



Efficient generation of highly immunocompetent dendritic cells from peripheral blood of patients with hepatitis C virus-related hepatocellular carcinoma ☆,☆☆

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ABSTRACT

Background & aims: Immunotherapy using dendritic cells (DCs) is a promising cancer therapy. The success of this therapy depends on the function of induced DCs. However, there has been no consensus on optimal conditions for DC preparation in vitro for immunotherapy of hepatocellular carcinoma (HCC) patients. To address relevant issues, we evaluated the procedures to induce DCs that efficiently function in hepatitis C virus (HCV)-related HCC. **Methods:** We studied immunological data from 14 HCC patients. The DC preparation and the surface markers were assessed by flow cytometric analysis. Four different additional activation stimuli (Method I, medium alone; Method II, with OK-432; Method III, with IL-1 β + IL-6 + TNF- α ; Method IV, with IL-1 β + IL-6 + TNF- α + PGE2) were tested and the functions of DCs were confirmed by examination of the ability of phagocytosis, cytokine production and allogeneic mixed lymphocyte reaction (MLR).

Results: The numbers of DCs induced and their cytokine production ability were not different between healthy controls and HCC patients. T-cell stimulatory activity of DCs in MLR was significantly lower in HCC patients than in healthy controls. The maturation of DCs with OK-432 boosted production of cytokines and chemokines, such as IL-2, IL-12p70, IFN- γ , TNF- α , IL-13 and MIP1 α , and restored T-cell stimulatory activity of DCs in MLR.

Conclusions: The clinically approved compound OK-432 is a candidate for highly immunocompetent DC preparation and may be considered as a key drug for immunotherapy of HCV-related HCC patients.

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1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the fourth leading cause of cancer-related deaths in Japan [1]. Hepatitis C virus (HCV) infection is a common cause of

chronic liver disease and leads to liver cirrhosis, contributing to the incidence of HCC [2]. Although some therapies for HCC exist, tumor recurrence rates are extremely high in these patients after curative treatments, including hepatic resection and radiofrequency ablation (RFA) [3]. In terms of the reason of this, HCV-related chronic hepatitis and cirrhosis are known to have carcinogenic potential for the development of HCC [4]. For the secondary chemoprevention of HCC patients with HCV-related cirrhosis, recent studies showed the efficacy of long-term and low-dose interferon- α therapy [5]. Among many strategies for HCC recurrence, immunotherapy is considered to be an attractive strategy to eradicate tumor cells completely [6].

Until now, different immunotherapeutic approaches have been tested for patients with HCC [7]. However, tumors have evolved numerous immune escape mechanisms, including the generation of cells with immune suppressor functions, such as Tregs and myeloid-derived suppressor cells [8]. Dendritic cells (DCs) are the most potent professional antigen-presenting cells with the unique ability to initiate and maintain adaptive immunity, and are considered

Abbreviations: DC, dendritic cell; HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell.

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to overcome the immune-suppressive environment produced by tumors [9,10]. DCs are also known to enhance antitumor immunity by activating the functions of endocytosis, trafficking, maturation and cytokine production. Numerous studies have shown that DCs from peripheral blood of HCC patients are reduced in number, have an immature phenotype and an impaired function [11]. Moreover, to date, there is no consensus on optimal conditions for DC preparation in vitro for immunotherapy of HCC patients.

Recently, we have developed the combined immunotherapy of transcatheter hepatic arterial embolization (TAE) with infusion of immature and mature monocyte-derived DCs (MoDCs) for HCC [12, 13]. In this study, we evaluated the procedures to induce MoDCs that efficiently function in the immune-mediated treatments for HCC.

2. Materials and methods

2.1. Patients and healthy controls

Fourteen patients (four women and ten men) attending Kanazawa University Hospital (Ishikawa, Japan) between September 2007 and December 2008 were enrolled in this study. All patients were serologically positive for HCV. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI) and CT angiography. Blood samples were taken from 14 patients with HCC and 14 healthy controls having no hepatitis history and serologically negative for both hepatitis B and C. The clinical profiles of the patients and controls analyzed in the present study are shown in Table 1. All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

2.2. Preparation of immature DCs

Immature DCs were separated from peripheral blood mononuclear cells (PBMCs) of patients and healthy controls. PBMCs were isolated by centrifugation using Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). The cells were resuspended in serum-free medium (GMP CellGro DC Medium; CellGro, Manassas, VA) and allowed to adhere to 6-well tissue culture dishes (Costar, Cambridge, MA) at 1.4×10^7 cells in 2 mL per well. After 2 h at 37 °C, non-adherent cells were removed and adherent cells were cultured in the medium with 50 ng/mL recombinant human IL-4 (GMP grade; CellGro) and 100 ng/mL recombinant human GM-CSF (GMP grade; CellGro) for 5 days to generate immature DCs.

Table 1
Characteristics of healthy controls and patients.

