Analysis of Methods for the Generation of Dendritic Cells from Human Peripheral Blood Monocytes

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───── Abstract ──

Dendritic cells (DC) are highly efficient antigen-presenting cells that initiate the primary immune response. Several laboratories have developed culture systems for human DC from peripheral blood monocytes. Most of these studies have used fetal calf serum (FCS) containing culture conditions that are inappropriate for human application. GM-CSF and IL-4 were used to make immature DC. The monocyte-conditioned medium (MCM) was used to induce the final maturation of DC. Using the previously described methods, the quality of MCM has unpredictable variations. Therefore using a defined cocktail of growth factors for the generation of mature DC would be advantageous for experimental as well as clinical purposes. In this study, it is suggested that combinations of both GM-CSF/ IL-4 or GM-CSF/IL-13 could be used as the first-step culture to produce immature DC, and that cytokine cocktail (GM-CSF, IL-4, IL-1 β, TNF- α, IL-6, PGE$_2$) was as efficient as MCM for the second step-culture to produce fully maturated DC. Here, we have generated an easily reproducible culture system for DC that allows for the generation of large amounts of immature and mature DC, and we also now have established the method in a FCS-free system that is suitable for clinical use.

Key Words: Dendritic cell culture, maturation, cytokine cocktail, CD83 monocyte conditioned medium

INTRODUCTION

Dendritic cells (DC) are highly efficient professional antigen presenting cells (APCs) capable of stimulating resting T-cells in the primary immune response, and they are more potent APCs than peripheral blood monocytes or B cells. DC are also critically involved in autoimmune disease, graft rejection, and human immunodeficiency virus infection, as well as the stimulation of nave and memory B and T lymphocytes. They capture and process antigens in non-lymphoid tissues, then migrate to T-cell dependent areas of secondary lymphoid organs through afferent lymph or blood stream to prime nave T-cells and initiate the immune response. During this process, DC lose their Ag-capturing/processing ability as they differentiate into mature, fully stimulatory APCs.[1,2] Maturation is typically marked by the upregulation of MHCII, costimulatory molecules and dendritic cell-restricted molecules, CD83.[3]

Several laboratories have developed culture systems that allow for the generation of comparatively large numbers of DC from CD34+ stem cells of blood and bone marrow.[4,5] However, several problems still need to be addressed before the widespread clinical use of these cells will be feasible. First, almost all the protocols published have used culture conditions with xenologous sera such as fetal calf serum (FCS) which might be a problem in clinical use. Second, the frequency of CD34+ cells in human blood is very low, so this practice is less practical. Another alternative approach for DC culture is to use peripheral blood monocytes as starting cells. As the result, the two step culture system for mature DC generation from monocytes was established.[6-8] In the first step, monocytes were grown in granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 for 1 week to generate immature DC. In the second step, the so-called monocyte-conditioned medium (MCM) was used to induce the final maturation of DC. The major advantage of MCM is that it does not contain any foreign serum and can be obtained from

autologous human blood. However, the composition of peripheral blood mononuclear cells in the producing MCM varies considerably and the quality of MCM also has unpredictable variations.[9] Therefore, using a defined cocktail of growth factors for the generation of mature DC would be advantageous, but it has been reported that the cytokine cocktail was less effective than MCM in mediating the terminal maturation of DCs.[10]

In this study, we generated monocyte-derived DC according to the two-step culture system and we compared the effects of IL-13 and c-kit to make immature DC in the first step of culture and compared the effects of MCM and cytokine cocktail (GM-CSF, IL-4, IL-1β, TNF-α, IL-6, PGE$_2$) in the second step of culture system.

MATERIALS AND METHODS

Media and cytokines

Culture media were RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) or X-vivo (Bio-Whittaker, Walkersville, Maryland, USA), 2 μM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 5×10^{-5} M 2-β mercaptoethanol (Merk, Munchen, Germany), 1% autologous serum. The following human recombinant cytokines were used: 800 U/ml GM-CSF (Novartis, Frimley, UK) and 1,000 U/ml IL-4 (PBH, Hanover, Germany), 50 ng/ml IL-13 (Pharmingen, San Diego, CA, USA), 20 ng/ml c-kit ligand (Amogen Biologicals, Thousand Oaks, CA, USA), 1,000 U/ml IL-6 (PBH), 10 ng/ml IL-1β (PBH), 10 ng/ml TNF-α (PBH), 1 μg/ml prostaglandin E$_2$ (PGE$_2$) (Sigma Chemical Co., St. Louis, MO, USA).

