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 μL of serum-free medium (GMP CellGro DC Medium) and co-cultured in 96-well round-bottomed microplates. After 2 days at 37 °C, 1 μCi 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 ± 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

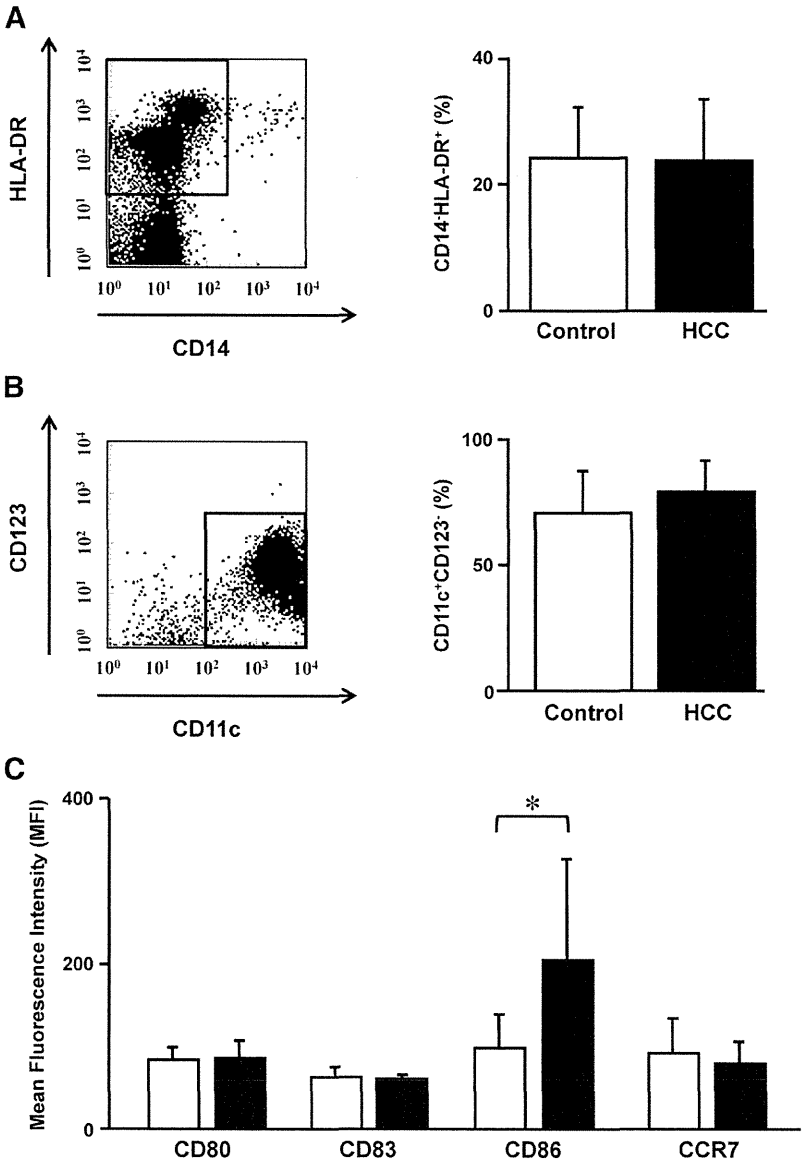

Fig. 2. The yield and characteristics of DCs derived from PBMCs in the presence of IL-4 and GM-CSF (Method I). (A) The yield of CD14$^-$HLA-DR$^+$ DCs in healthy controls and HCC patients, which was calculated as a percentage of the cells gated on side and forward scatter. (B) The yield of CD11c$^+$CD123$^-$ 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) ± 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 ($lin1^{-}HLA\text{-}DR^{+}$) 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 [^{3}H]-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 $CD14^{-}HLA\text{-}DR^{+}$ 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