	Controls	HCC patients	P
No. of patients	14	14	
Age (years)	42.6 ± 14.9	68.8 ± 7.6	<.05
Gender (M/F)	7/7	10/4	n.s.
WBC ($\times 10^3/\mu\text{L}$)	ND	43.5 ± 15.4	n.s.
PLT ($\times 10^4/\mu\text{L}$)	ND	13.1 ± 6.0	n.s.
PT (%)	ND	85.2 ± 13.3	n.s.
ALT (IU/L)	ND	59.7 ± 46.8	n.s.
Alb (g/dL)	ND	3.3 ± 0.6	n.s.
T-Bil (mg/dL)	ND	0.8 ± 0.4	n.s.
Histology of non-tumor liver			
Chronic hepatitis	ND	8	n.s.
Cirrhosis (Child-Pugh A/B/C)	ND	6 (5/1/0)	n.s.
TNM stage (I/II/IIIa/IIIb/IIIc/IV)	ND	0/11/1/2/0/0	

Data are expressed as the mean ± SD.
ND: not determined, n.s.: not significant.

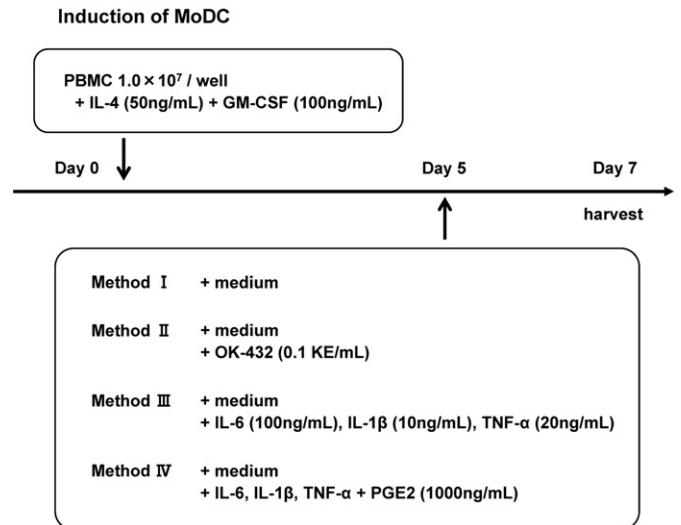


Fig. 1. Protocols for preparation of DCs. DCs, which were derived from PBMCs in the presence of IL-4 and GM-CSF for 5 days, were cultured for 2 additional days with the serum-free medium only (Method I), OK-432 (Method II), and cytokine cocktails consisting of IL-6, IL-1 β and TNF- α without (Method III) or with (Method IV) PGE2. On day 7, these cells were harvested and evaluated.

2.3. Activation of immature DCs

Several activation stimuli were tested (Fig. 1). After 5 days of culture, the immature DCs induced by the above method were cultured for 2 additional days in the serum-free medium (Method I) or stimulated with 0.1 KE/mL OK-432 (Chugai Pharmaceuticals, Tokyo, Japan) (Method II), 10 ng/mL IL-1 β (GMP grade; CellGro), 100 ng/mL IL-6 (GMP grade; CellGro) and 20 ng/mL TNF- α (GMP grade; CellGro) (Method III), and IL-1 β , IL-6, TNF- α and PGE2 (Kaken Pharmaceuticals, Tokyo, Japan) (Method IV). On day 7, the cells were harvested.

2.4. Antibodies

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-lin1 (lineage cocktail 1; CD3, CD14, CD16, CD19, CD20 and CD56)-FITC, anti-HLA-DR-PerCP and -FITC (L243), anti-CD11c-APC (S-HCL-3), anti-CD123-PE (9F5), anti-CCR7-PE (3D12), anti-CD14-APC (M ϕ P9) (BD Biosciences Pharmingen, San Jose, CA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA).

2.5. Flow cytometric analysis

Surface markers on DCs were evaluated using flow cytometric analysis. Cells were analyzed on a FACSCalibur™ for four-color flow cytometry. Data analysis was performed using CELLQuest™ software (Becton Dickinson, San Jose, CA).

2.6. Endocytosis assay

The endocytic capacity of DCs was assessed by measurement of FITC dextran (Sigma-Aldrich, St. Louis, MO) uptake. DCs were incubated for 30 min at 37 °C in the presence of 1 mg/mL FITC dextran, washed three times and analyzed using a FACSCalibur™ cytometer.

2.7. Cytokine production assay

The concentrations of cytokines and chemokines in the supernatants of culture medium were measured using enzyme-linked immunosorbent assay (ELISA) kit for IL-12p40 and IFN γ (Biosource International,

Camarillo, CA) and the Bioplex assay (Bio-Rad, Hercules, CA) according to the manufacturers' instructions.