Antibodies and antibody-coated magnetic beads

Primary antibodies used for flow cytometric analyses are listed in Table 1. Conjugated secondary reagents were FITC-conjugated anti-mouse Ig (Biosource International, Camarillo, CA, USA), PE-conjugated anti-mouse Ig (Amersham International, Amershams, UK). For the depletion of T and B cells in the peripheral blood mononuclear cell (PBMC) preparations, pan-T-CD2- and pan-B-CD19 Dynabeads (Dynal AS, Oslo, Norway) were used.

Generation of MCM

MCM was prepared as follows: 5×10^7 PBMC were incubated for 1h at 37°C in 10 ml complete culture medium on bacteriologic 10 ml plates coated with 10 mg/ml human IgG (Dong Shin Pharmacy, Seoul, Korea). Non-adherent cells were rinsed off and adherent monocytes were cultured at 37°C for 24h. Conditioned medium was then collected and frozen at -20°C until use.

Dendritic cell culture

a) **Preparation of PBMC**: Two-step centrifugation was done in order to minimize the number of contaminating platelets; After underlying 30 ml of diluted blood (20 ml PBS containing 5 U/ml heparin (20 μl) +2 mM EDTA and 10 ml blood) with 15 ml of Ficoll/Hypaque (density: 1.0777 g/ml) in a 50 ml tube, the sample was centrifuged for 20 min, 200 $\times$ g (1,200 rpm) at room temperature. The top 20 − 25 ml of plasma (with the most platelets) was carefully removed. The centrifugation was then continued for another 20 min. at room temperature at 400 $\times$ g (1,700 rpm). Interphases were harvested and washed five times (2,500 rpm, 10min) with cold PBS + 5 mM EDTA (PBS, 100 ml + EDTA, 0.1861 g).

b) **Preparation of plasma**: Using the removed plasma, plasma was heat-inactivated at 56°C for 30 min, then it was centrifuged at 1,000 $\times$ g (2,500 rpm) for 10 min. The platelet-free supernatant was used as autologous plasma.

c) **Monocyte separation by the depletion of T- and B-cells**: We used two different methods; one was immunomagnetic bead separation, another was cell attachment assay for the depletion of T- and B cells.

Immunomagnetic bead separation; Dynabeads Pan-B-CD19 and Pan T- CD2 were washed 4 times with PBS with 2% human serum albumin. PBMC (3 −5 $\times 10^7$/ml) were incubated with the magnetic beads at a ratio of 1 : 1 : 1 (PBMC : CD-2beads : CD-19-beads) at 4°C for 45 min. using a Dynal mixer (Dynal AS). Lymphocytes were then depleted in a Dynal magnet (Dynal AS). Residual non-adherent cells were used for culture.

Cell attachment assay; PBMC were attached to 6-well plates for 40 minutes. The supernatant and the floating cells were discarded and the attached cells

were used for culture.

d) **Culture and generation of DC from mono-cytes in two steps**

First step (culture of immature DC); Mono-cytes were plated in 6-well plates at a final concentration of 3×10^6 cells in 3 ml culture medium supplemented with 1% heat-inactivated autologous plasma, 800 U/ml GM-CSF and 1,000 U/ml IL-4. Cells were fed every other day (± 1 ml) with fresh complete medium containing 1,600 U/ml GM-CSF and 1,000 U/ml IL-4. Alternative cytokine combinations were used for the first-step culture: IL-4 was substituted by 50 ng/ml IL-13 or 20 ng/ml c-kit ligand was added to GM-CSF and IL-4.

Second step (culture of mature DC); At day 7, non-adherent cells were harvested from the wells, washed twice, and replated into new 6-well plates in complete culture medium containing 800 U/ml GM-CSF and 1,000 U/ml IL-4, then stimulated with various combinations of cytokine cocktails and MCM.

Morphologic observation of DC

The morphology of DC was observed by inverted microscopy, scanning electron microscopy and transmission electron microscopy during the culture periods.

Flow cytometry analysis

Cell staining was performed using the panel of the monoclonal antibodies (Table 1). Cells were incubated

Table 1. List of Primary Antibodies

Specificity	Clone/name	Ig class	Source
HLA-DR	L243	Mouse IgG2a	BD
CD1a	OKT-6	Mouse IgG1	Ortho
CD3	SK7	Mouse IgG1	BD
CD4	SK3, SK4	Mouse IgG1	BD
CD8	SK1	Mouse IgG1	BD
CD14	UCHM1	Mouse IgG2a	Ancell
CD19	4G7	Mouse IgG1	BD
CD45	RA3-6B2	RatIgG2a	PhaMingen
CD80	L307.4	Mouse IgG1	BD
CD83	HB-15A17.11	Mouse IgG2b	Serotec
CD86	BU63	Mouse IgG1	Ancell

