

Mature dendritic cells generated from patient-derived peripheral blood monocytes in one-step culture using streptococcal preparation OK-432 exert an enhanced antigen-presenting capacity

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Abstract. Dendritic cells (DCs) have been shown to be potent in inducing cytotoxic T cell (CTL) response leading to the efficient anti-tumor effect in active immunotherapy. Myeloid DCs are conventionally generated from human peripheral blood monocytes in the presence of interleukin (IL)-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF). Streptococcal preparation OK-432, which is known to be a multiple cytokine inducer, has been extensively studied as to its maturation effects on immature DCs using an *in vitro* culture system. The purpose of this study was to examine whether it could be possible to generate mature DCs directly from peripheral monocytes using OK-432. We specifically focused on the possibility that recombinant cytokines, which are considered to be essential for *in vitro* DC generation, could be substituted by OK-432. Human peripheral monocytes, which were obtained from patients with advanced cancer, were cultured with IL-4 and OK-432 for 7 days. Cultured cells were compared with DCs generated in the presence of IL-4 and GM-CSF with or without OK-432 with regard to the surface phenotype as well as the antigen-presenting capacity. As a result, the culture of monocytes in the presence of IL-4 followed by the addition of OK-432 on day 4 (IL-4/OK-DC) induced cells with a fully mature DC phenotype. Functional assays also demonstrated that IL-4/OK-DCs had a strong antigen-presenting capacity determined by their enhanced antigen-specific CTL response

and exerted a Th1-type T cell response which is critical for the induction of anti-tumor response. In conclusion, human peripheral blood monocytes cultured in the presence of IL-4 and OK-432 without exogenous GM-CSF demonstrated a fully mature DC phenotype and strong antigen-presenting capacity. This one-step culture protocol allows us to generate fully mature DCs directly from monocytes in 7 days and thus, this protocol can be applicable for DC-based anti-tumor immunotherapy.

Introduction

One major aim of active cancer immunotherapy is the successful induction of anti-tumor cytotoxic T lymphocyte (CTL) responses resulting in the prolongation of survival of patients with cancer. The dendritic cell (DC) is the most potent antigen-presenting cell (APC) that can initiate the primary T cell immune response (1) and is considered to be one of the most promising adjuvants for cancer vaccine (2). DCs can be derived from multiple sources such as the CD34⁺ bone marrow cells, cord blood stem cells, peripheral blood precursor cells (3-8), and non-proliferative CD14⁺ blood monocytes (9-11), and can also be generated *in vitro* using different cytokine combinations including granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor- α (TNF- α), interleukin (IL)-4, Flt3 ligand (Flt3L), CD40 ligand (CD40L) and IL-3. Among these cell sources, peripheral blood monocytes have been widely used for DC generation in clinical trials utilizing the cell culture system with GM-CSF and IL-4 because of its accessibility (12). To conduct an efficient DC-based active immunotherapy study, the protocol needs to be well designed in terms of the cell culture conditions as well as the maturation status of DCs. Mature DCs have the potential to induce strong anti-tumor CTL responses *in vivo* and are thus considered to be a suitable adjuvant for tumor vaccine (2). Indeed, the administration of immature DCs that were generated from monocytes with GM-CSF and IL-4 did not lead to marked immune responses, but in contrast, the administration of mature DCs in the same patient generated the desired response (13).

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Therefore, it will be very important to establish an optimal protocol to generate mature DCs that can lead to effective anti-tumor CTL responses and no induction of regulatory T cells, which have immune-suppressive effects. Several inducers for DC maturation, such as TNF- α , lipopolysaccharide (LPS), CD40L, poly I:C (a synthetic double-stranded RNA), interferon- α (IFN- α), and conditioned medium of adherent autologous monocytes in culture, termed monocyte-conditioned medium (MCM), have been reported so far (14-18). As for monocyte-derived DCs, the combination of IL-1 β , IL-6, TNF- α and prostaglandin E₂ (PGE₂) as a maturation stimulus is considered to be a current gold standard (19). However, the use of many recombinant cytokines in the DC generation cultures may pose a potential hazard because of the possibilities of the increased chance of contamination and also the labor-intensiveness.

Recently, we have demonstrated that OK-432 (Picibanil, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), a penicillin-inactivated, lyophilized preparation of an avirulent human strain of *Streptococcus pyogenes* (group A), stimulates monocyte-derived immature DCs to acquire a mature phenotype and to produce significant amounts of T helper 1 (Th1)-type cytokines, such as IL-12 and IFN- α (20). OK-432 is an immunomodulatory agent that has potential therapeutic properties for use in cancer treatment as a biological response modifier (BRM) and is available in GMP quality in Japan (21,22). Previous reports have indicated that OK-432 activates human peripheral blood mononuclear cells (PBMCs) and causes them to induce multiple cytokines, including IL-1, IL-2, IL-6, TNF- α , IFN- α , GM-CSF and IL-12 (23-28), some of which are important for differentiation as well as the maturation of DCs.

In the current study, we hypothesized that it may be possible to allow monocytes to differentiate into DCs and to induce the maturation of DCs in the same culture by utilizing the endogenous cytokines produced by the monocytes themselves in the culture under the presence of OK-432 without exogenous help of some of the recombinant cytokines that are generally required. Herein, we developed a novel method to generate DCs with fully matured phenotypes by the use of OK-432 and IL-4 without GM-CSF in a 7-day culture *in vitro* directly from human peripheral blood monocytes. We also examined whether these DCs could promote Th1-type immune responses and the efficient CTL responses against peptide antigens that are critical to induce an effective anti-tumor response.

Materials and methods

Peripheral blood and isolation of monocytes. Buffy coats derived from metastatic cancer patients participated in a clinical trial for DC-based active immunotherapy (11 colorectal, 2 gastric, 5 lung cancer) were obtained and a part of them were used in this study under written informed consent (29). PBMCs were separated by density centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Sweden). The PBMCs obtained were cryopreserved in a freezing medium, CP-1 (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) mixed with an equal volume of RPMI-1640 (Nikken, Kyoto, Japan), at a concentration of 5×10^7 cells/ml until use. CD14⁺ monocytes were isolated from PBMCs with anti-CD14 microbeads (Miltenyi Biotech, Sunnyvale, CA) according to the

manufacturer's instructions. The purities of the CD14⁺ cells determined by flow cytometry were constantly >95%.

