR. Autologous T lymphocytes were purified with a nylon cotton column. After treating

with mitomycin for 30 min, DCs were mixed with 100 μl of T lymphocytes (1×10^6 ml^{-1}) in a U-bottom 96 well plate. The ratio of DCs:T cells was 1:10 and 1:100. The total volume was adjusted to 200 μl/well, and the cells were cultured for four additional days; 15 μl/well tetrazolium salt (MTT) (5 g/L) was added into the cells four hours before the ending of culture, then 150 μl DMSO was added into the medium just before the end of culture. Optical density (O.D.) was measured by Elx800 (Biotech, USA) at a wavelength of 570 nm.

Data analysis

Data are expressed as mean±*SE*. Parameters collected in autologous MLR were analyzed using one-way ANOVA test. $P<0.05$ was considered significant.

RESULTS

Morphologic analysis

In this study, identification of DCs relied on light microscopy, transmission electron microscopy and flow cytometry analysis. Initial studies showed that the appropriate concentration of mononuclear cells for DC proliferation is $1\sim2\times10^7$ ml^{-1}. A higher concentration of mononuclear cells increased loss of monocytes and decreased harvest rate. A lower concentration caused less clustering and inhibited DCs proliferation. The suspension cells should be gently washed away to avoid the loss of monocytes, but the lymphocytes should be washed away completely to increase the purity of DCs.

DCs were grown in RPMI 1640 in 10% FBS supplemented every three days with 30 ng/ml GM-CSF and 20 ng/ml IL-4. Under these conditions, cell clusters could be seen clearly two hours after the addition of cytokines. On Day two or Day three, many proliferation clusters began to adhere loosely to the bottom of the culture plate while irregular cells began to be suspended. Conversion of adherent monocytes to cells with typical DC morphology, i.e. great increase in size, irregular shape and multiple dendritic projections, could be seen on Day six by light microscopy (Fig.1). The average yield of DCs in this condition was $(8.2\pm0.9)\times10^6$ ml^{-1}, the percentage was 61.5%±5.7%.

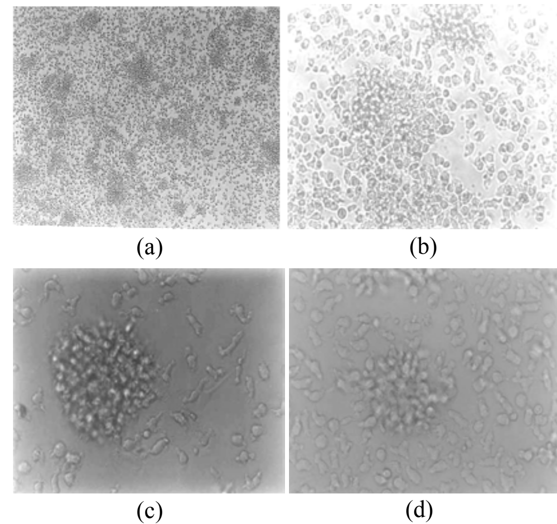

Fig.1 Morphological characteristics observed by phase contrast microscopy of PBMCs that were placed into culture medium (RPMI 1640) containing 10% FBS and 30 ng/ml GM-CSF and 20 ng/ml IL-4 for two hours (a), two days (b), three days (c) and six days (d). Cytokines were changed on the third day. Original magnifications were 10× in (a) and 40× in others

The morphologic characteristics of DCs collected on the sixth day were further studied by transmission electron microscope. The cells had abundant cytoplasmic projections with irregular nuclei. There were incisions on the nuclear membrane and many membrane apparatuses, such as endoplasm, Golgi complex, Birbeck-like granules (Grassi *et al*., 1998) and rare mitochondria (Fig.2).

Phenotype analysis of DCs

DCs collected on the sixth day were stained with monoclonal antibodies and then subjected to flow

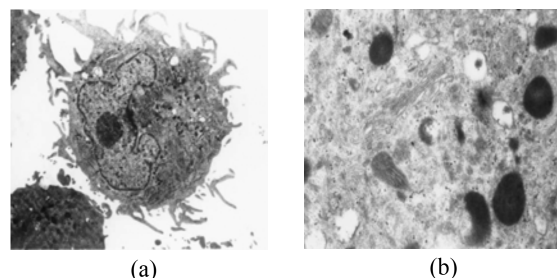

Fig.2 Morphologic characteristics of DCs in transmission electron microscope. Typical images show that the migrating cells have an oval nucleus and dense cytoplasm; many of the cells have very dark staining vesicles in the cytoplasm. DCs have a more complex structure of dendrites or veils and a more open and lobed nucleus. Original magnifications were 4000× in (a) and 25000× in (b)

cytometry analysis. More than 10 000 cells were analyzed each time. According to FS/SS (forward scattering/side scattering) gating, the cells were crowded together very well (Fig.3a), suggesting good homogeneity of DCs. In terms of FITC and PE negative controls (Fig.3b), the phenotype of DCs was CD1a+, CD80+, CD86+, CD83−, CD14+, CD56−, CD3− and CD19− (Fig.3c).