Abbreviations: BD, Becton Dickinson, San Jose, CA, USA; Ortho, Raritan, NJ, USA; Ancell, Ancell Co., Bayport, MN, USA; Serotec, Serotec Ltd. Kidlington, England.

for 30 min at 4°C with MoAbs in PBS and 2% FCS. After washing, cells were restained with fluorescent (FITC, PE) conjugated secondary Abs for 30 min at 4°C. After washing, cells were resuspended in PBS, 1% paraformaldehyde and analyzed with a FACScan (Beckton Dickinson, Mountain View, CA, USA). Marker expression was evaluated as a percentage of positive cells in the relevant population defined by forward scatter/side scatter characteristics. Expression levels were evaluated by assessing mean fluorescence intensity (MFI) indexes calculated by relating the MFI noted with the relevant mAb to the control mAb fzor samples labeled in parallel and acquired using the same setting.

Allogeneic MLR

T cells (1×10^5/well), enriched using nylon wool columns as described, were stimulated with cytokine cocktail-induced mature DC. Cells were cultured in flat-bottom microtiter plates in a final volume of 2 μl/well in RPMI + 5% FCS for 4 days and additional 16h in the presence of [3H] thymidine (1 Ci/well = 37 kBq/well, Amersham, Braunschweig, Germany). [3H] thymidine incorporation was measured using a liquid scintillation counter (Beckman, Palo Alto, CA, USA).

Endocytosis assay

FITC-conjugated Dextran (Sigma) was added to the final concentration of 1mg/ml to the cultured DC and incubated for 60 minutes at 37°C or 4°C. After the incubation, DC were washed 3 times with ice-cooled PBS and analyzed by flow cytometry.

RESULTS

Surface molecules expression of monocytes according to the separation methods

The phenotypic expression of monocytes separated from PBMC by the depletion of T- and B-cells showed no significant differences according to the separation methods: cell attachment assay and immunomagnetic bead separation (Fig. 1). We preferred cell attachment assay because it was a simpler and less time-consuming method.
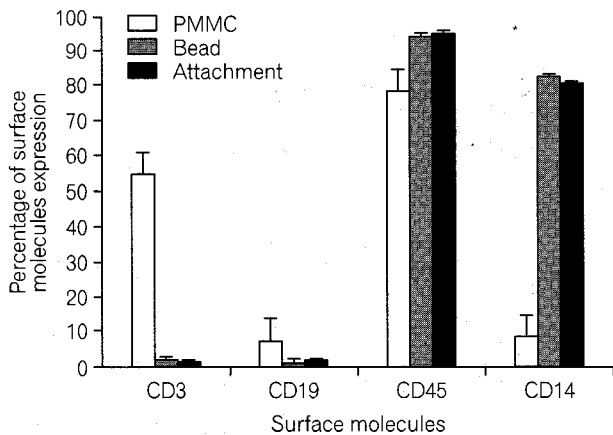
Fig. 1. The phenotypic characteristics of monocytes between separation methods: Monocytes were separated from peripheral blood mononuclear cells by two methods-cell attachment assay and immunomagnetic bead separation. There were no significant differences between the two methods (p <0.05 by t test). The error bars represent SD for 5 samples.

Surface molecules expression of DC according to the alternative cytokine combination in the first-step culture

In the first-step culture, three cytokine combinations were used for DC culture: GM-CSF, IL-4 were added to culture media. In another combination, IL-4 was substituted by IL-13, while in another combination, c-kit ligand was added to GM-CSF, IL-4. The expression of surface molecules (CD14, B7-2, CD83) among the 3 groups showed no significant differences (Fig. 2). After full maturation by MCM in the second-step culture, the expression of surface molecules showed no differences among the 3 groups (data not shown)

Surface molecules expression of DC according to the alternative cytokine combinations in the second-step culture