Generation of DCs. Monocytes were seeded into 6-well plates at a density of 5×10^5 cells/ml in 3 ml AIM-V (Life Technologies, Inc., Grand Island, NY) containing 2% human AB serum and 25 ng/ml IL-4 (Peprotech, London, UK) with 50 ng/ml GM-CSF (Peprotech). Culture plates were incubated in a 5% CO₂ incubator at 37°C, and an additional 3 ml of medium containing cytokines was added to the culture on day 4. After 7 days of culture, non-adherent and loosely adherent cells were harvested from the plates and referred to as GM/IL-4/DC. OK-432 was used in some experiments to activate GM/IL-4/DC as described previously (20). Briefly, OK-432 (0.1 KE/ml) was added to monocyte culture, initiated under the presence of IL-4 and GM-CSF, on day 4. Stimulated DCs were harvested on day 7 and referred to as GM/IL-4/OK-DC. Monocytes were also cultured only with either IL-4 (IL-4/OK/DC) or GM-CSF (GM/OK-DC) initially, and then stimulated with OK-432 (0.1 KE/ml) on various days between day 0 and day 7.

Flow cytometric analysis. Phenotypic analysis of cells was carried out by flow cytometry. Cells were incubated with fluorescence-conjugated mAbs in PBS containing 1% BSA for 30 min at 4°C. FITC-conjugated mAbs specific for CD14, CD40, CD80, CD83, CD86 and CD1a (BD PharMingen, San Diego, CA) for cell staining, and isotype-matched immunoglobulins for negative controls were used. Anti-Su streptococcus antibody (Su strain) (anti-Su antibody) (Chugai Pharmaceutical Co.), which is a polyclonal antibody that recognizes OK-432, derived from the sera of the rabbits immunized with streptococcus pyogenes with Freund's complete adjuvant, was used to measure the density of OK-432 components on the surface of DCs. Cells were incubated with anti-Su antibody as a primary antibody for 2 h at 4°C and then with the secondary antibody, FITC-conjugated swine anti-rabbit immunoglobulin for 30 min at 4°C. Analyses were performed with a FACSCalibur (Becton Dickinson) using the CellQuest software.

Allogeneic mixed lymphocyte reaction. To evaluate the immuno-stimulatory function of the DCs, allogeneic mixed lymphocyte reaction (allo-MLR) was performed. Irradiated DCs (25 Gy) were cultured with 1×10^5 allogeneic T cells in 200 μ l AIM-V containing 2% heat-inactivated human AB serum for 6 days in 96-well flat-bottomed plates, and during the final 16 h each well was pulsed with 1 μ Ci of [³H]-thymidine. The radioactivity incorporated into the DNA, which correlates with cell proliferation, was measured by scintillation counting using a TopCount scintillation counter (Packard Instruments, Meriden, CT).

Cytokine production assay. The concentrations of cytokines in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using the kits for IL-1 β , IL-6, IL-10, IL-12 p70, IFN- α , GM-CSF and TNF- α (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

The detection of intracellular cytokines in T cells primed with autologous DCs and stimulated with mitogens. T cells were

co-cultured with DCs in a 24-well culture plate at a 10:1 ratio (1×10^6 T cells; 1×10^5 DCs) for 5 days in AIM-V containing 5% heat-inactivated human AB serum. Thereafter, CD4⁺ T cells were harvested using anti-CD4 microbeads (Miltenyi Biotech) and stimulated with 25 ng/ml of PMA and 1 μ g/ml of ionomycin for 48 h. The supernatants were harvested and the concentrations of cytokines (IFN- γ and IL-4) were measured by ELISA. T cells, which were activated with PMA and ionomycin in the presence of Golgistop for the final 4 h, were subjected to intracellular cytokine staining. Cells were permeabilized using a Cytotfix Cytoperm kit (BD PharMingen) and stained with PE-conjugated anti-IL-4 mAb and FITC-conjugated anti-IFN- γ mAb.

Induction of antigen-specific T cells using peptide-pulsed DCs. HLA-A24-restricted Flu peptide (RFYIQMCTEL) (kindly provided by Dr K. Takesako, Takara Bio., Shiga, Japan) derived from influenza nucleoprotein was used as a model antigen for CTL induction. Generated DCs obtained from a HLA-A24⁺ donor determined by FACS using anti-HLA-A24 mAb were pulsed with 40 μ g/ml peptide in the presence of 3 μ g/ml β_2 -microglobulin for 4 h at 37°C. These peptide-pulsed DCs were irradiated (25 Gy) and co-cultured in a 24-well plate with autologous T cells, obtained by positive selection with anti-CD3 microbeads (Miltenyi Biotech). Each well contained 1×10^5 peptide-pulsed DCs and 1×10^6 T cells in 2 ml AIM-V with 5% heat-inactivated human AB serum and 10 ng/ml IL-7. After 2 days of culture, 20 units/ml IL-2 was added to the culture medium. On day 7, the cultures were re-stimulated with autologous peptide-pulsed DCs, and IL-2 was added on day 9. Cells were harvested on day 14 for CTL assay. Antigen-specific cytotoxic assays were determined by the detection of IFN- γ production by T cells upon antigen-stimulation using an IFN- γ ELISA kit. Briefly, the T cells were stimulated with TISI cells (HLA-A24⁺; provided by Dr K. Takesako, Takara Bio.) pulsed with or without Flu peptide at effector:target ratio of 10:1. Forty-eight hours later, the supernatants were harvested, and the concentration of IFN- γ was measured.

