Generation of mature dendritic cells fully capable of T helper type 1 polarization using OK-432 combined with prostaglandin E$_2$

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Dendritic cell (DC) administration appears to be a very promising approach for the immunotherapy of cancer. The results of clinical studies have suggested that the nature and the magnitude of antitumor immune responses are critically affected by DC functions, including production of T helper type 1 (Th1)-inducing cytokines, activation of T cell subsets and natural killer (NK) cells, and migration from peripheral tissues to the T cell area of the draining lymph nodes. Administration of immature DCs could fail to fully stimulate antigen-specific immune responses and might induce tolerance under some conditions. In this study, we developed a method to obtain fully mature DCs, and we compared in detail the DCs thus obtained with those obtained using a maturation stimulus termed monocyte-derived medium (MCM)-mimic, which is a mixture of recombinant cytokines and prostaglandin E$_2$ (PGE$_2$) mimicking the components of monocyte-conditioned medium. Using DCs derived from monocytes of advanced cancer patients in this study, we found that DCs stimulated with OK-432 alone showed phenotypes similar to those of mature DCs induced using MCM-mimic, though with better secretion of IL-6 and IL-12. However, these DCs were found to have poor migratory capacity associated with the marginal expression of CCR7. When OK-432 was combined with PGE$_2$, the CCR7 expression and migratory capacity of DCs were significantly improved without impairing other immuno-stimulatory functions. These results suggest that stimulation with the combination of OK-432 and PGE$_2$ could be applicable as an alternative to MCM-mimic in clinical trials which require fully matured DCs to induce Th1-type immune responses against tumor cells even in patients with advanced cancer. (Cancer Sci 2003; 94: 1091–1098)

Dendritic cells (DCs) are very potent antigen-presenting cells, which play central roles in bridging between innate and acquired immunity via direct cell-cell interactions and/or cytokine production.[1–4] Since these DC functions could be used to induce potent immune responses against certain antigens presented on DC, clinical application of DCs has been initiated as a cellular immunotherapy against cancer.[5] The early-phase clinical studies have shown that both the induction of tumor-specific T cells and objective clinical responses could be achieved, at least in some patient populations, with the administration of mature DCs generated *in vitro* from subsets of peripheral blood including adherent mononuclear cells, CD14$^+$ monocytes, and CD34$^+$ precursor cells. Strategies to load DCs with tumor antigens include pulsing peptides, proteins[6, 7] or tumor cell lysate, and transfecting tumor-derived RNA. Analysis of the clinical results strongly suggests that the maturation status of DCs used in such protocols greatly affects the immune response that follows the treatments.[8]

Multiple reagents have been reported to induce DC maturation. The known DC maturation stimuli include CD40 ligand (CD40L),[4, 9, 10] poly I:C (a synthetic double-stranded RNA),[11, 12]

interferon (IFN)-α, lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, and conditioned medium of adherent autologous monocytes in culture, termed monocyte-conditioned medium (MCM). Among these currently available reagents, MCM has been considered to be one of the best stimuli. Analysis of MCM has shown that it is a mixture of prostaglandin E$_2$ (PGE$_2$) and inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-α.[13] Although MCM is a potent stimulant for DC maturation, MCM has some obvious disadvantages, including variable efficacy in maturation from blood donor to donor, the necessity for cumbersome procedures to isolate autologous monocytes and plasma, and the difficulties in quality assurance. As an alternative to MCM, some investigators have been using a cocktail of defined cytokines corresponding to those found in MCM.[14] This cytokine cocktail, termed "MCM-mimic," contains PGE$_2$ and recombinant cytokines such as TNF-α, IL-1β, IL-6. Although MCM-mimic has been considered to be a good stimulus for DC maturation, it has significant drawbacks, including cumbersome preparation, difficulties in purchasing recombinant cytokines prepared under good manufacturing practice (GMP) and modest expression of IL-12, a critical cytokine for the induction of T helper type 1 (Th1) responses from the stimulated DCs.

Recently, we have reported that OK-432 (Picibanil) could be used for stimulating the maturation of monocyte-derived human DCs.[15] OK-432 is a biological response modifier (BRM) derived from the weakly virulent Su strain of *Streptococcus pyogenes*.[16] It is available in GMP quality and has been used in Japan for more than 20 years as a non-specific immuno-adjuvant to treat various types of cancers. Our previous study, described above, indicated that OK-432 stimulates immature DCs to acquire mature phenotype and to produce significant amounts of Th1-type cytokines such as IL-12 and IFN-γ without interfering with the presentation of pulsed epitope-peptides.

In this report, we examined in detail the functions of OK-432-stimulated DCs derived from the monocytes of advanced cancer patients, compared with those of MCM-mimic-stimulated DCs. The results suggest that OK-432, when combined with PGE$_2$, is as effective a stimulus as MCM-mimic in almost every respect, including migratory capacity. It is even superior to MCM-mimic in terms of production of IL-12, a critical cytokine to induce cellular immune response.