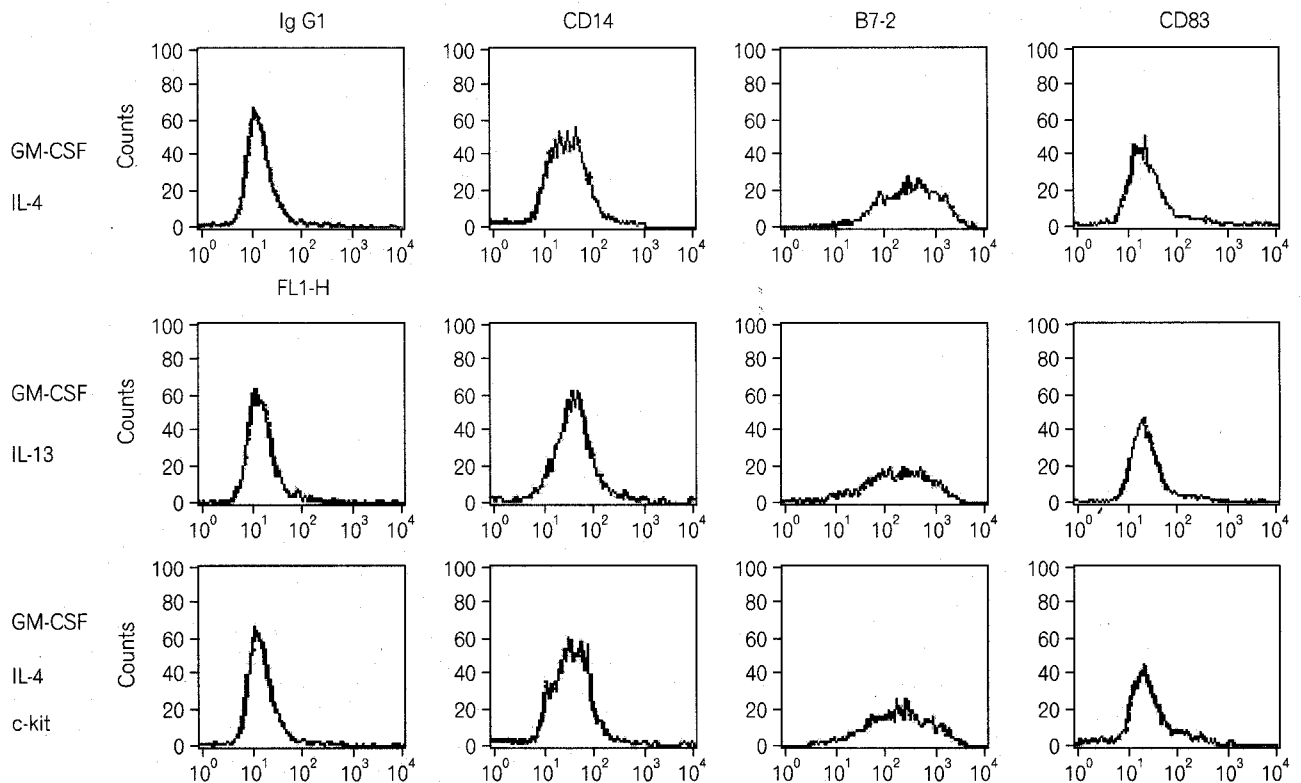In the second step culture, mature DC cultured by