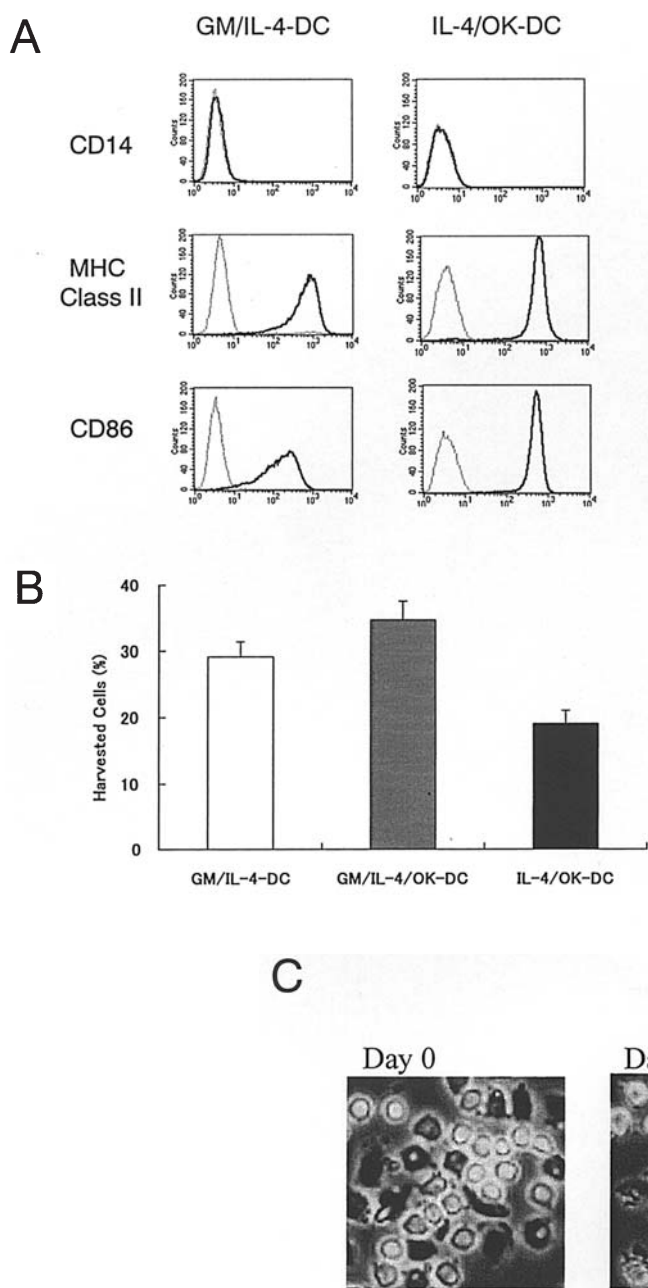


Figure 1. Phenotypic and morphological change and yields of DCs grown in the presence of IL-4 and OK-432 without exogenous GM-CSF. (A) Phenotypic analysis with regard to the expressions of surface markers, including CD14, CD86 and MHC class II molecules. The phenotype expression pattern of IL-4/OK-DC was identical to that of GM/IL-4-DC. (B) Yields of GM/IL-4-DC, GM/IL-4/OK-DC and IL-4/OK-DC. The numbers of monocytes plated on day 0 was set equal to 100%. Percentages indicate the numbers of recovered cells on day 7. (C) Peripheral blood monocytes (left) were cultured solely with IL-4 for 4 days (middle), followed by a further 3 days culture in the presence of OK-432 (right). Magnification $\times 200$.

Results

Cells generated in the presence of IL-4 and OK-432 demonstrate the characteristics of monocyte-derived DCs in terms of the phenotypic expression and morphology. First, we tried to address whether it could be possible to generate DCs using OK-432 instead of recombinant cytokines including IL-4 and GM-CSF. Human peripheral blood monocytes were cultured in the presence of either IL-4 (IL-4/OK-DC) or GM-CSF (GM/OK-DC) on day 0 and followed by the addition of OK-432 on various days. DCs induced in the presence of both IL-4 and GM-CSF (GM/IL-4-DC) on day 0 were used as the control for conventional monocyte-derived DCs. Cells were harvested on day 7 and phenotypic analysis was performed with regard to the expressions of surface markers including CD14, CD86 and MHC class II molecules to see if generated cells meet the requirements to be identified as DC. As shown in Fig. 1A, the phenotype expression pattern of monocytes

initially cultured with IL-4 on day 0 and then stimulated with OK-432 on day 4 (IL-4/OK-DC) was identical to that of GM/IL-4-DC, although GM/OK-DCs did not comply with the phenotypic features of a typical population of monocyte-

derived DCs due to the higher yield of CD14⁺ cells (data not shown). When OK-432 was added between day 0 and day 3, the yields of DCs on day 7 strikingly decreased because of the apparent cell death possibly induced by the cytotoxicity of OK-432 itself, or by over stimulation by OK-432 and exogenous cytokines at the same time just after the thawing of the cells (data not shown). The addition of OK-432 on day 4 resulted in up-regulation of the expression of CD86 at maximum level as compared to day 5 or later (data not shown). The purities of the DCs determined by the surface phenotype as well as the macroscopic findings both of GM/IL-4-DC and IL-4/OK-DC were as >90%, although the cell yields of IL-4/OK-DC measured with the trypan blue staining test were slightly lower than GM/IL-4-DC (Fig. 1B). The overall cell yield of IL-4/OK-DC and GM/IL-4-DC (the number of DCs/the number of PBMCs used for CD14⁺ cells purification) were 2-3% and 3-5%, respectively. Morphologically, monocytes cultured in the presence of IL-4 alone for 4 days showed a faintly veiled structure, and they came to appear as loosely adherent clumps with some isolated floating large cells having prominent dendrites and small clusters after an additional 3 days of culture in the presence of OK-432 (Fig. 1C). Taken together, IL-4/OK-DCs obtained by day 7 after the addition of OK-432 on day 4 seem to differentiate into cells that meet the requirements, the loss of CD14 and the up-regulation of MHC class II, to be identified as dendritic cells, thus the addition of GM-CSF can be substituted by OK-432 for the generation of monocyte-derived dendritic cells.