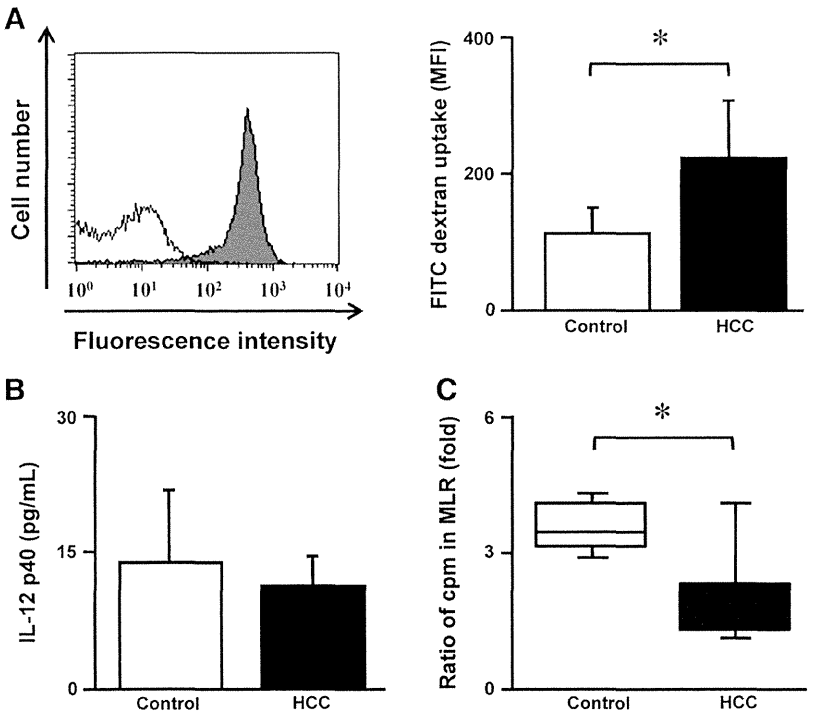

Fig. 3. Analysis of functions of DCs. (A) The phagocytic capacity of DCs was assessed by measurement of FITC dextran uptake by flow cytometry. A representative analysis is shown in the left panel. The shaded curves indicate MFIs of DCs incubated with FITC dextran for 30 min at 37 °C, and the unshaded 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 ± 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. *$P < .05$.