Fig. 2. The characteristics of DC between alternative cytokine combinations in the first-step culture. In the first-step culture, there was no differences in the expression of surface molecules on culture day 7 among the various cytokine combinations.
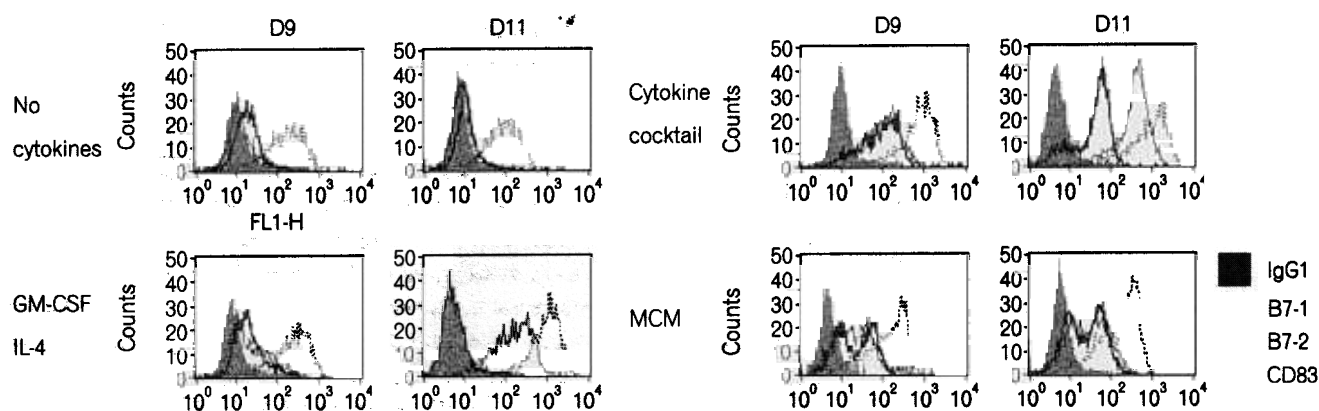
Fig. 3. The characteristics of DC between alternative cytokine combination in the second step culture. DC cultured by cytokine cocktail (GM-CSF, IL-4, IL-1β, TNF-α, IL-6, PGE2) in the second-step culture showed a higher expression of CD83, B7-1, and B7-2 compared to GM-CSF/IL-4 generated DC which expressed little or no expression of CD83. The maturation-specific marker CD83 expression showed no significant difference between MCM and cytokine cocktail induced mature DCs. The expression of surface molecules showed no difference between MCM and cytokine cocktail after 2 days of further culture without adding cytokines.
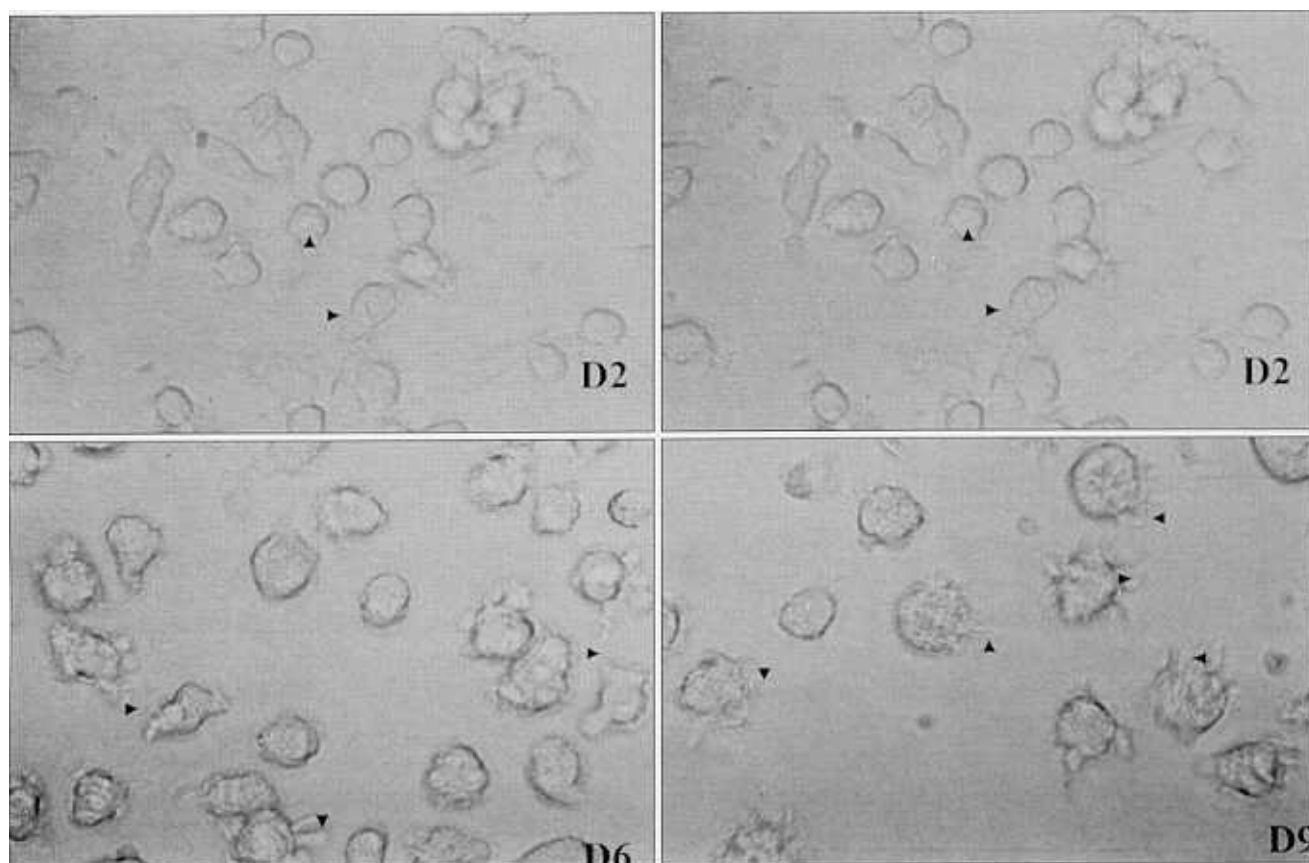