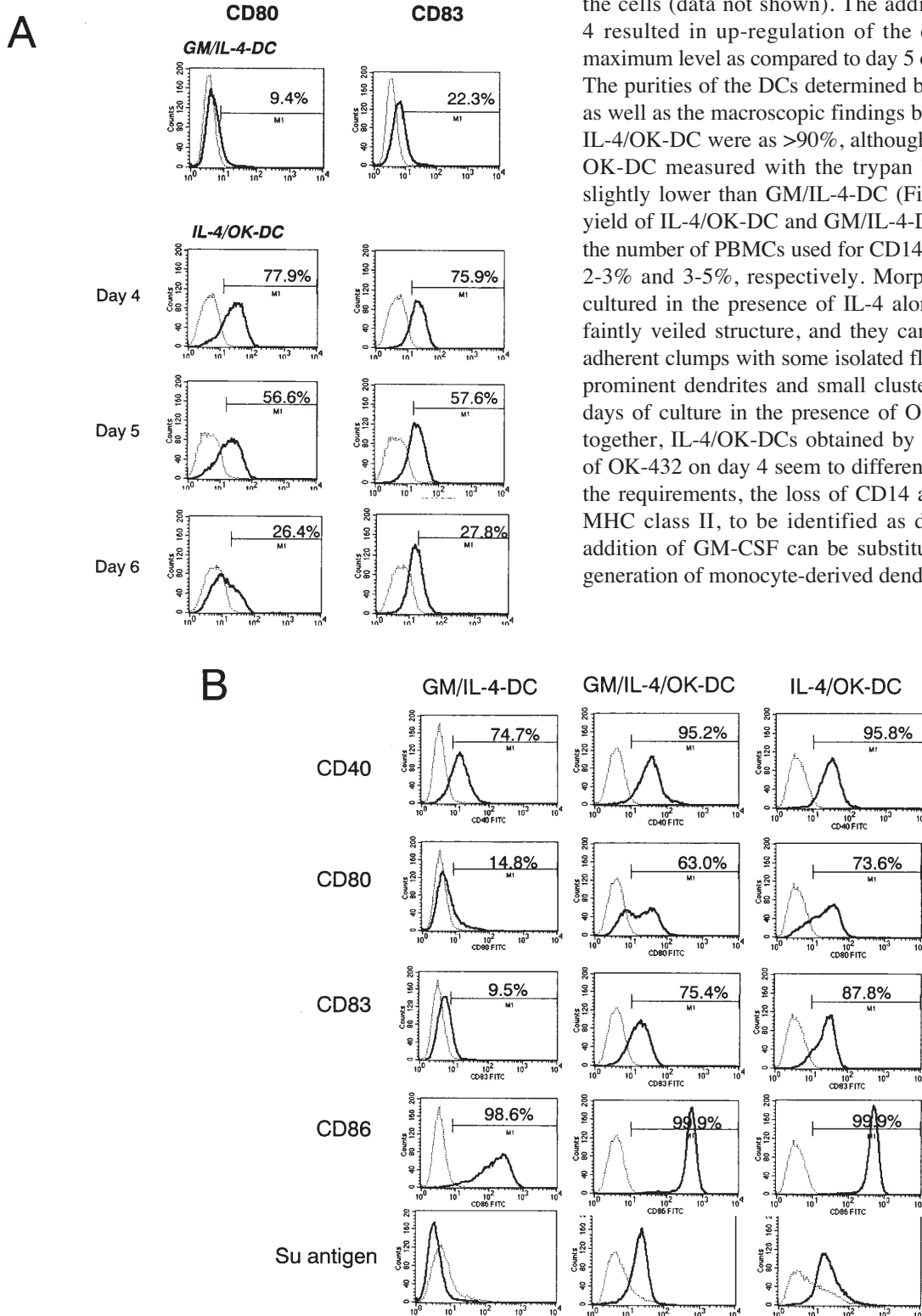


Figure 2. (A) Kinetics of the expression of costimulatory molecules on the cell surface of IL-4/OK-DCs stimulated with OK-432 (0.1 KE/ml) on days 4, 5 and 6. DCs were stained with FITC-conjugated anti-CD80 and anti-CD83 mAbs and cell surface expressions were analyzed by FACS. Data shown are from two separate representative experiments. The phenotype of GM/IL-4-immature DCs is shown as the control. (B) Comparative cytofluorographic analysis of GM/IL-4-DC, GM/IL-4/OK-DC and IL-4/OK-DC. Large cells (i.e., DC) were gated. The fluorescence of gated cells is depicted in the histograms. The thin line in each panel represents staining with isotype-matched irrelevant control Igs. Each row of histograms represents FACS profiles of one individual side-by-side comparative experiment.

Table I. Cell surface expression of DCs (MFI).

	CD80	CD83	CD86
GM/IL-4-DC	2.37±0.72	3.08±1.05	253.72±26.89
GM/IL-4/OK-DC	16.29±2.17 ^a	12.48±0.84 ^a	346.35±40.30
IL-4/OK-DC	12.18±3.69 ^a	12.98±3.12 ^a	337.03±53.86

^aP<0.05 compared with GM/IL-4-DC.

Similar maturation effects shown in GM/IL-4/OK-DC and IL-4/OK-DC. In a previous study, we observed that mature DCs could be effectively induced by OK-432 (20) and they were identical to cells referred to as GM/IL-4/OK-DC in the current study. We have also demonstrated that GM/IL-4/OK-DC was phenotypically and functionally mature and thus

comparable to mature DCs induced with the use of the combination of recombinant cytokines. Consistent with these data, the results from phenotypic analysis shown in Fig. 1A indicated that IL-4/OK-DC revealed a higher mean fluorescence intensity of CD86 as compared to GM/IL-4-DC, suggesting that IL-4/OK-DC possesses a mature phenotype. Here, we tried to confirm if this maturation effect could also be detected in the different surface markers including CD80, CD83 and CD40. Anti-Su antigen Ab was also used to determine the internalization of OK-432 and the expression of its antigenic legion on the cell surface. OK-432 was added on various days between 4 and 6 after the initiation of culture in the presence of IL-4 alone. Fig. 2A shows that the expressions of CD80 and CD83 were maximally up-regulated when OK-432 was added to the culture on day 4. In this optimized culture condition, maturation markers became similarly up-regulated in both GM/IL-4/OK-DC and IL-4/OK-DC as compared to GM/IL-4-DC with regard to the % positive cells (Fig. 2B), as well as mean fluorescence intensity (Table I).