Materials and Methods

Patients. The following experiments were performed using peripheral blood mononuclear cells (PBMCs) donated by the patients enrolled in a phase I DC vaccination trial approved by

Table 1. Clinical profiles of the patients

Patients (Age and sex)	Disease	Stage	Treatment	Metastasis
Patient 1 (42, F)	Melanoma	IV	Chemotherapy IFN-β Peptide	Liver, spleen, intradermal, bone, brain, right ventricle
Patient 2 (50, M)	Melanoma	IV	Surgery Chemotherapy	Lung, bone, spleen, intradermal, lymph nodes
Patient 3 (61, F)	Breast cancer	IV	Surgery Radiation Chemotherapy	Lung, bone, intradermal, lymph nodes
Patient 4 (64, M)	Stomach cancer	IV	Surgery Chemotherapy	Lymph nodes

the Institute Review Board of the Institute of Medical Science, The University of Tokyo (Table 1). All the patients had given written informed consent, and each experiment was performed using PBMCs harvested from the patients prior to the initiation of the treatment. In this paper, we present representative results from particular patients, but this is done only when consistent findings were obtained in all four patients tested.

Generation of immature DCs from advanced cancer patients. White blood cells from the patients were harvested by leukapheresis using a Cobe Spectra (Cobe BCT, Lakewood, CO).[17, 18]

PBMCs were separated from leukapheresis products by means of standard density gradient centrifugation using Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden). Then, they were cryopreserved in a freezing medium that consists of 10% DMSO (Sigma, St. Louis, MO), 20% heat-inactivated autologous serum, and 70% RPMI-1640 medium (Invitrogen, Carlsbad, CA) at 2.5×10^7 cells/ml until assay. Monocyte-derived DCs were generated from cryopreserved PBMCs using the standard method described elsewhere[17] with minor modifications. In brief, cryopreserved PBMCs were thawed in a water bath at 37°C, then supplemented with RPMI-1640 medium, centrifuged once at 1500 rpm×5 min at 4°C, and were plated in 100×20 mm Primaria tissue culture dishes (Becton Dickinson (BD), Franklin Lakes, NJ) at the density of 2.0×10^7 cells per dish in 5 ml of RPMI-1640 medium without serum. They were allowed to adhere to the dish at 37°C in 5% CO_2 for 3 h. Non-adherent cells were removed carefully. The remaining cells were cultured for 7 days in 5 ml of complete medium, which consists of RPMI-1640 supplemented with 2% heat-inactivated autologous human serum, 500 U/ml of recombinant human (rh) GM-CSF (PeproTech Ec, Ltd., London, UK) and 250 U/ml of rhIL-4 (PeproTech Ec, Ltd.).

Activation of immature DCs. After culture for 7 days, non-adherent and loosely adherent cells were harvested from the dishes, counted, and placed in 6-well plates (Corning Costar, Cambridge, MA) at the density of 1.0×10^6 cells/well in 3 ml of complete medium supplemented with 10 μg/ml of OK-432 alone, 10 μl/ml of OK-432 and 1 μg/ml of PGE_2, or MCM-mimic consisting of 10 ng/ml of rhTNF-α, 10 ng/ml of rhIL-1β, 1000 U/ml of rhIL-6 and 1 μg/ml of PGE_2.[19] Forty-eight hours after the stimulation, the cells were harvested and analyzed. The OK432 was provided by Chugai Pharmaceutical Co., Ltd. (Tokyo). The cytokines, such as rhIL-1β, rhIL-6, and rhTNF-α, were purchased from PeproTech Ec, Ltd. PGE_2 was purchased from Sigma.

Flow cytometry. The phenotypic analysis of DC preparations was performed on day 7 and on day 9 using a FACSCalibur (Becton Dickinson, Mountain View, CA) with Cell Quest software. Dead cells and small cells in the cell preparations were gated out on the basis of the light-scattering properties for this analysis, and the remaining cells were analyzed for CD11c, CD80, CD83, CD86, and HLA-DR expression (all PE or FITC-conjugated mAbs were purchased from BD PharMingen, San Diego, CA). In the examination of CCR7 expression, anti-CCR7 mAb and FITC-conjugated goat anti-mouse IgM mAb purchased from BD PharMingen were used.

Cytokine measurement. Seven days after the initiation of culture, DCs were incubated with the stimuli, the culture superna-

tants were collected, and the concentrations of IL-6 and IL-12 (p70 and p40) in the samples were examined using ELISA kits (Endogen, Rockford, IL) specific to the respective cytokines.

In vitro chemotaxis assay. The in vitro chemotaxis assay was performed with minor modifications as described previously.[20] In brief, recombinant chemokine (rhMIP-3β/CCL19) purchased from Genzyme/TECHNE (Minneapolis, MN) was diluted to appropriate concentrations with the serum-free assay medium to a final volume of 600 μl and applied to 24-well tissue culture plates (Corning Costar). Transwell culture inserts (Corning Costar) of 6.5 mm diameter with 5.0-μm pores were inserted into each well, and DCs (5.0×10^5 cells per each well) were added to the top chamber in assay medium at a final volume of 100 μl. The plates were incubated for 4 h, then the cells in the bottom chambers were recovered. Harvested cells were counted, and an aliquot was stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD86 mAbs (BD PharMingen) for analysis by FACSCalibur. The numbers of migrating DCs were calculated from the number of all migrating cells and the percentages of the cells positive for both CD11c and CD86.

Allogeneic mixed lymphocyte reaction (allo-MLR). To examine the immuno-stimulatory function of the DCs after different treatments, allo-MLR was performed. Immature DCs were stimulated with mitomycin-C (Kyowa Hakko, Tokyo) for 45 min at 37°C. They were added to 2.0×10^4 to 2.0×10^5 allogeneic PBMCs obtained from healthy volunteers in 96-well flat-bottomed plates. Then the mixture was co-incubated for 2 days in complete medium supplemented with 5% heat-inactivated autologous serum. Two days after the initiation of the co-culture, the cells and the culture supernatant were harvested. Concentrations of IFN-γ in culture supernatants were determined using ELISA kits (Endogen), and the surface expression of CD69 expression (BD PharMingen) on CD4+ T cells, CD8+ T cells or CD56+ NK cells was examined using double color staining. All mAbs were purchased from BD PharMingen. In some experiments, anti-IL-12 mAb (20 μg/ml; BD PharMingen) was used to block IL-12 effects and mouse IgG₁ (BD PharMingen) was used as a control antibody. The NK activity of the resultant lymphoid cells of MLR was tested using a standard 4-h ^{51}Cr-release assay against K562.