2.8. Allogeneic mixed lymphocyte reaction (allo-MLR)

To evaluate the immune-stimulatory function of DCs after different stimulations, allogeneic mixed lymphocyte reaction (allo-MLR) was performed. 1×10^4 irradiated DCs (25 Gy) and 1×10^5 allogeneic PBMCs from a healthy donor were suspended in 200 μ L of serum-free medium (GMP CellGro DC Medium) and co-cultured in 96-well round-bottomed microplates. After 2 days at 37 °C, 1 μ Ci of [3 H]-thymidine (Amersham Pharmacia Biotech, Piscataway, New Jersey) was added to each well. The amount of incorporated [3 H]-thymidine was counted using a liquid scintillation counter (Beckman Coulter, Palo Alto, California). Results are expressed as the stimulation index (counts per minute in the presence of DCs divided by counts per minute in the absence of DCs).

2.9. Statistical analysis

Results are indicated as means \pm SD. The statistical significance of differences between groups was determined by applying the Mann–Whitney *U* test and unpaired *t* test. Any *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Yield and phenotype of DCs in patients and healthy controls

Adherent cells separated from PBMCs were cultured in the presence of IL-4 and GM-CSF, and harvested on day 7 (Method I). These harvested cells, which showed high levels of MHC class II (HLA-DR) and the absence of marker for mature monocytes (CD14), were consistent with the cell surface markers of DCs. The yield of DCs was variable, ranging from 23% to 28% of the initial PBMC population, and indicated no

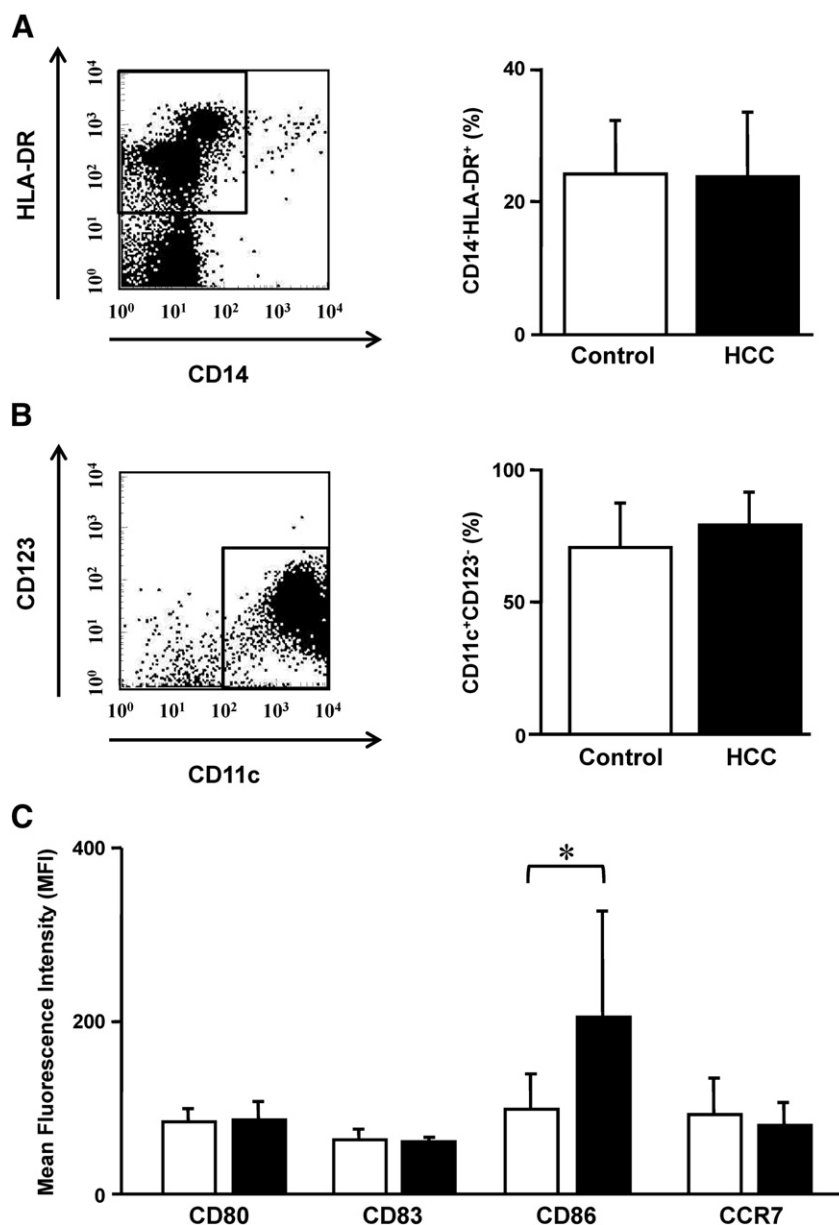


Fig. 2. The yield and characteristics of DCs derived from PBMCs in the presence of IL-4 and GM-CSF (Method I). (A) The yield of CD14⁺HLA-DR⁺ DCs in healthy controls and HCC patients, which was calculated as a percentage of the cells gated on side and forward scatter. (B) The yield of CD11c⁺CD123⁺ myeloid DCs in healthy controls and HCC patients, which was calculated as a percentage in lin1⁺HLA-DR⁺ cells. (C) Analysis of cell surface markers of lin1⁺HLA-DR⁺ DCs in healthy controls and HCC patients. Data are expressed as mean fluorescence intensity (MFI) \pm SD. White and black bars indicate healthy controls and HCC patients, respectively. **P* < .05.

significant difference between healthy controls and HCC patients (Fig. 2A). We next analyzed the surface markers, which classified DCs as myeloid or plasmacytoid DCs, on these lineage marker (lin1⁻-negative and HLA-DR-positive (lin1⁻HLA-DR⁺) DCs. In both patients and controls, the majority of DCs expressed CD11c and the percentages of the DCs classified under the myeloid subset (CD11c⁺CD123⁻) were not different among the two groups (Fig. 2B).