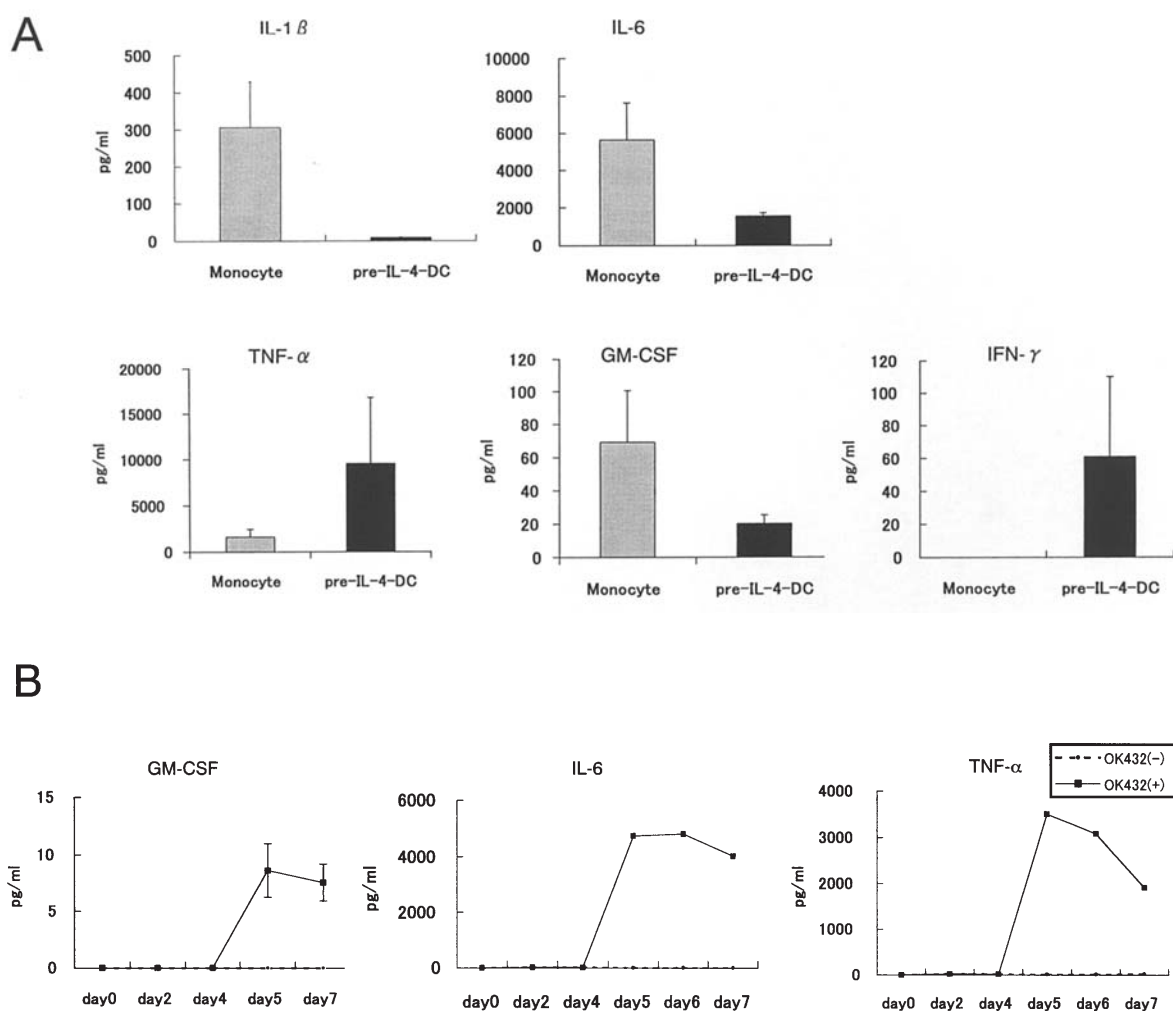


Figure 3. (A) Cytokine secretion by pre-DCs and monocytes. Pre-DCs were induced in the presence IL-4 only (pre-IL-4-DC) for 4 days and harvested. These pre-DCs and monocytes were plated at 2×10^5 cells/ml in the presence or absence of OK-432 (0.1 KE/ml) for another 48 h, and culture supernatants were assayed for IFN- γ , GM-CSF, TNF- α , IL-1 β and IL-6. Each sample was tested in duplicate and the data are shown as the mean \pm SD of 3 independent experiments. (B) Changes of concentrations in culture supernatants of GM-CSF, TNF- α and IL-6 were measured. Monocytes were seeded at a density of 5×10^5 cells/ml on day 0 in the presence of IL-4 alone, followed by the addition of OK-432 on day 4. In culture of IL-4/OK-DC, a sizeable amount of GM-CSF, IL-6 and TNF- α emerged in the culture supernatants after the addition of OK-432 on day 4.