Fig. 4. The light microscopic feature of cultured DC. The dendritic processes (◄) were first seen on culture day 1−2; they have increased in size and number by day 6, and they were larger and more numerous after full maturation by day 9.
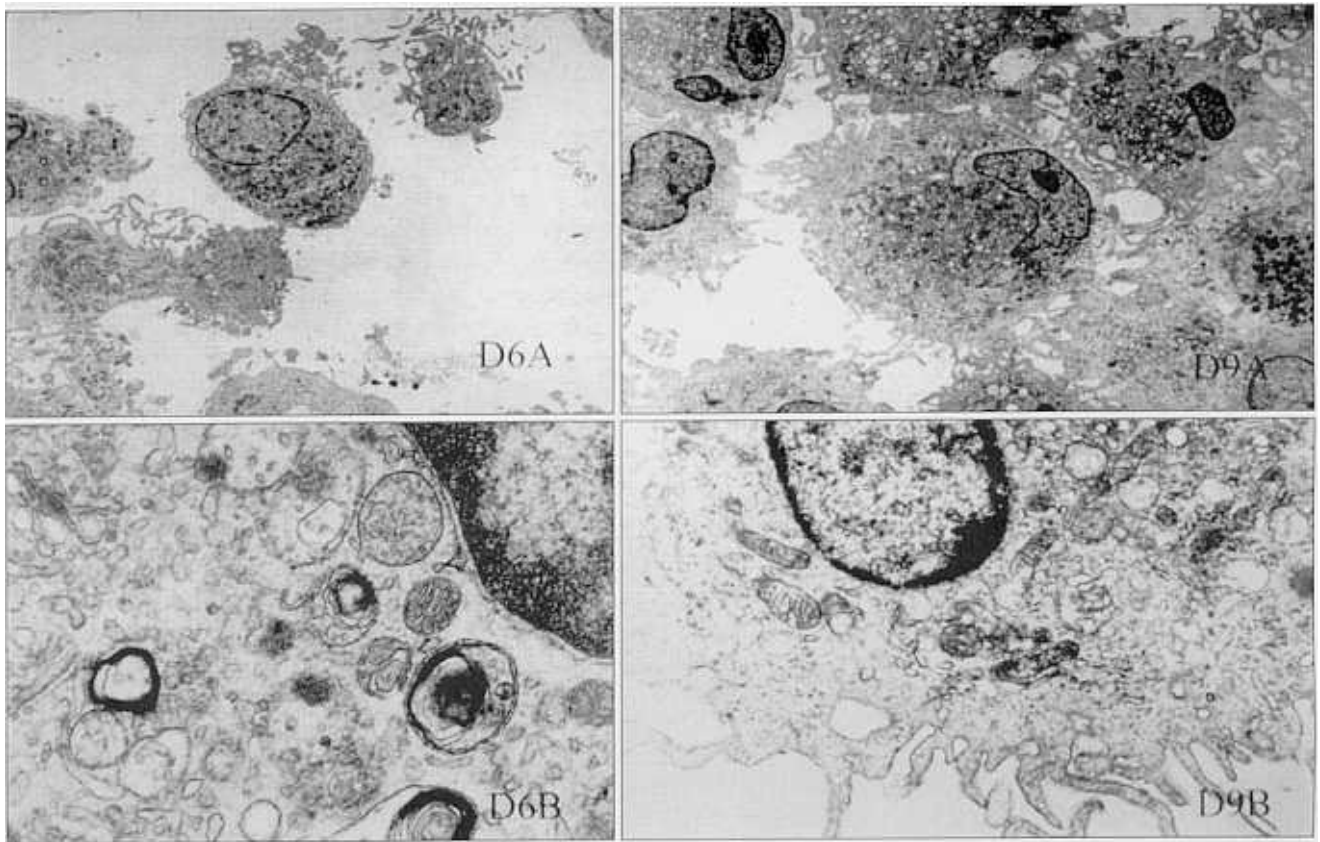
Fig. 5. The transmission electron microscopic feature of cultured DCs. The well-developed dendritic process was seen at culture day 6 (D6), The dendritic process was larger and more numerous after full maturation (D9). There were no Berbeck granule-like structures in the immature and mature DC (D6A; x4,400, D6B; ×57,000, D9A; ×3,300, D9B; ×17,800).

cytokine cocktail of GM-CSF, IL-4, IL-1 β, TNF-α, IL-6, and PGE$_2$ showed a higher expression of CD83, B7-1 and B7-2 compared to GM-CSF, IL-4 generated DC which expressed little or no expression of CD83. The CD83 expression between MCM and cytokine cocktail induced mature DC showed no significant difference. Both MCM and cytokine cocktail in the maturation of DC showed stable expression of CD83 for 2 days in a further culture without adding cytokines (Fig. 3).

Morphologic features of cultured DC

In inverted microscopic study, the dendritic process was first seen at culture day 1-2. It had increased in size and number at day 7, and it was larger and more numerous after full maturation at day 9 (Fig. 4). There were no morphologic differences between MCM and cytokine cocktail in the maturation of DC.

Scanning and transmission electron microscopic findings showed similar features of the dendritic process compared to inverted microscopic findings (data not shown). Transmission electron microscopy showed no Birbeck granule-like structures in the immature and mature DC (Fig. 5).

Characteristics of mature DC cultured by cytokine cocktail in the second step

Stability of cytokine cocktail induced mature DC: The stability of CD83 expression of cytokine cocktail inducing mature DC was evaluated in the cytokine depleted culture condition. The mature DC showed similar stable CD83 expression for 2 days in the cytokine depleted or contained medium (Fig. 6).