Subsequently, we analyzed the expression of co-stimulatory molecules, including B7-1 (CD80), B7-2 (CD86), an activation marker (CD83) and a chemokine receptor (CCR7) by recording geometric mean fluorescence intensities (MFIs) (Fig. 2C). Notably, the expression level of CD86 was significantly increased in HCC patients (204.5 ± 120.5 , $P < .05$) compared with that of healthy controls (97.7 ± 42.3). The expression levels of CD80, CD83 and CCR7 were similar in the two groups.

3.2. Functions of DCs in patients and healthy controls

The endocytic and phagocytic capacities of DCs were assessed by measurement of FITC dextran uptake using flow cytometry. A representative result is shown in Fig. 3A. MFIs of DCs induced from PBMCs of HCC patients indicate more FITC dextran uptake than those from healthy controls (223.3 ± 82.9 vs. 113.1 ± 35.4 , $P < .05$).

Next, we analyzed their ability to produce inflammatory cytokines that influence T-cell function. The spontaneous IL-12p40 production of DCs was measured using an enzyme-linked immunosorbent assay (ELISA) (Fig. 3B). The concentrations of IL-12p40 in the supernatant did not differ between HCC patients and healthy controls (11.4 ± 3.2 vs. 14.2 ± 7.5 , $P = .29$).

In contrast, regarding T-cell stimulatory activity of DCs in allogeneic MLR, which was expressed as stimulation index value of [³H]-thymidine

incorporation at the T-cell/DC ratio of 10/1, the index values were significantly lower in HCC patients than in healthy controls (1.9 ± 1.1 vs. 3.5 ± 0.5 , $P < .05$) (Fig. 3C).

3.3. Yield and phenotype of DCs with different stimulations

Considering effective antitumor immunity of DC-based immunotherapy, maturational status and T-cell stimulatory potential of DCs are important. Therefore, we next examined the effects of different maturation stimuli on the phenotypes and the functions of DCs induced from PBMCs in HCC patients. As shown in Fig. 1, PBMCs of HCC patients were differentiated into immature DCs in the presence of IL-4 and GM-CSF on day 5 and then harvested on day 7 after culturing for two additional days in several activation stimuli.

First, the expressions of HLA-DR, various costimulatory molecules and chemokine receptor, which were the indicators of DC maturation, were assessed (Fig. 4). By culturing with the additional stimuli (Methods II, III and IV), the percentage of CD14⁻HLA-DR⁺ DCs was significantly increased compared with that in the medium alone (Method I) (Fig. 4A). However, the percentage of CD11c⁺CD123⁻ DCs was not different in all groups.

In the next step, we assessed the geometric mean fluorescence intensities of CD80, CD83, CD86 and CCR7 (Fig. 4B). The expressions of CD80 and CD86 of DCs with the stimulation (Methods II, III and IV) increased significantly compared with those with medium alone (Method I). Furthermore, the DCs stimulated with OK-432 (Method II) or cytokine cocktail with PGE2 (Method IV) showed a significant increase of CD80 and CD86 in comparison with those stimulated with cytokine cocktail without PGE2 (Method III). The expression level of CD83 was increased by OK-432 (Method II) and cytokine cocktail with PGE2