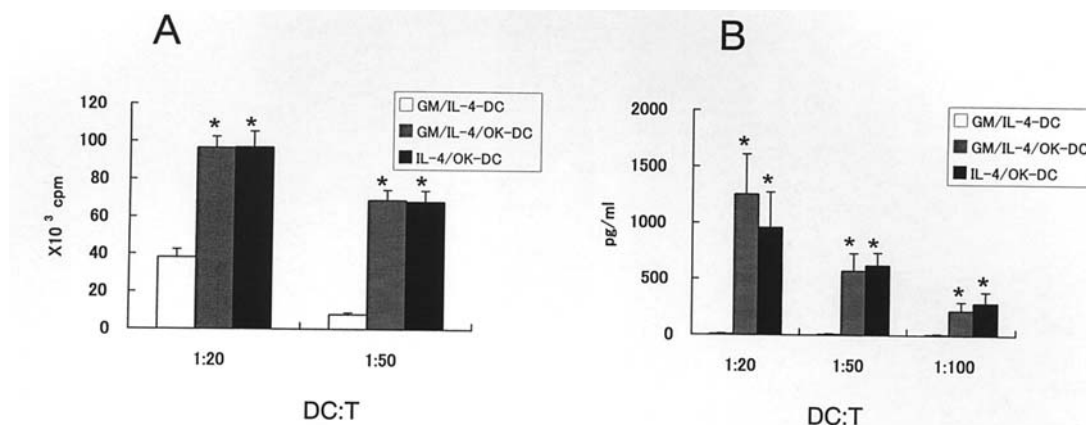


Figure 4. The immunostimulatory functions of DCs were determined by allogeneic MLR. (A) Irradiated DCs were cultured with allogeneic CD14⁺ T cells for 6 days. Proliferation was determined by [³H]-thymidine incorporation by the addition of [³H]-thymidine during the last 16 h of the culture period. Cultures were set up in triplicate, and results representing the mean \pm SD were obtained from 2 individual experiments. (B) Another similar set of cultures were set up for IFN- γ determination, and the concentrations of IFN- γ in culture supernatants were determined using ELISA kits. Each sample was tested in duplicate, and the data are given as the mean \pm SD of 3 independent experiments. Asterisk indicates significant difference between GM/IL-4/OK-DC or IL-4/OK-DC and GM/IL-4-DC ($P < 0.05$).

Cells cultured with IL-4 produce various inflammatory cytokines upon stimulation with OK-432. Since OK-432 is known to induce multiple cytokines from immune cells, such as monocyte-derived DCs and macrophages (20,30), it is conceivable that the addition of OK-432 into the culture may induce cytokines that are required for the differentiation of monocytes into DCs, as well as for the maturation of differentiated DCs. To address this question, monocytes cultured with IL-4 alone for 4 days (pre-IL-4-DC) were stimulated with OK-432 and the level of IFN- γ , TNF- α , IL-1 β , IL-6 and GM-CSF secreted in the culture supernatants were measured by ELISA. As expected, GM-CSF, TNF- α , IL-6 and IFN- γ were detected in the culture supernatant after 48 h (Fig. 3A). In the series of experiments to examine the kinetics of cytokines produced in the supernatants, we found that the cytokines, IL-6, TNF- α and GM-CSF, became detectable on day 5 and maintained a certain level of concentration until day 7 (Fig. 3B). In conclusion, the supernatant in the culture of IL-4/OK-DC contains various cytokines required for both the generation of DCs from monocytes and the induction of maturation of immature DCs.

IL4/OK-DC has a strong immunostimulatory capacity determined by the mixed lymphocyte reaction. Primary allo-MLR was carried out to assess the functional capacity of IL-4/OK-DC to present antigens to T cells. As shown in Fig. 4A, the T cell proliferation activities were augmented by the use of IL-4/OK-DC and GM/IL-4/OK-DC as APCs compared to the non-activated GM/IL-4-DC). The production of IFN- γ was also increased markedly when T cells were stimulated with IL-4/OK-DC and GM/IL-4/OK-DC (Fig. 4B).

T cell primed with autologous IL-4/OK-DC induces a Th1-type response upon mitogenic stimulation. DCs that induce a Th1-type response have been reported to be a key factor for the efficient induction of the anti-tumor immune response. T cells stimulated by autologous IL-4/OK-DC were subjected to cytokine assays by ELISA to examine if they could induce

a Th1-type response or not. Generated DCs were cultured for 5 days in the presence of autologous CD4⁺ T cells purified from PBMCs with magnetic isolation and the T cells were subsequently activated with PMA and ionomycin. T cell-derived cytokine productions were measured by ELISA and intracellular FACS analyses. As shown in Fig. 5A, T cell responses in the culture with IL-4/OK-DC were skewed to higher Th1/Th2 ratio due to the increased production of IFN- γ . In contrast, GM/IL-4-DC without OK-432 stimulation induced a low Th1/Th2 response because of the lower production of IFN- γ and higher production of IL-4 and IL-10. The polarized cytokine production profiles induced by GM/IL-4-DC, GM/IL-4/OK-DC and IL-4/OK-DC were also confirmed by the detection of intracellular cytokines by means of flow cytometry. Consistent with the results from ELISA, more IFN- γ producing cells were detected in T cells stimulated by IL-4/OK-DC or IL-4/GM/OK-DC than IL-4/GM-DC (Fig. 5B).

IL-4/OK-DC effectively induces an enhanced peptide-specific T cell response in vitro. For the further evaluation of the antigen-presenting capacity of DCs, we performed CTL induction experiments using the peptide derived from influenza nucleoprotein. Flu peptide-pulsed DCs were co-cultured with T cells for 7 days. Responder cells were then re-stimulated for 7 more days and peptide-specific IFN- γ production was examined on day 14. As shown in Fig. 6, the peptide-specific response induced by IL-4/OK-DC was stronger than that induced by GM/IL-4-DC or GM/IL-4/OK-DC.