Allogeneic mixed lymphocyte reaction (MLR): In MLR, cytokine cocktail-induced mature DC were used as stimulators, and the stimulation index (SI)

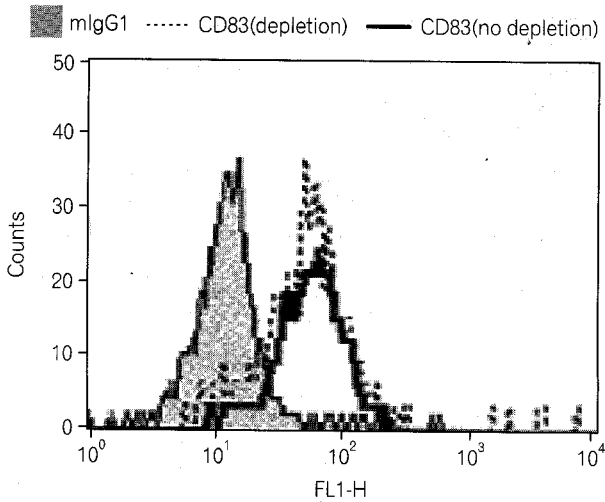**mIgG1**   ······· CD83(depletion)   ——— CD83(no depletion)

Fig. 6. *The stability of mature DC. Mature DC cultured with cytokine cocktail showed stable expression of CD83 for 2 days after cytokine cocktail depletion by washing the culture media on day 9.*
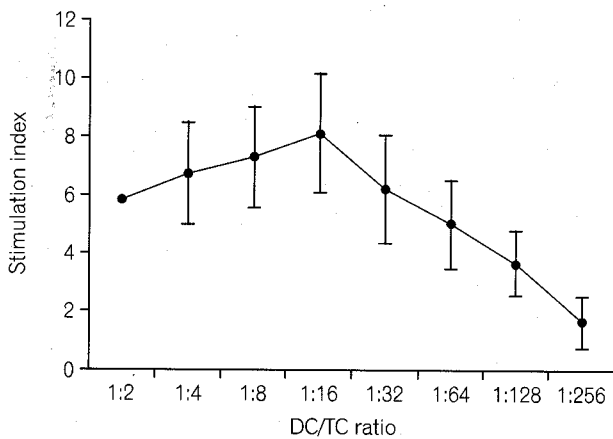

Fig. 7. *T cells were stimulated with cytokine cocktail-induced mature allogeneic DC. Cells were co-cultured for 4 days and the stimulation index was 5 or more. The error bar represents SD for 3 samples.*

$$SI = \frac{MLR \text{ at each } DC/TC \text{ ratio}}{MLR \text{ without stimulator cell}}$$

was calculated as follows.

$$SI = \frac{MLR \text{ at each } DC/TC \text{ ratio}}{MLR \text{ without stimulator cell}}$$

The stimulation index was 5 or more at the DC : TC ratio of 1 : 2, and peaked at the ratio of 1 : 16 (Fig. 7).

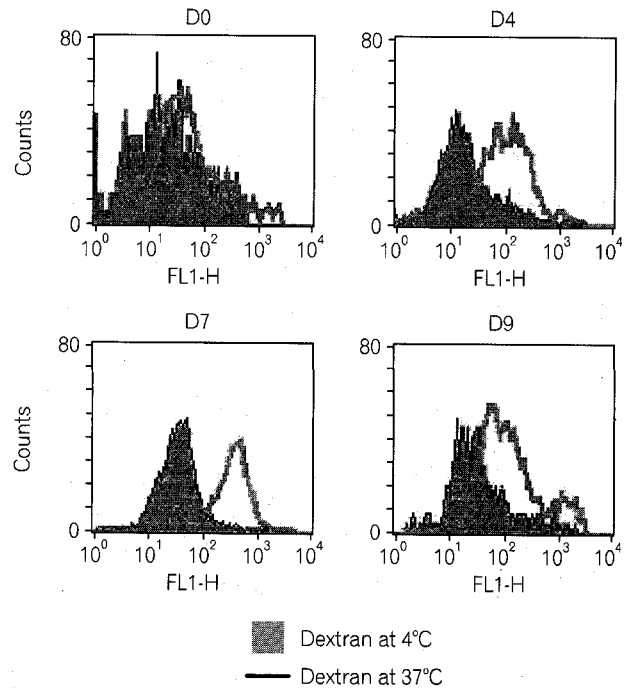**Dextran at 4°C**
——— Dextran at 37°C

Fig. 8. *The change of endocytic capacity during DC maturation. Endocytosis capacity of dextran was increased in immature DC (D7), and decreased after full maturation (D9).*

Endocytosis assay: Endocytosis assay was done by adding dextran to the DC at culture day 0, 2, 4, 7, 9. Endocytosis activity was increased at first-step culture, reaching a peak at day 7, then decreased after full maturation at day 9 (Fig. 8).