Influence of DC functions on Th1 differentiation. The CD4+ CD45RO− cells were isolated as naïve Th cells from PBMCs of healthy donors by magnetic cell sorting (MACS) using Midi-MACS separation columns (Miltenyl Biotec, Sunnyvale, CA). In brief, CD4+ cells, separated using a CD4 Multisort Kit (Miltenyl Biotec), were mixed with microbeads conjugated with anti-CD45RO antibody, and applied to a MidiMACS LS+ column to remove CD45RO+ cells. The percentage of CD4+ CD45RO− cells in the harvested population was more than 95% throughout this study. Allogeneic CD4+ CD45RO− cells were cultured with 1.0×10^5 immature DCs (iDC), OK-DCs, OK-P-DCs or MCMm-DCs for 7 days in the presence of rIL-2 (100 U/ml) (Teceleukin; Biogen, Inc., Cambridge, MA). After the culture, the cells were harvested and examined for intracellular cytokine production with FACSCalibur. In brief, the cells were stimulated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma) and 100 ng/ml of A23187 (Sigma) for 4 h in the presence of brefeldin A (Sigma), stained with PerCP-conjugated anti-CD4 mAb, fixed with 4% paraformaldehyde, treated

with permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN_3 and 0.5% Triton X-100, pH 7.5), and then incubated on ice with PE-conjugated anti-IL-4 mAb and FITC-conjugated anti-IFN-γ mAb for 45 min. The percentage of cells expressing cytoplasmic IL-4 or IFN-γ was determined by FACSCalibur. PerCP-conjugated anti-CD4 mAb, PE-conjugated anti-IL-4 mAb and FITC-conjugated anti-IFN-γ mAb were purchased from BD PharMingen.

Results

DCs stimulated with OK-432 alone (OK-DCs) showed phenotypic characteristics similar to those of DCs stimulated with MCM-mimic (MCMm-DCs), except for migratory capacity associated with modest CCR7 expression. Characteristics of the DCs stimulated with OK-432 (OK-DCs) were examined and directly compared with those of the DCs stimulated with MCM-mimic (MCMm-DCs)

using immature DCs derived from monocytes obtained from stage IV cancer patients (Table 1). Immature DCs were generated with rhGM-CSF plus rhIL-4 as described in "Materials and Methods," and OK-432 was used under the optimal conditions determined in our previous study.[15] The cells with no stimulation showed sparse and short dendrites typical of iDC. The cells stimulated with OK-432 and MCM-mimic showed morphologies consistent with those of mature DCs (Fig. 1). Flow-cytometric analysis of OK-DCs showed up-regulated surface expression of CD83, CD80, CD86, and HLA-DR at similar levels to those on MCMm-DCs (Fig. 2A). The expression of CD40 was also up-regulated on OK-DCs as well (data not shown). These results suggest that OK-DCs have all the phenotypic characteristics of fully matured DCs induced with MCM-mimic.

We also examined cytokine production of OK-DCs compared with that of MCMm-DCs (Fig. 2B). The OK-DCs produced IL-
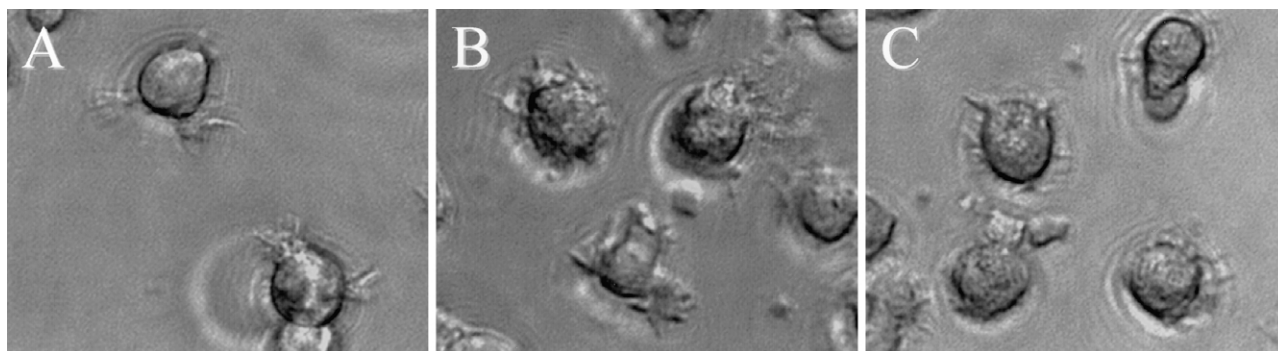