TNFα induced the maturation of DCs

DCs on the sixth day were stimulated by TNFα 30 ng/ml and cultured for 48 more hours and then subject to phenotype analysis. Flow cytometry analysis results showed that the expression of CD80, CD86 and CD83 by DCs increased significantly, and the CD83 expression had the greatest increase.

Functional activity of DCs assessed by autologous MLR

After stimulation by TNFα or LPS for 24 h, DCs were treated with mitomycin for 30 min and then mixed with autologous T lymphocyte with the ratio of 1:10 or 1:100. Compared to the T cell and DCs controls, autologous MLR of DCs was significantly augmented after TNFα as well as LPS stimulations (P<0.05, n=6, Fig.4), suggesting that DCs stimulated by TNFα and LPS could promote the proliferation of T lymphocytes.

DISCUSSION

This study developed a simple and easy way to generate DCs from PBMCs exposed to rhGM-CSF and rhIL-4. The DCs produced by this method acquired morphologic and antigenic characteristics of DCs. In addition, TNFα induced the maturation of DCs and the presence of TNFα or LPS during differentiation of DCs significantly stimulated T cell proliferation.

DCs are known to be the most potent APCs till date, and only APCs can stimulate an undifferentiated T lymphocyte. The importance of DCs in the regulation of immune responses is gradually receiving more attention with thorough studies on DCs. Although widely distributed, the number of DCs is rather low in

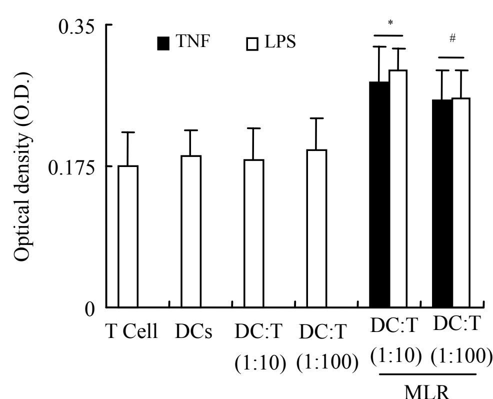

Fig.4 The functional activity of DCs was assayed by autologous MLR. 1×10^6 ml^{-1} DCs were cultured with the medium containing TNFα 30 ng/ml or LPS 100 ng/ml respectively for 24 h, then DCs were collected and autologous MLR was performed. The T cell, DC and DC:T mixtures that were not activated with LPS or TNF were considered as controls. * and # P <0.05 vs T cell control and DC control

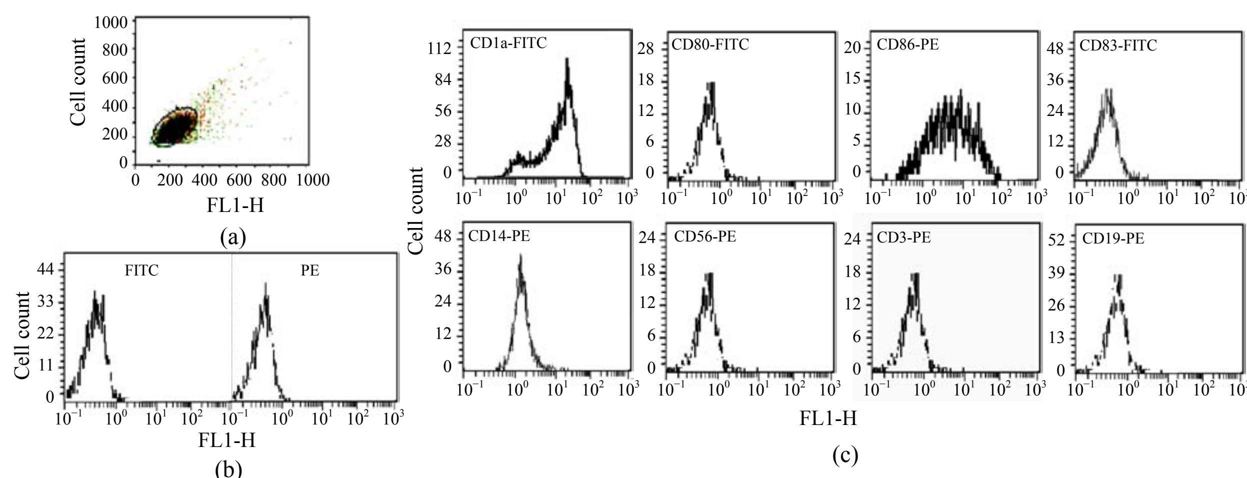

Fig.3 Phenotype of DCs by flow cytometry. Panel (a) shows the FS/SS characteristics of the DC population, this population was further evaluated for expression of single-cell surface markers; Panel (b) shows negative control of FITC staining and PE staining; Panel (c) shows representative single-cell surface-marker histograms from flow cytometric analysis of DCs on Day six

human. PBMCs, an aggregation of abundant immunological competent cells, such as T lymphocytes, natural killer cells and lymphokine activated killer cells, probably play an important role in immunological reactions.