Influence of culture media on the expression surface molecules of DC

The culture medium RPMI 1,640 and X-VIVO15 influenced the expression of cell surface molecules in different fashions. There was a great difference in CD1a expression between culture media. Only DC cultured in RPMI 1,640 medium expressed CD1a, whereas there was no expression of CD1a in X-VIVO15. CD83 and B7-1 expression showed a slightly different pattern, but it was insignificant (data not shown).

DISCUSSION

The best characterized DC are epidermal Langerhans cells (LC).[11] They constitutively express MHC II

molecules and are defined by their dendritic morphology and the presence of a Birbeck granule, functionally specialized for antigen capture and processing.[11] During 2−3 days of LC culture, maturated LC undergo dramatic phenotypic and functional changes.[12,13] Their capacity for antigen uptake and processing is reduced, but their surface molecules expression of MHC II, B7-1, and B7-2 are increased.[14] Maturation is usually defined as the irreversible acquisition of several properties: dendritic morphology, non-adherence to plastic, upregulation of MHC and costimulatory molecule, and the expression of the DC-restricted molecule, CD83.

Many of the changes described appear to be dependent on the influence of cytokines. In the epidermis, keratinocytes are the principal cells that provide the necessary microenvironment to induce differentiation and migration (maturation) of LC by producing cytokines.[15] Most of these cytokines are not constitutively expressed in vivo, but can be induced by a variety of stimuli including inflammatory agents, ultraviolet (UV) irradiation, tumor-promoting agents and several types of injury.[16-18]

A major advance over the past few years has been the development of methods for growing DC from progenitors. In humans, the cooperation between GM-CSF and TNF-α is crucial for the generation of DC from CD34+ progenitors in cord blood and adult bone marrow.[4,5] Since CD34+ stem cells are rare in normal bone marrow and peripheral human blood, it is more desirable to generate DC from CD34− peripheral blood precursors. Generation of human DC from PBMC seems to be dependent on the combination of GM-CSF and IL-4.[8] GM-CSF induces the formation of small DC progenitor aggregates in human blood,[19] whereas IL-4 inhibits the formation of macrophage clones and allows extensive DC growth and maturation.[20] In the first step culture of this study, IL-4 was substituted by IL-13 and c-kit ligand was added to GM-CSF and IL-4. There were no differences of surface molecule expression according to the alternative cytokine combinations.

In the FCS-containing culture system, CD14+ PBMC can be differentiated to CD83+ DC in the presence of GM-CSF, IL-4, and TNF-α.[21] However, these studies using FCS-containing culture conditions were inappropriate for clinical application. Culturing lymphocyte-depleted PBMC using GM-CSF and IL-4 in FCS-free conditions for 7-days resulted in the generation of DC precursors with high levels of MHC II molecules. Yet these cells were immature, lacked the DC-specific marker CD83 and were unstable on the withdrawal of cytokines, resulting macrophage like cells. In FCS-free culture condition MCM has been used to induce full maturation of DC in the GM-CSF and IL-4 cultured immature DC. As MCM is a rather undefined cocktail, the yield and quality of DC generated with MCM varies substantially. Therefore, defined cocktail has been used in the maturation of DC. The possible responsible factors in the MCM-induced DC maturation were TNF-α, IL-1 β, IL-6, and INF-α, because these could be partially substituted for MCM-induced DC maturation. Inflammatory signals including TNF-α, IL-1, IL-6 and LPS were known to induce the maturation of DC in vivo and in vitro. Thus, pro-inflammatory cytokines seem to be of vital importance for the maturation of DC. It was reported that cytokine cocktail was less effective than MCM to make mature DC.[10] But, our data demonstrated that under FCS-free conditions, the pro-inflammatory cytokine cocktail was as efficient as MCM in the final maturation of the immature DC to the mature immunostimulatory DC.

From the results, it is suggested that the combinations of both GM-CSF/IL-4 or GM-CSF/IL-13 could be used as the first-step DC culture, and that cytokine cocktail of GM-CSF, IL-4, IL-1 β, TNF-α, IL-6, PGE$_2$ was as efficient as MCM for the second step-culture to produce the fully mature DC. Here, we have generated an easily reproducible culture system for DC that allows for the generation of large amounts of immature and mature DC, and we have now established the method in a FCS-free system that is suitable for clinical use.

ACKNOWLEDGMENTS

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