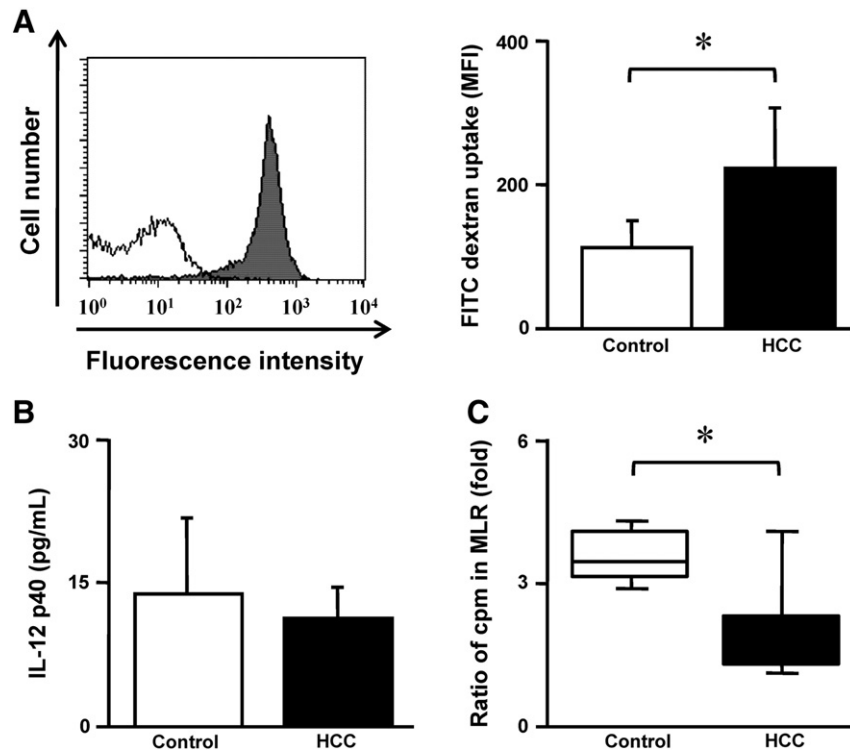


Fig. 3. Analysis of functions of DCs. (A) The phagocytic capacity of DCs was assessed by measurement of FITC dextran uptake by flow cytometry. A representative analysis is shown in the left panel. The shaded curves indicate MFIs of DCs incubated with FITC dextran for 30 min at 37 °C, and the unshaded curves are those of control DCs incubated without FITC dextran at the same time. MFIs of positive cells in healthy controls (white bar) and HCC patients (black bar) are indicated in the right panel. Data are expressed as MFI \pm SD. (B) The capacity of cytokine production of DCs in healthy controls and HCC patients. (C) T-cell stimulatory capacity of DCs was evaluated by allogeneic MLR. White and black bars indicate healthy controls and HCC patients, respectively. * $P < .05$.

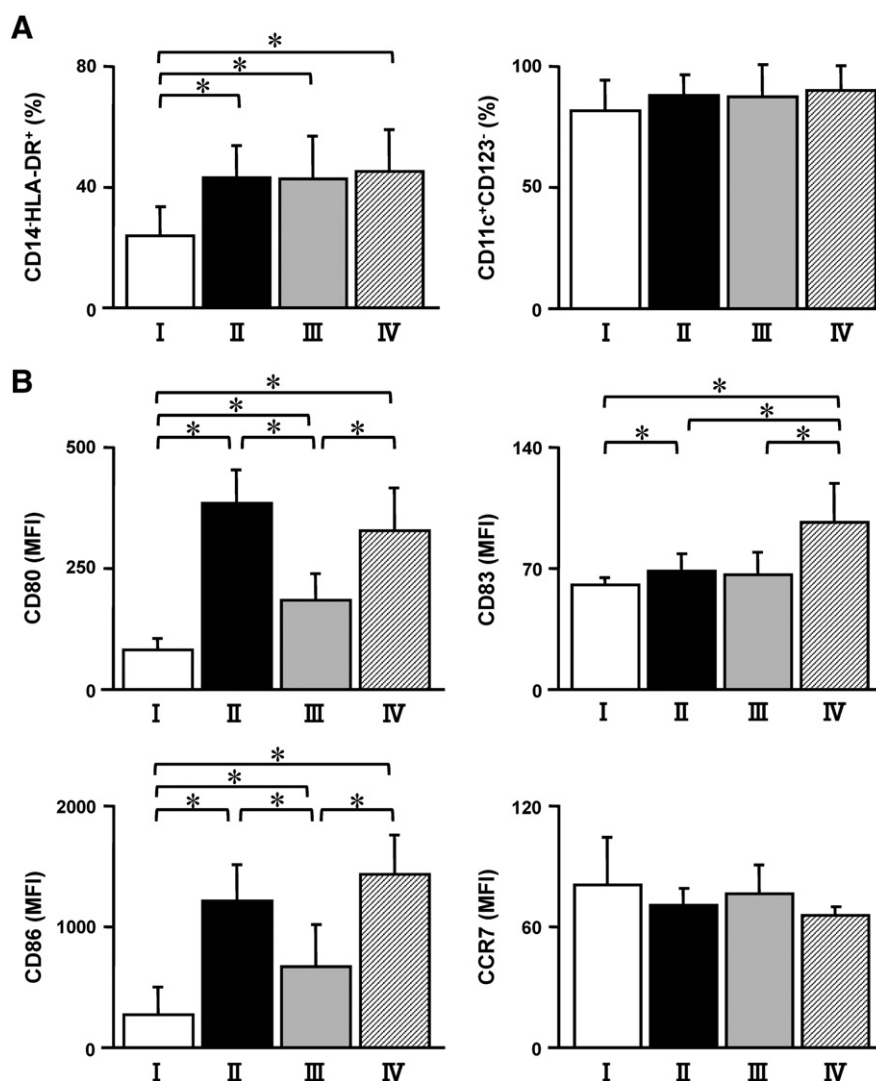


Fig. 4. Comparison of the yield and phenotype of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The yields of CD14⁺HLA-DR⁺ and CD11c⁺CD123⁻ DCs are shown in left and right panels, respectively. (B) The surface markers consisting of CD80, CD83, CD86 and CCR7 on DCs were evaluated by flow cytometry. White, black, gray and hatched bars indicate the methods of DC maturation with methods I, II, III and IV, respectively. The methods of DC maturation are shown in Fig. 1. **P* < .05.