PBMCs can be made to differentiate into DCs by the stimulation of some pathogens or inflammatory factors (Zhou and Tedder, 1996). Therefore, this study tried to establish a simplified and easy method to generate DCs in vitro and determine their immunobiological characteristics. Many studies have already established methods to generate DCs in vitro, although in most of these studies, the whole blood sample in which both red blood cell and platelets were wasted and discarded was used. On the other hand, monocytes were finally isolated by negative selection, using a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, anti-IgE antibodies and MACS microbeads coupled to the anti-hapten monoclonal antibody. These magnetically labelled cells were depleted by retaining them on a MACS column in the magnetic field of the MidiMACS, whereas monocytes were eluted from the column using several washes (Duperrier *et al*., 2000; Hashimoto *et al*., 1999; Matteo Rigolin *et al*., 1999). The procedure for isolation of PMBCs is very expensive. In the present study, we modified and simplified these methods. First, we isolated PBCs from white blood cells obtained by blood separation that did not waste RBCs and platelets; second, we purified monocytes by the simple patching method that is much cheaper than purification by magnetic beads. The yield of PBCs was 95% in the present study. We also found that the appropriate mononuclear cell concentration was 1×10^7~2×10^7 ml^{-1} for purifying monocytes and is important for the proliferation of DCs.

The morphologic characteristics of DCs were identified by phase contrast microscopy and transmission electron microscopy. After culturing for six days, DCs developed specific growth characteristics and morphologic features of dendritic cells. The abundant membrane-like apparatuses in the cytoplasm are essential for the function of DCs. It is especially important to note that the purity of DCs obtained by our method is very high. This is one of the advantages of PBMCs-derived DCs.

CD1a is the characteristic molecular marker on DCs derived from the monocyte and related to the antigen-presenting procedure of DCs. CD83 is the specific marker of mature DCs, CD14 is the marker of monocytes whose expression decreases gradually during DC differentiation from monocytes. CD80 and CD86 are important co-stimulating factors in the T lymphocyte proliferation effect of DCs (Bender *et al*., 1996; Duperrier *et al*., 2000; Ebner *et al*., 2001; Labeur *et al*., 1999). According to Steinman and colleagues, the phenotypic characteristics of the most mature DC population are complete loss of CD14 and increased expression of CD1a, MHC Class II receptor, CD80 and CD86 (Banchereau and Steinman, 1998). Therefore, DCs generated by our methods had low expression of CD14, moderate expression of CD80, CD1a and CD86, and thus, would be considered relatively immature DCs when compared to DC generated by other methods (Zhou and Tedder, 1996; Morse *et al*., 1998). Other reports have shown that on Day six the phenotype of DCs derived from PBMCs in vitro by stimulation of GM-CSF and IL-4 includes CD1a+, CD80+, CD86+, CD14+ (but at lower levels than in monocytes), CD83+/− and no expression of the specific molecular marker of T & B lymphocyte, non-killing cell (NK). Similar results were obtained in the present study. TNFα has been used to mimic inflammation or antigenic exposure occurring in vivo, and can cause maturation of DCs differentiated in vitro. With the additional stimulation of TNFα for two days, the DCs with immature phenotype will mature to CD83+, accompanied with increased CD80 and CD86 expressions, suggesting that TNFα could stimulate the maturation of DCs. DCs are specialized APCs that are responsible for the activation of undifferentiated T cells and the generation of primary T-cell responses (Cella *et al*., 1997). The functional activity of DCs was evaluated by autologous MLR. We found that DCs generated by this method were able to promote T cell proliferation after TNFα or LPS stimulations that mimic inflammation or antigenic exposure.

CONCLUSION

This study demonstrated the morphological characteristics and functional activity of PBMC-derived DCs generated in vitro. The present in vitro DC models will be particularly useful for studies on the

role of DC in the immune response against infectious agents, allow-antigens and xenon-antigens. The ability to generate DCs in vitro not only offers possibilities to study immunological responses to vaccines, but could also provide a relevant model for human vaccine research.

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