Fig. 1. Morphological characteristics of the iDCs (panel A), OK-DCs (panel B) or MCMm-DCs (panel C) (×400 magnification).
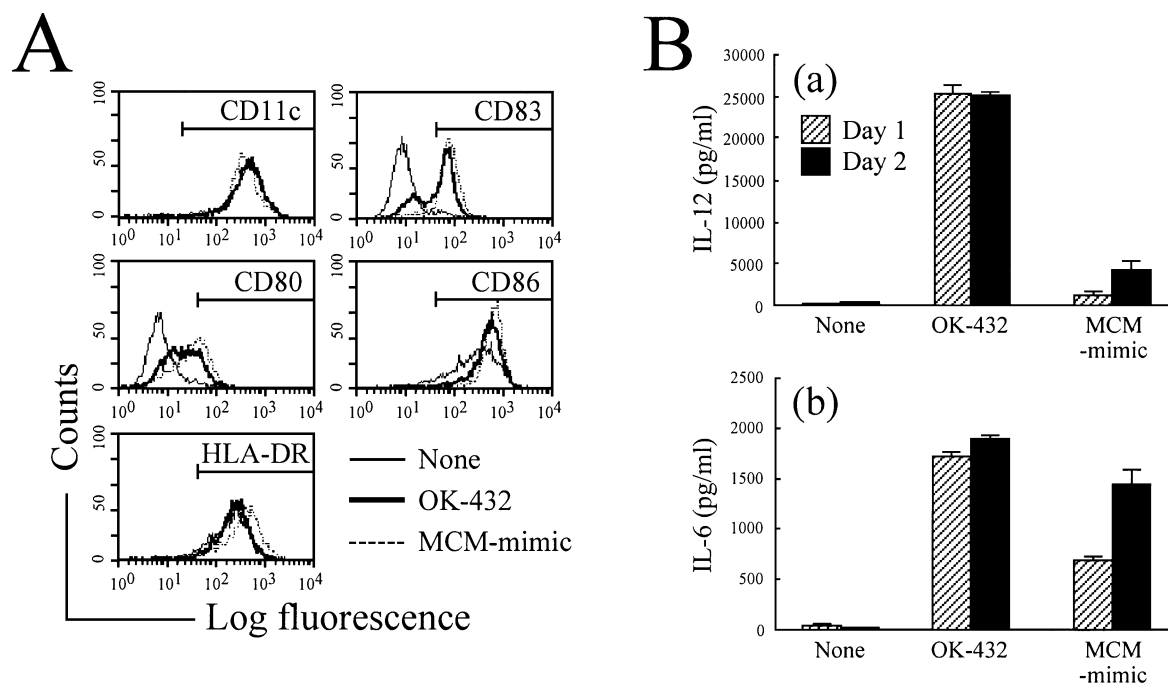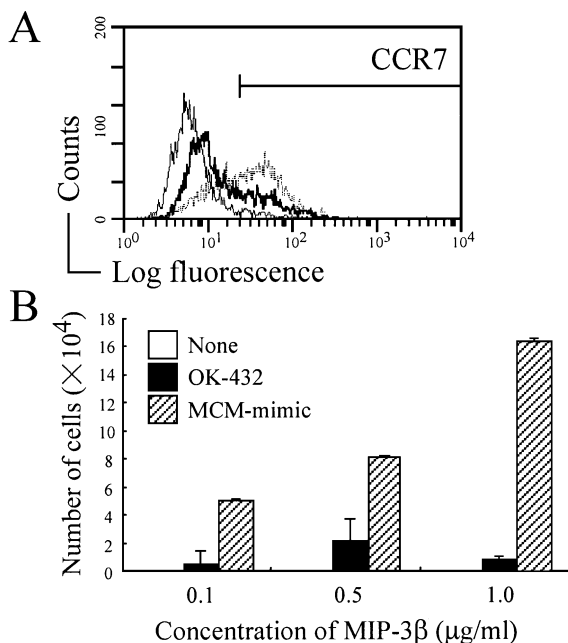

Fig. 2. DCs stimulated with OK-432 alone showed phenotypic characteristics similar to those of DCs stimulated with MCM-mimic and produced large amounts of IL-12. Immature DC, OK-DCs or MCMm-DCs were induced as described in "Materials and Methods," and their phenotypic characteristics were determined by FACSCalibur 9 days after the initiation of the culture. The fine solid, bold solid and dotted lines represent the staining patterns of iDCs, OK-DCs and MCMm-Dcs, respectively. The bars indicate the area considered to be positive judging from the patterns stained with PE- or FITC-labeled isotype control IgG (panel A). Production of IL-12 (a) and IL-6 (b) by iDCs, OK-DCs and MCMm-DCs was examined. Day-7 DCs were stimulated with none, OK-432 or MCM-mimic for 2 days. IL-12 and IL-6 activities in culture supernatant of the DCs were determined with the respective ELISA kits (panel B). Similar data were obtained in three additional cancer patients.

12, which is a critical cytokine for the induction of Th1-type responses, at much higher levels than those of MCMm-DCs (Fig. 2B-a). Although IL-10, a cytokine with immunosuppressive effects, was not produced by either type of DCs (data not shown), IL-6 was produced by OK-DCs at a level higher than or equivalent to the concentration contained in the medium of MCMm-DCs on the tested days (Fig. 2B-b).


Fig. 3. DCs stimulated with OK-432 alone have up-regulated CCR7 expression, but at a level much lower than that of DCs stimulated with MCM-mimic. Immature DCs, OK-DCs or MCMm-DCs were induced as described in "Materials and Methods," and their phenotypic characteristics were determined by FACSCalibur 9 days after the initiation of culture. The fine solid, bold solid and dotted lines represent the staining patterns of iDCs, OK-DCs and MCMm-Dcs, respectively. The bars indicate the areas considered to be positive (None, 3.7%; OK-432, 14.8%; MCM-mimic, 41.7%) judging from the patterns stained with PE- or FITC-labeled isotype control IgG (panel A). Immature DCs, OK-DCs or MCMm-DCs were induced as described in "Materials and Methods" and their migratory capacities were examined in terms of their chemotactic responses to MIP-3β (0.1, 0.5 and 1.0 μg/ml). Although iDCs and OK-DCs showed no or marginal migration, MCMm-DCs showed potent migrating capacity (panel B). Similar data were obtained in three additional cancer patients.