(Method IV) stimulation. The expression of CCR7 was not different among the groups.

3.4. Function of DCs with different stimulations

Initially, the change of phagocytic capacity of DCs induced from PBMCs in HCC patients was assessed by the same protocol (shown above) (Fig. 5A). The uptake of FITC dextran of DCs stimulated with OK-432, cytokine cocktail with and without PGE2 (Methods II, III and IV) was decreased significantly compared with that of DCs cultured in medium alone (Method I) (*P* < .05).

Next, we also examined cytokine production, such as IL-12p40 and IFN- γ , of DCs by ELISA (Fig. 5B). The DCs stimulated with OK-432 (Method II) produced much more IL-12p40 and IFN- γ than the DCs stimulated with cytokine cocktail with and without PGE2 (Methods IV and III, respectively) or medium alone (Method I) (*P* < .05). In the analysis of IFN- γ production, the DCs stimulated with OK-432 (Method II) produced the largest amount of IFN- γ among the groups (*P* < .05).

3.5. Allo-stimulatory capacity of DCs with different stimulations

To assess whether the enhanced expression of costimulatory molecules reflects the antigen presentation capacity, we studied the function

using an allo-MLR (Fig. 5C). Stimulation with OK-432 (Method II) or cytokine cocktail with PGE2 (Method IV) was more efficient in inducing T-cell proliferation than that with cytokine cocktail without PGE2 (Method III) or medium alone (Method I). Moreover, the index value of DCs stimulated with OK-432 was significantly higher than that of DCs stimulated with cytokine cocktail with PGE2 (9.9 ± 3.9 vs. 4.7 ± 1.7 , *P* < .05).

To evaluate the mechanism of strong allo-stimulatory capacity of DCs induced by OK-432 in HCC patients, the cytokine levels in allogeneic MLR supernatant were examined using Bioplex assay (Fig. 6). The levels of cytokines in the medium containing DCs with OK-432 stimulation (Method II), such as IL-2, IL-12p70, IFN- γ , TNF- α , IL-13 and MIP1 α , were significantly higher than those in the medium containing DCs with medium alone (Method I) (*P* < .05) (Fig. 6A). In addition to an increase of these cytokines and chemokines, other cytokines including IL-4, IL-10 and IL-17 were also significantly increased in the medium in which DCs with OK-432 stimulation and PBMCs were co-cultured (Fig. 6B).

4. Discussion

Immunotherapy is a promising therapy for HCC patients and a number of the therapies have been evaluated [14]. Among the numerous