Discussion

In the present study, we showed that fully mature DCs (IL-4/OK-DC) could be generated in the culture of human peripheral blood monocytes initiated in the presence of IL-4 alone and followed by the additional OK-432 on day 4 without GM-CSF. This novel method enables us to generate mature DCs, demonstrating not only the phenotype but also the functional abilities, which are identical to DCs matured by the additional

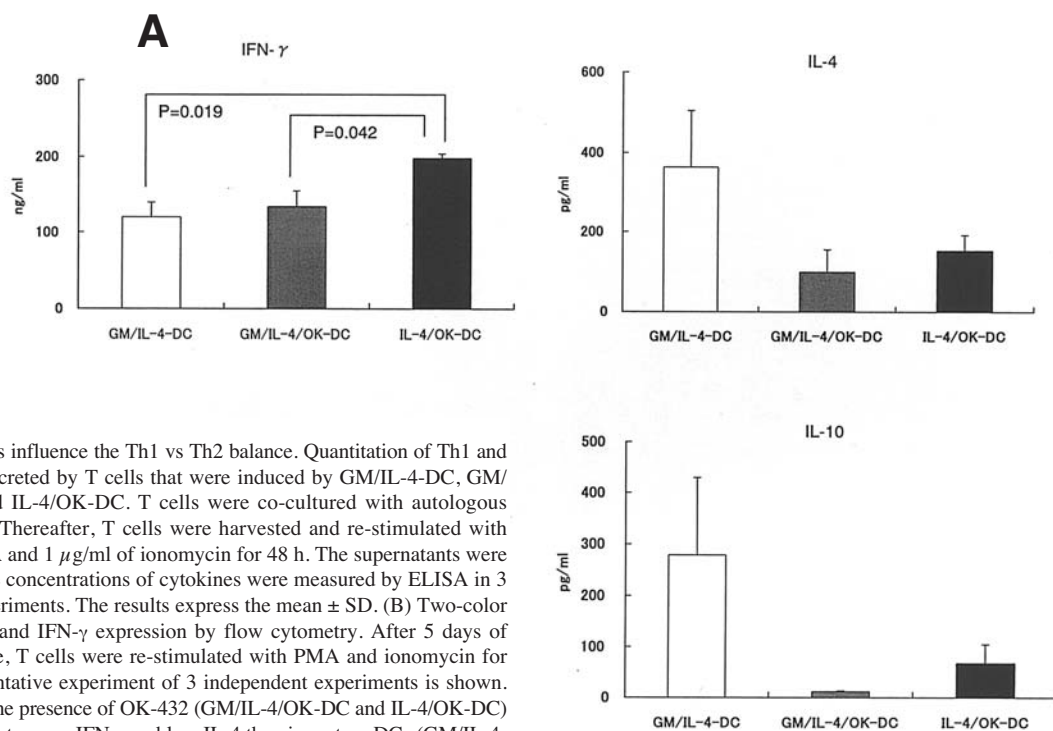
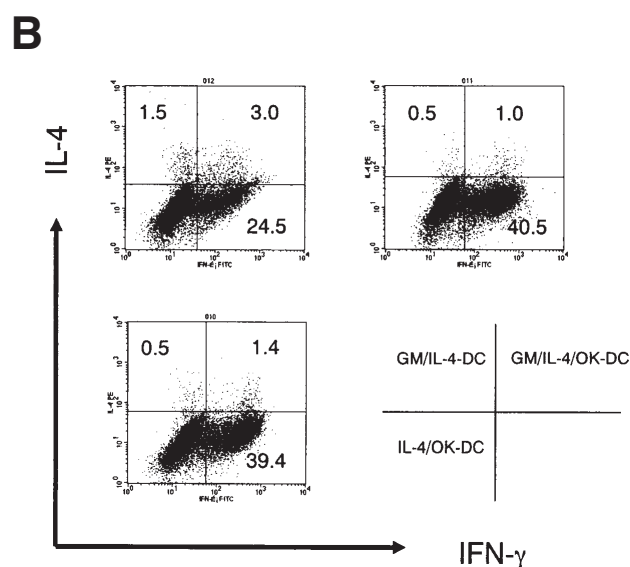


Figure 5. (A) DCs influence the Th1 vs Th2 balance. Quantitation of Th1 and Th2 cytokines secreted by T cells that were induced by GM/IL-4-DC, GM/IL-4/OK-DC and IL-4/OK-DC. T cells were co-cultured with autologous DCs for 5 days. Thereafter, T cells were harvested and re-stimulated with 25 ng/ml of PMA and 1 μ g/ml of ionomycin for 48 h. The supernatants were harvested and the concentrations of cytokines were measured by ELISA in 3 independent experiments. The results express the mean \pm SD. (B) Two-color analysis of IL-4 and IFN- γ expression by flow cytometry. After 5 days of DC-T cell culture, T cells were re-stimulated with PMA and ionomycin for 4 h. One representative experiment of 3 independent experiments is shown. DCs cultured in the presence of OK-432 (GM/IL-4/OK-DC and IL-4/OK-DC) made T cells secrete more IFN- γ and less IL-4 than immature DCs (GM/IL-4-DC). Quadrants were set according to the fluorescence intensities of FITC-conjugated and PE-conjugated isotype-matched control Igs with irrelevant specificity. Parenthesis represents the percentage in each quadrant.

culture of immature DCs using recombinant cytokines, directly from PBMCs, and thus implies the possibility for the application in tumor immunotherapy in a clinical setting. So far, there have been numbers of reports describing methods to generate mature DCs derived from peripheral blood monocytes, especially in the context of the use for clinical studies (19). To generate human DCs from monocytes, both IL-4 and GM-CSF have been used in many studies (31,32). According to our results, the combination of GM-CSF and OK-432 did not allow the monocytes to fully differentiate into DCs, assumed because of the lack of IL-4 in the culture. This result is consistent with the previous reports that indicated that IL-4 appeared to down-regulate the expression of CD14 (33) and thus increased the purity of DCs. IL-4 has also been implicated to up-regulate MHC class II molecules and to enhance the allo-stimulatory properties of monocytes (34). However, in contrast to IL-4, GM-CSF appears not to be essential for the generation of human monocyte-derived DCs (35,36). Brossart *et al* demonstrated that Flt3L and IL-4 could induce immature DCs (10). They showed that even CD40L alone was able to differentiate the monocytes into DCs without any other cytokines. Ebner *et al* also showed that monocytes could differentiate into DCs in the presence of IL-3 and IL-4, although these DCs produce less IL-12 and therefore they could be tolerogenic rather than immunogenic (11). Consistent with these reports, our results indicate that the addition of GM-CSF could be abandoned for the induction of DCs when IL-4 and OK-432 were present in the culture. However, a previous study has shown that OK-432 stimulates monocytes and produces a variety of inflammatory cytokines including GM-CSF (23) and thus, exogenous GM-CSF might be simply substituted by an endogenous cytokine produced