Furthermore, we examined the expression and the function of CCR7, which is associated with DC maturation and migration, on the DCs. The expression of CCR7, which is expressed on the surface of mature DCs, was up-regulated on OK-DCs but at a much lower level than that of MCMm-DCs (Fig. 3A). *In vitro* chemotaxis assay using MIP-3β, a CCR7 ligand, showed marginal migratory capability of OK-Dcs, consistent with the intensity of CCR7 surface expression (Fig. 3B). In contrast, MCMm-DCs showed very good migratory capability.

Supplementation of PGE$_2$ up-regulates CCR7 expression on OK-DCs without affecting their other surface phenotypes or the secretion of IL-6 and IL-12. The migration of antigen-loaded DCs into draining lymph nodes is considered to be a critical event for efficient induction of T cell immune responses. Thus, low migratory capacity could impair efficient antigen-presentation of OK-Dcs, especially *in vivo*. To improve the migratory capability of OK-DCs, we added PGE$_2$, which has recently been shown to be a key factor for inducing CCR7 expression on DCs,[21, 22] to OK-DCs during the stimulation. The DCs stimulated with OK-432 and PGE$_2$ (OK-P-DCs) showed up-regulated CCR7 expression at a level almost identical with that of MCMm-DCs (Fig. 4A), and good migration in chemotaxis assay (Fig. 4B). Thus, OK-P-DCs have as good a migratory capacity as that of MCMm-DCs.

In order to examine the influence of PGE$_2$ on DC functions other than migration, the surface phenotype and cytokine secretion of OK-DCs, OK-P-DCs, and MCMm-DCs were examined (Fig. 5). OK-P-DCs showed a surface phenotype almost identical to that of MCMm-DCs, which have higher expression of CD80 and HLA-DR than OK-DCs. Furthermore, OK-P-DCs secreted IL-12 and IL-6 at levels much higher than those of MCMm-DCs and equivalent to those of OK-DCs (Fig. 6).

OK-P-DCs stimulate IFN-γ production in allo-MLR more potently than MCMm-DCs. The potency of the OK-P-DCs in T or natural killer (NK) cell stimulation was evaluated by testing their ability to stimulate allogeneic bulk PBMCs in mixed lymphocyte reaction (allo-MLR). OK-DCs and OK-P-DCs induced IFN-γ at high levels in allo-MLR (Fig. 7A). In contrast, MCMm-DCs induced IFN-γ at a modest level, which was far lower than those induced with OK-DCs or OK-P-DCs. The IFN-γ induction with OK-DCs and OK-P-DCs was almost completely abrogated when IL-12 in the allo-MLR was blocked using an antibody specific to IL-12. We also examined which cell population is responsible for the IFN-γ response. In the lymphoid cells of allo-MLR stimulated with OK-DCs or OK-P-DCs, expression of CD69 on CD56$^+$ NK cells was drastically up-regulated (Fig. 7B). Up-regulation of CD69 expression was also noted on the NK cells contained in allo-MLR stimulated with MCMm-DCs.
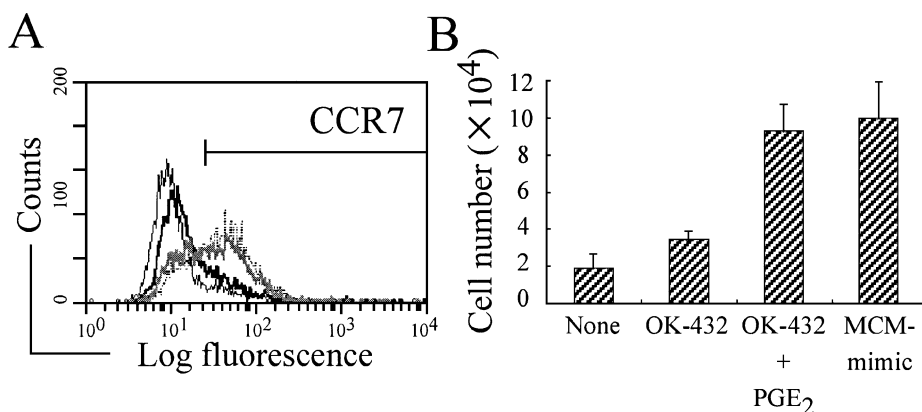
Fig. 4. Supplementation of PGE$_2$ up-regulates CCR7 expression and the migrating capacity of OK-DCs. Immature DCs, OK-DCs, OK-P-DCs or MCMm-DCs were induced as described in "Materials and Methods" and their phenotypic characteristics were determined by FACSCalibur 9 days after the initiation of culture. The fine solid, bold solid, gray bold solid and dotted lines represent the staining patterns of iDCs, OK-DCs, OK-P-DCs and MCMm-DCs respectively. The bars indicate the areas considered to be positive judging from the patterns stained with PE- or FITC-labeled isotype control IgG (panel A). Immature DCs, OK-DCs or MCMm-DCs were induced as described in "Materials and Methods," and their migratory capacity was determined 9 days after the initiation of culture. DCs were analyzed for their chemotactic responses to MIP-3β (0.5 μg/ml) (panel B). Similar data were obtained in three additional cancer patients.