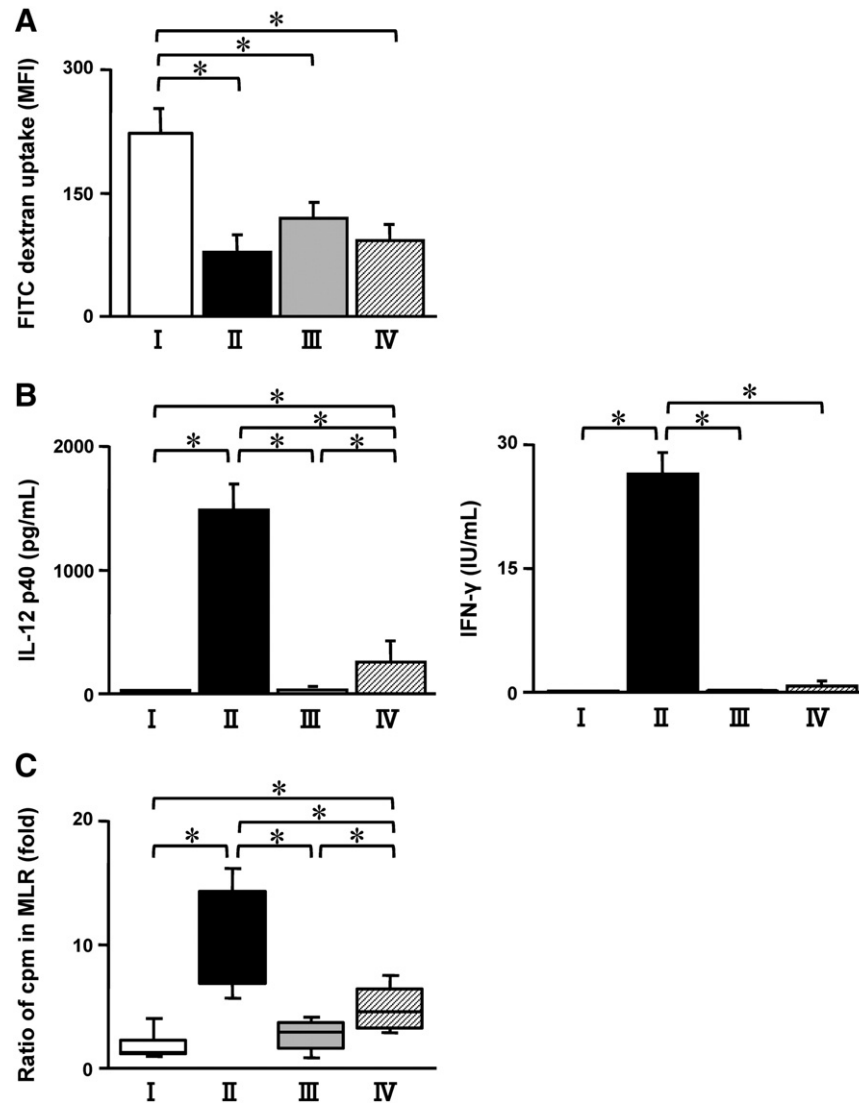


Fig. 5. Comparison of the functions of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The phagocytic capacity of DCs was evaluated by the uptake of FITC dextran using flow cytometry. (B) The capacity of cytokine production of DCs was measured by ELISA. (C) T-cell stimulatory capacity of DCs was evaluated by allogeneic MLR. White, black, gray and hatched bars indicate the methods of DC maturation with methods I, II, III and IV, respectively. The methods of DC maturation are shown in Fig. 1. * $P < .05$.

immune cells, DCs are the most potent type of antigen-presenting cells in the human body. However, impaired function of DCs has been implicated in the escape of the tumor from immune control in cancer patients [15]. In chronic HCV-infected patients, recent studies have shown that the function of MoDCs is not necessarily impaired [16], while several groups have reported a maturation defect and impaired function in HCC patients [17].

In the present study, the number and cell surface maturation markers of harvested DCs derived from PBMCs of HCC patients are not different from those of healthy controls. In particular, the results that the percentage of CD11c⁺CD123[−] myeloid DCs was not different suggested that the culture method using IL-4 and GM-CSF is suitable for the induction of effective DCs in HCC immunotherapy. Because myeloid DC is a main player that produces cytokines such as IL-12, induces T-helper type 1 (Th1) response and antigen-specific cytotoxic T-cell immunity [18].

In the analysis of maturation markers, the DCs in HCC patients showed similar expression levels of CD80, CD83, CCR7 and even a high expression level of CD86 compared with the DCs in healthy donors. However, the result of FITC dextran uptake indicated that the DCs in

HCC patients showed high ability, suggesting that their functional phenotype is still immature. Consistent with these results, stimulatory capacity of these DCs in MLR was lower than that of healthy controls, suggesting that additional treatment is required for optimal DC preparation.

Unfortunately, there is still no consensus on the optimal procedure for preparation of DCs using the drugs with good manufacturing practice (GMP) grade. For the clinical application of DC-based immunotherapy, it is desirable to use maturation agents with GMP grade for the safety of patients. Therefore, in this study, we evaluated the procedures using GMP-grade compounds. OK-432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyogenes*, was reported to be immunomodulatory and have potential therapeutic properties for cancer immunotherapy [19,20]. DCs stimulated with OK-432 have been suggested to acquire a mature phenotype, produce a significant amount of T-helper type 1 (Th1) cytokines such as IL-12 and IFN-γ and enhance cytotoxic T-lymphocyte activity [21]. Otherwise, many cytokines, often containing pro-inflammatory mediators, or their combinations have been tested for DC maturation. Since 1997, cytokine cocktails containing TNF-α, IL-1β, IL-6 and PGE2 have been shown to induce DC maturation