by monocytes upon stimulation with OK-432 in the current study. Interestingly though, the GM-CSF detected in our culture system was much lower (20-30 pg/ml) in concentration as compared to the optimal concentration (10-100 ng/ml) which has been used in many protocols (Fig. 3A) (31,32). According to our results regarding the cytokine production profile of monocytes cultured with IL-4 and stimulated with OK-432, either IFN- γ , IL-6, TNF- α , IL-10 (data not shown), IL-12 (data not shown) or any combination of these besides GM-CSF may be involved in the differentiation of monocytes into DCs (Fig. 3A). Therefore, experiments using the combination of these recombinant cytokines or antibodies to neutralize these cytokines for the investigation of the contribution of each of these cytokines will be required to elucidate the detailed mechanism.

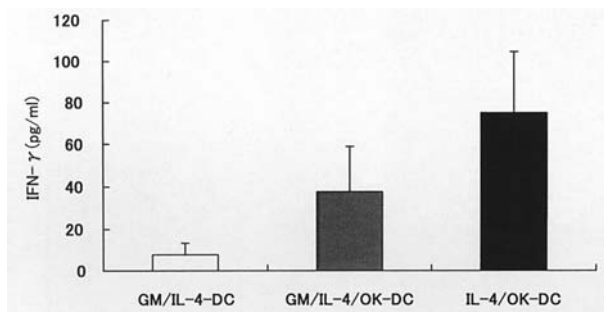


Figure 6. CTL induction experiment using Flu peptide derived from influenza nucleoprotein was performed. Flu peptide pulsed DCs were co-cultured with T cells for 7 days. Responder cells were then re-stimulated for a further 7 days and the peptide-specific IFN- γ production was examined on day 14. The background levels of peptide-specific IFN- γ production detected in response to TSI cells without peptide-pulse were subtracted to obtain the values shown in the figure. Results representing the mean \pm SD were obtained from 3 individual experiments.

Most of the protocols as to the preparation of human monocyte-derived DCs reported so far require 10-14 days to generate mature DCs using multiple recombinant cytokines. Romani *et al* reported that mature DCs could be generated using the monocyte-conditioned medium, which is considered to be a cytokine cocktail containing TNF- α , IL-1 β and IL-6 at a final yield of 0.8 to 3.3 $\times 10^6$ cells out of 40 ml of the blood (16). As shown in Fig. 1B in the current study, our protocol resulted in the yield of mature DCs ranging from 2 to 3% of the starting material, PBMCs. Therefore, approximately 1 $\times 10^6$ mature DCs will be generated from 10 ml of the blood according to the calculation based on the observation that about 10% of PBMCs are CD14 $^+$ cells (data not shown). Taken together, our protocol seems to be attractive in terms of cost- and labor-effectiveness for clinical trials because this method makes it possible to generate mature DCs directly from peripheral monocytes in a 7-day one-step culture using less cytokines resulting in a high purity and high yield, comparable to previous reports, although there may still be some aspects regarding the *in vivo* effectiveness for CTL induction that need to be explored before materializing this protocol in a clinical study.

It is most likely that cytokines produced endogenously in the culture play a critical role not only for differentiation of monocytes into DCs but also for inducing the maturation of generated DCs in our system. Indeed, as mentioned before, cytokine assays showed that IFN- γ , TNF- α and IL-6, which have been shown to be key factors for DC maturation, were detected in the culture after the stimulation with OK-432 (Fig. 3A). Moreover, the internalization of OK-432 itself may also have a direct effect on DC maturation. We showed that DCs stimulated with OK-432 express Su antigen on their cell surfaces (Fig. 2B), which indicates that OK-432 was internalized by the DCs. It was also reported that the activation of NF- κ B, one of the intracellular signaling pathways of DC maturation (35), was induced by OK-432, and this effect was inhibited with cytochalasin B, an inhibitor of endocytosis (30). These findings suggest that the maturation of DCs may be dependent, at least in part, on the internalization of OK-432 by immature DCs. The involvement of toll like receptors (TLRs), which are key molecules that link innate

and acquired immunity and are also involved in the maturation signaling for DCs, in the recognition of OK-432 by immune cells has been suggested by Okamoto *et al* (37). They have shown that OK-PSA, a high-performance liquid chromatography-purified fraction of OK-432 to lipoteichoic acid is capable of IFN- γ production by immune cells via the signaling through TLR4 (37). However, Nakahara *et al* demonstrated that β 2-integrin, but neither TLR2 nor TLR4, could mediate the IL-12 production of DCs by OK-432 (38). Thus, the mechanism by which DCs can be activated by OK-432 related products appears to remain unclear.

For efficient anti-tumor immunity, generation of DCs that induce Th1-type response is important (19). In this context, IL-4/OK-DC that has Th1 dominant pattern of cytokine expression and higher ability to induce peptide-specific CTL response is considered to be favorable for cancer vaccine therapy. IFN- γ has been shown to be one of the key factors for the induction of Th1 by DC besides IL-12 and costimulation mediated via CD28/B7 (39). We have demonstrated that OK-432 induces the production of IL-12 by DC (20), and IFN- γ was also detected in the culture of IL-4/OK-DC (Fig. 5). Thus, Th1-oriented profile of cytokine production by DCs shown herein may be critically involved in the efficient induction of Th1 response as well as CTL response.

In conclusion, our findings provide a new possible application of OK-432 for the generation of fully matured DCs directly from human peripheral blood monocytes *in vitro*. The DC population generated in the presence of IL-4 and OK-432 without exogenous GM-CSF can induce as strong a Th1 response in MLR as conventional mature DCs generated with multiple cytokines do. Further studies as to the effect of these DCs *in vivo* are warranted to clarify their usefulness as potent adjuvants for cancer vaccine therapy.

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