However, the extent of the up-regulation was less than that with OK-DCs or OK-P-DCs. The levels of CD69 expression on CD4^{+} and CD8^{+} T cells were not significantly different. Furthermore, NK activities of the lymphoid cells in allo-MLR stimulated with OK-DCs and OK-P-DCs were significantly higher than those in the case of MCMm-DCs (Fig. 7C).

These results suggest that OK-DCs and OK-P-DCs can induce potent IFN-γ response and NK activity in allo-MLR, mainly through IL-12 production.

Both OK-DCs and OK-P-DCs equally promote the Th1 polarization of allogeneic naïve T cells. The influence of OK-DCs and OK-P-DCs on Th1 differentiation was examined using allogeneic
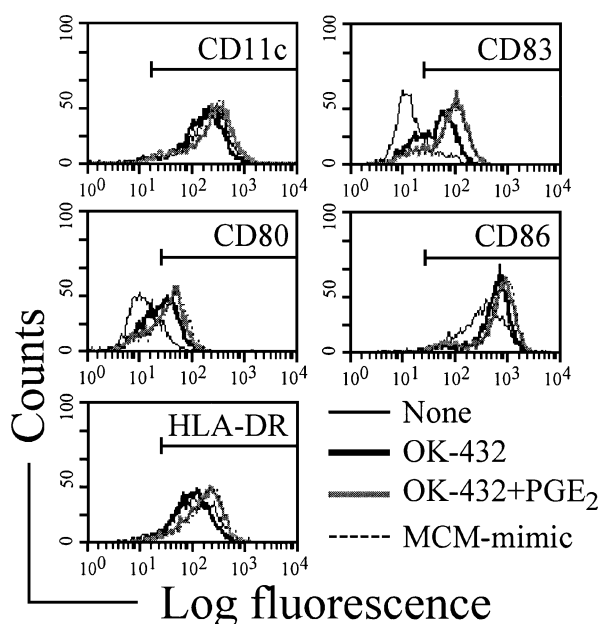

Fig. 5. Supplementation of PGE$_2$ on OK-DCs did not affect their surface phenotypes. Immature DCs, OK-DCs, OK-P-DCs or MCMm-DCs were induced as described in "Materials and Methods" and their phenotypic characteristics were determined by FACSCalibur 9 days after the initiation of culture. The fine solid, bold solid, bold gray solid and dotted lines represent the staining patterns of iDCs, OK-DCs, OK-P-DCs and MCMm-Dcs, respectively. The bars indicate the areas considered to be positive judging from the patterns stained with PE- or FITC-labeled isotype control IgG. Similar data were obtained in three additional cancer patients.

naïve Th cells (Fig. 8). Allogeneic naïve CD4^{+} T cells were co-cultured with iDCs, OK-DCs, OK-P-DCs or MCMm-DCs in the presence of IL-2 (100 U/ml) for 7 days. As shown in Fig. 8, the percentage of the CD4^{+} cells with intracellular IFN-γ production was significantly and equally increased when they were stimulated with either OK-DCs or OK-P-DCs. These results strongly suggest that addition of PGE$_2$ to OK-432 does not affect Th1 polarization induced with OK-432 alone.
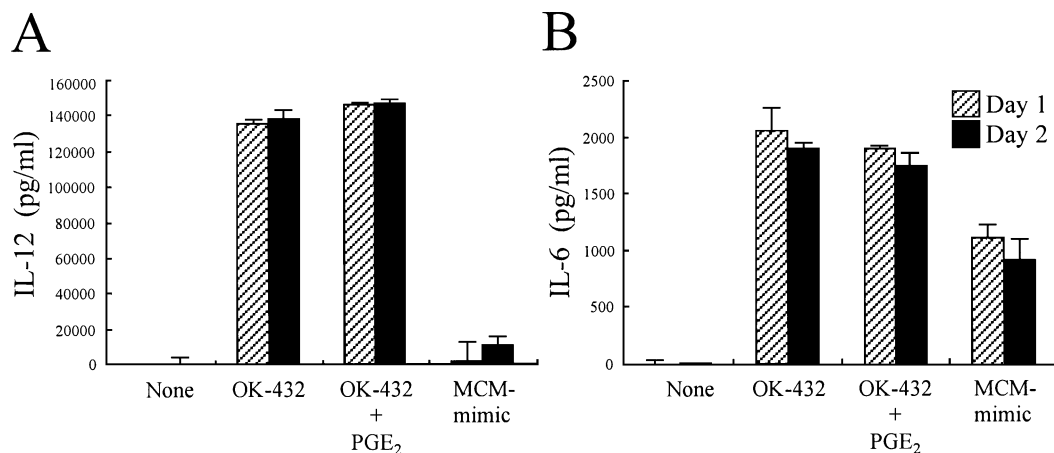
Discussion

In this study, we have examined our hypothesis that OK-432 could be used to stimulate DCs derived from the PBMCs harvested from stage IV cancer patients. DCs stimulated with OK-432 alone showed a phenotype similar to that of mature DCs stimulated with MCM-mimic, secreting large amounts of IL-12 and IL-6, but they had a relatively low migratory capacity associated with only marginal expression of CCR7. When OK-432 stimulation was combined with PGE$_2$, a component of MCM-mimic, the CCR7 expression and migratory capacity of DCs were significantly enhanced without any effect on other DC functions, including surface phenotype and IL-6 and IL-12 secretion. Furthermore, OK-P-DCs promoted NK activity, IFN-γ response and Th1 polarization in allo-MLR mainly through their IL-12 production.