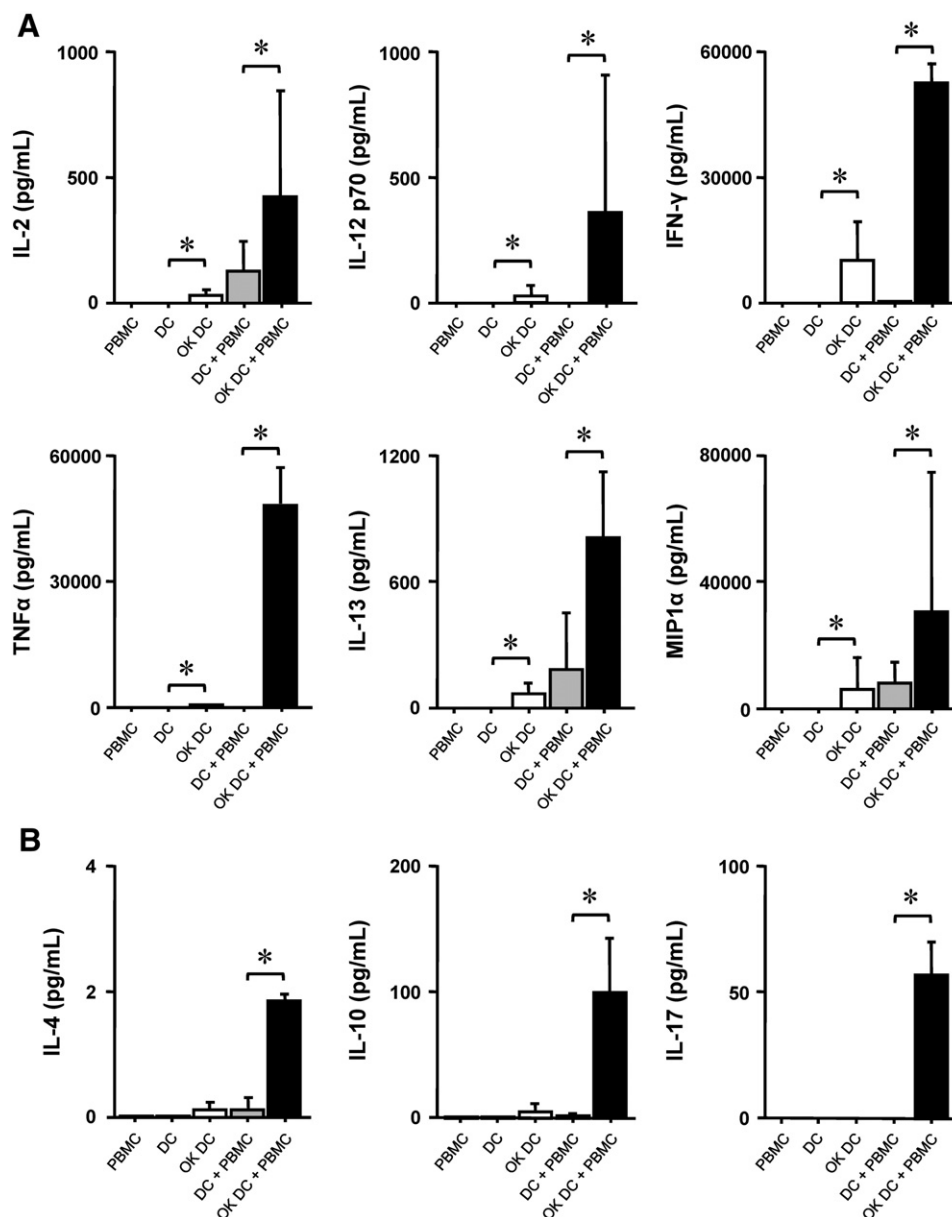


Fig. 6. Cytokine production of DCs induced from PBMCs of HCC patients with and without OK-432 stimulation in MLR. The cytokine levels of culture supernatants in allogeneic MLR were assessed by Bioplex assay. As controls, supernatants of medium cultured with PBMCs alone and DCs alone with and without OK-432 stimulation were used. (A) The concentrations of cytokines and chemokines such as IL-2, IL-12p70, IFN-γ, TNF-α, IL-13 and MIP1α in the medium cultured with 5 different conditions as indicated below. (B) The concentrations of cytokines such as IL-4, IL-10 and IL-17 were significantly higher in the medium cultured with OK-432-stimulated DCs and PBMCs than those with OK-432-unstimulated DCs and PBMCs. Dotted, hatched, white, gray and black bars indicate the cytokine levels in the medium cultured with PBMCs alone, OK-432-unstimulated DCs alone, OK-432-stimulated DCs alone, OK-432-unstimulated DCs with PBMCs and OK-432-stimulated DCs with PBMCs, respectively. * $P < .05$.

[22]. In addition, adding PGE2 to the maturation cocktail was reported to enhance further CCR7 expression, migration capacity and T-cell stimulatory activity of DCs even in patients with advanced cancer [23]. In this study, on the basis of these results, we analyzed DCs derived from PBMCs of HCV-related HCC patients by OK-432 or cytokine cocktails.

Both methods, using OK-432 or cytokine cocktails, had success in enhancing the expression levels of CD80, CD83 and CD86. The phagocytic capacity of the DCs induced by both methods was lower than that of the DCs induced by a standard method. On the other hand, the production of cytokines such as IL-12p40 and IFN-γ and allostimulatory capacity were excellent in DCs with OK-432 stimulation. These results suggest that both methods are useful for maturation, but OK-432 stimulation is the best method of DC preparation for immunotherapy in HCV-related HCC patients. Moreover, the types of cytokines and chemokines detected in allogeneic MLR were very similar to those

that we previously reported in serum of patients who received immunotherapy with OK-432-stimulated DCs [13]. Taken together with these results, the DCs stimulated with OK-432 may have immunological potential in not only local but also systemic responses through cytokine production.

In conclusion, the results of the present study suggest that the clinically approved compound OK-432 is a candidate for highly immunocompetent DC preparation in HCV-related HCC patients and should provide us with a novel insight for immunotherapy of HCC.

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