Cellular immunotherapy using DCs is now considered to be a promising strategy to treat cancer patients, judging from the positive results of early clinical trials. For instance, both immunological and objective clinical responses have been observed after the administration of immature monocyte-derived DCs pulsed with prostate-specific membrane antigen (PSMA) peptides combined with radiotherapy.[23, 24] However, significant concerns have been raised about treatment using immature DCs at the same time. Dhodapkar *et al*.[25] showed that the effector function of T cells specific to influenza matrix peptide was inhibited by the administration of immature DCs derived from monocytes in PBMCs of healthy volunteers. Administration of immature DCs has also been suggested to insufficiently stimulate antigen-specific immune response, and even to induce tolerance in some conditions.[5] These results collectively suggest that immune responses against the tumor can only be fully induced by the administration of mature DCs.

Our previous report[15] and other work[26] suggested that OK-432 might be a good stimulus for DC maturation. In our recent report,[15] we have shown that OK-DCs have a phenotype compatible with that of mature DCs, abundantly produce Th1-type

Fig. 6. Supplementation of PGE$_2$ did not affect secretion of IL-6 and IL-12 by OK-DCs. Day-7 DCs were stimulated with none, OK-432 or MCM-mimic for 2 days. IL-12 (panel A) and IL-6 (panel B) concentrations in culture supernatant of the DCs were determined by using ELISA kits specific to the respective cytokines. Similar data were obtained in three additional cancer patients.
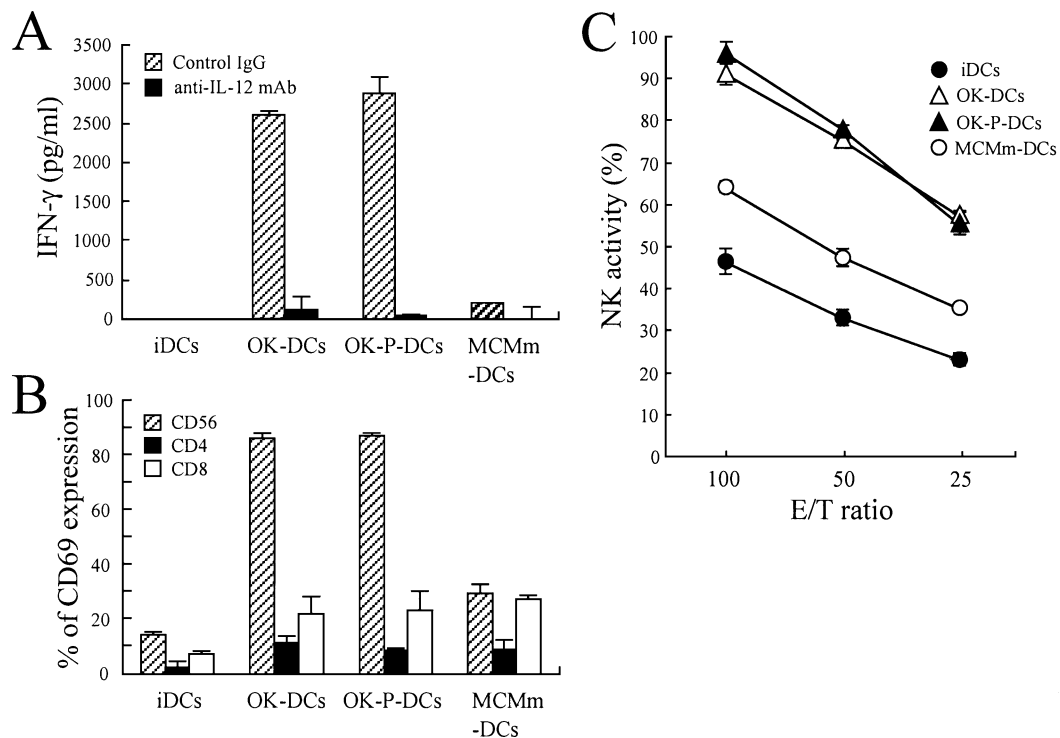
Fig. 7. The OK-DCs and OK-P-DCs stimulate IFN-γ production and NK activity in allo-MLR to levels higher than those in the case of MCMm-DCs. Freshly isolated PBMCs (1.0×10⁶ cells) obtained from healthy volunteers were stimulated with iDCs, OK-DCs, OK-P-DCs or MCMm-DCs (1.0×10⁵ cells) for 2 days. Cells and culture supernatant were harvested from the MLR. The culture supernatant was examined for IFN-γ activity using ELISA kits with or without anti-IL-12 blocking mAb (panel A). The CD69 expression pattern on CD4⁺, CD8⁺ T or CD56⁺ NK cells was examined by using a FACSCalibur (panel B). The NK activity against K-562 was also examined using 4-h ⁵¹Cr-release assay (panel C). Similar data were obtained in three additional cancer patients.

cytokines, and can efficiently induce potent CTLs specific to the pulsed epitope peptide. Furthermore, OK-432 appears to stimulate DCs through, at least in part, β₂ integrin, which is a receptor for various microbial antigens. In the current study, we have examined the characteristics of OK-DCs in detail, in comparison with those of MCMm-DCs. The results suggest that OK-432 could be a better stimulus for DC maturation than MCM-mimic, at least in the induction of IL-6 and IL-12, both of which have critical functions for inducing positive immune responses (Fig. 2).

Our results in allo-MLR suggest that IL-12 secreted by OK-DCs and OK-P-DCs is critically involved in the induction of IFN-γ response. Furthermore, OK-DCs and OK-P-DCs can activate NK cell functions, thereby playing an important role in regulating immune responses against various types of pathogens including tumor cells.

It also appears to be important that OK-432 can induce IL-6 production along with IL-12 production. IL-6 has multiple functions, particularly in the context of the acute phase response and B cell differentiation. Recently, Pasare and Medzhitov[27] demonstrated that cross-linking of pathogens to toll-like receptors (TLRs) on DCs triggers the activation of adaptive immune responses through at least two distinct mechanisms: the induction of costimulatory molecules on DCs and the production of IL-6, which renders pathogen-specific T cells refractory to the suppressive activity of CD4⁺ CD25⁺ T regulatory (T reg) cells, from DCs. Although TLR involvement in DC maturation with OK-432 is still controversial, our results suggest that OK-DCs are suitable for generating antigen-specific T cells efficiently by suppressing T reg cells on the tumor-specific T cells with IL-6.

Although OK-DCs have many characteristics compatible with those of fully matured DCs as shown above, we found that
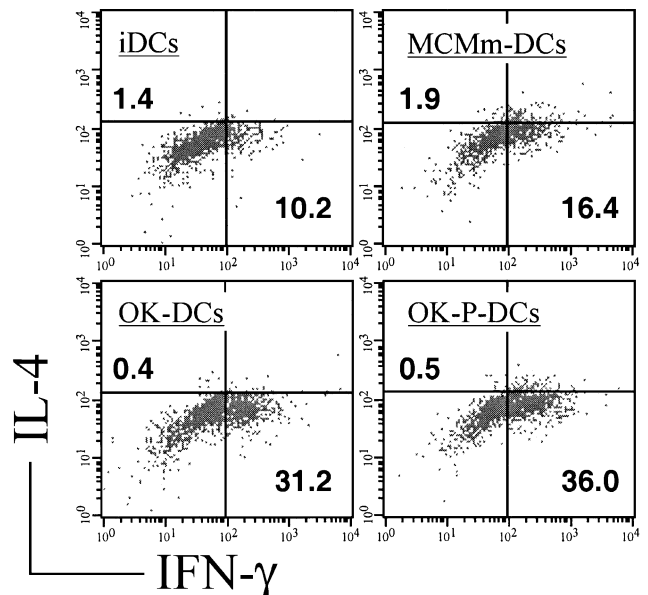
Fig. 8. Both OK-DCs and OK-P-DCs equally promote the Th1 polarization of allogeneic naïve CD4⁺ T cells. CD4⁺ CD45RO⁻ cells were obtained from healthy donors as naïve T cells by using magnetic beads selection. These cells were co-cultured with OK-DCs or OK-P-DCs derived from cancer patients and examined for intracellular IFN-γ and IL-4 expression using FACSCalibur after staining with PerCP-conjugated anti-CD4 mAb, PE-conjugated ant-IL-4 mAb, and FITC-conjugated anti-IFN-γ mAb. The percentage of positive cells in CD4⁺ T cells is indicated in figures. Similar patterns of expression were found in the experiments using DCs from three additional cancer patients.

the migratory capacity of OK-DCs is significantly inferior to that of MCMm-DCs. OK-DCs exhibit marginally up-regulated CCR7 expression when compared with that of immature DCs. This mediocre CCR7 expression of OK-DCs was associated with low migratory capacity toward MIP-3β *in vitro* (Fig. 3). Migration is an important function for DCs to locate themselves at the T cells of the lymph nodes, which is a suitable place to efficiently control immune responses. The migratory capacity of DCs is controlled by the expression of chemokine receptors, especially CCR7, on the surface of DCs. The ligands of CCR7, MIP-3β (CCL19) and SLC (CCL21), are expressed in lymph nodes and influence DC migration.[28, 29] In this study, we showed that migratory capacity of OK-DCs could be significantly improved by the addition of PGE$_2$, which up-regulates CCR7 expression (Fig. 4). Recent studies have demonstrated that chemokines play a major role in the migration of DCs.[30–32] Luft *et al.*[21] and Scandella *et al.*[22] have recently shown that PGE$_2$ regulates the migratory capacity of monocytes-derived DCs. However, PGE$_2$, mainly produced by APCs, has been shown to suppress Th1-related immune responses through multiple pathways, including the inhibition of IL-12 secretion, IL-12 receptor expression, and IFN-γ production. Furthermore, PGE$_2$ was shown to augment Th2-related immune responses as well, through multiple mechanisms including enhancement of the production of IL-10.[33, 34] To examine whether PGE$_2$ addi-

tion would affect OK-DC functions related to Th1 polarization, surface phenotypes, cytokine secretion, and IFN-γ response of allogeneic PBMCs primed with OK-P-DCs were evaluated (Fig. 7 and Fig. 8). All of the results consistently suggested that addition of PGE$_2$ does not significantly suppress any of the OK-DC functions mediating T cell and NK cell activation, including Th1 polarization.

These results strongly suggest that the simple combination of OK-432 and PGE$_2$, both of which are readily available in GMP quality, could induce full maturation of DCs, including migratory capacity. Furthermore, OK-DCs secrete IL-12 and IL-6, critical cytokines for stimulating Th1-type immune responses, at much higher levels than those of MCMm-DCs. Thus, OK-P-DCs appear to be a good alternative to MCMm-DCs, or even better in some respects, for use in clinical DC therapy to induce strong Th2-type immune responses specific to tumor cells.

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