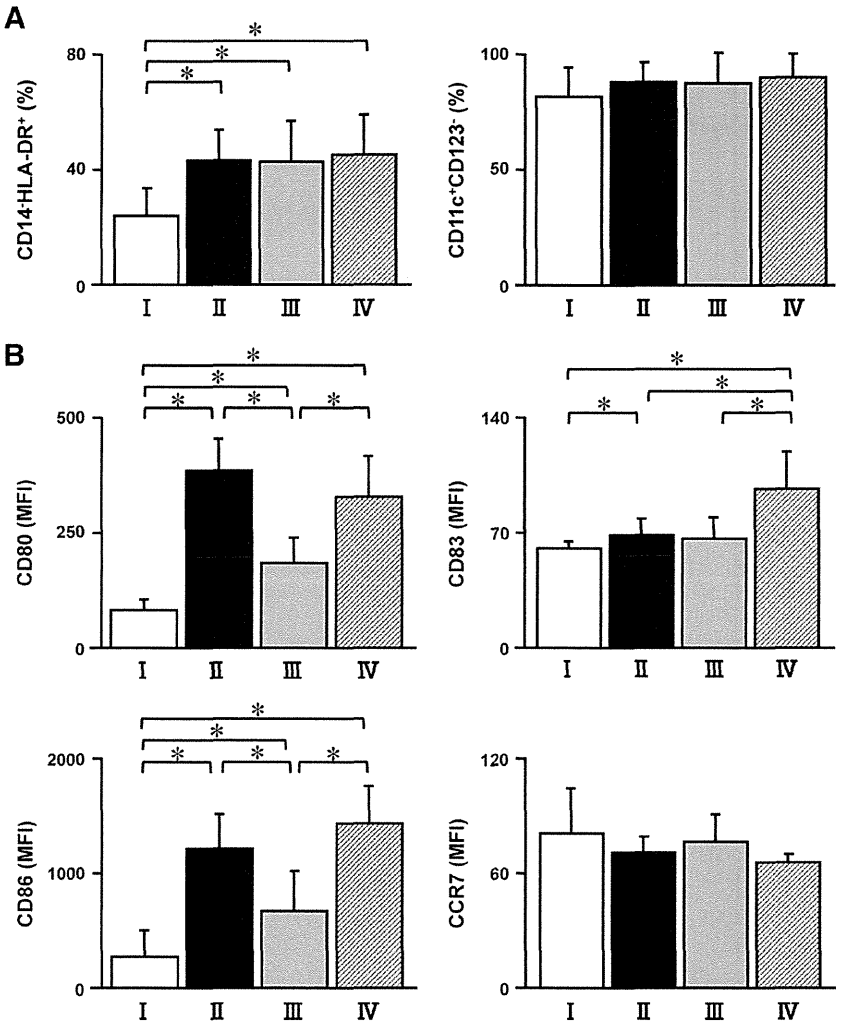

Fig. 4. Comparison of the yield and phenotype of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The yields of $CD14^-HLA\text{-}DR^+$ and $CD11c^+CD123^-$ DCs are shown in left and right panels, respectively. (B) The surface markers consisting of CD80, CD83, CD86 and CCR7 on DCs 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 MIP1α, 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

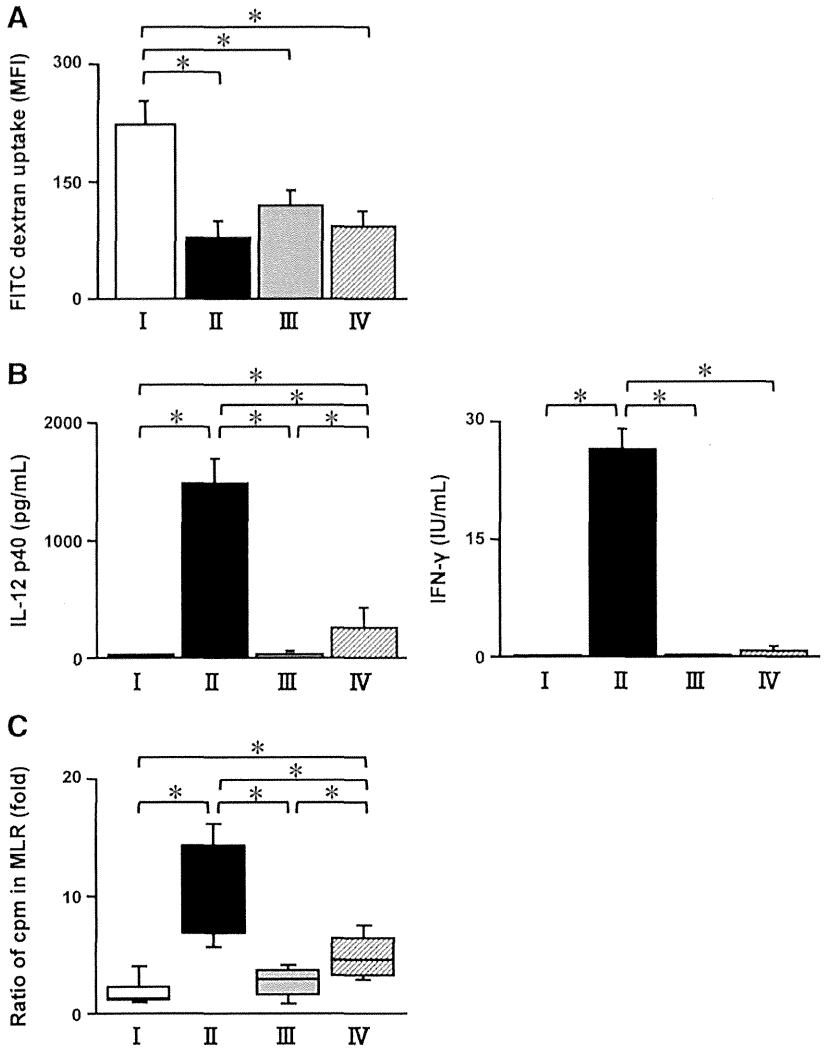

Fig. 5. Comparison of the functions of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The phagocytic capacity of DCs was evaluated by the uptake of FITC dextran using flow cytometry. (B) The capacity of cytokine production of DCs was measured by ELISA. (C) T-cell stimulatory capacity 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 pyrogenes*, 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 IFNγ 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

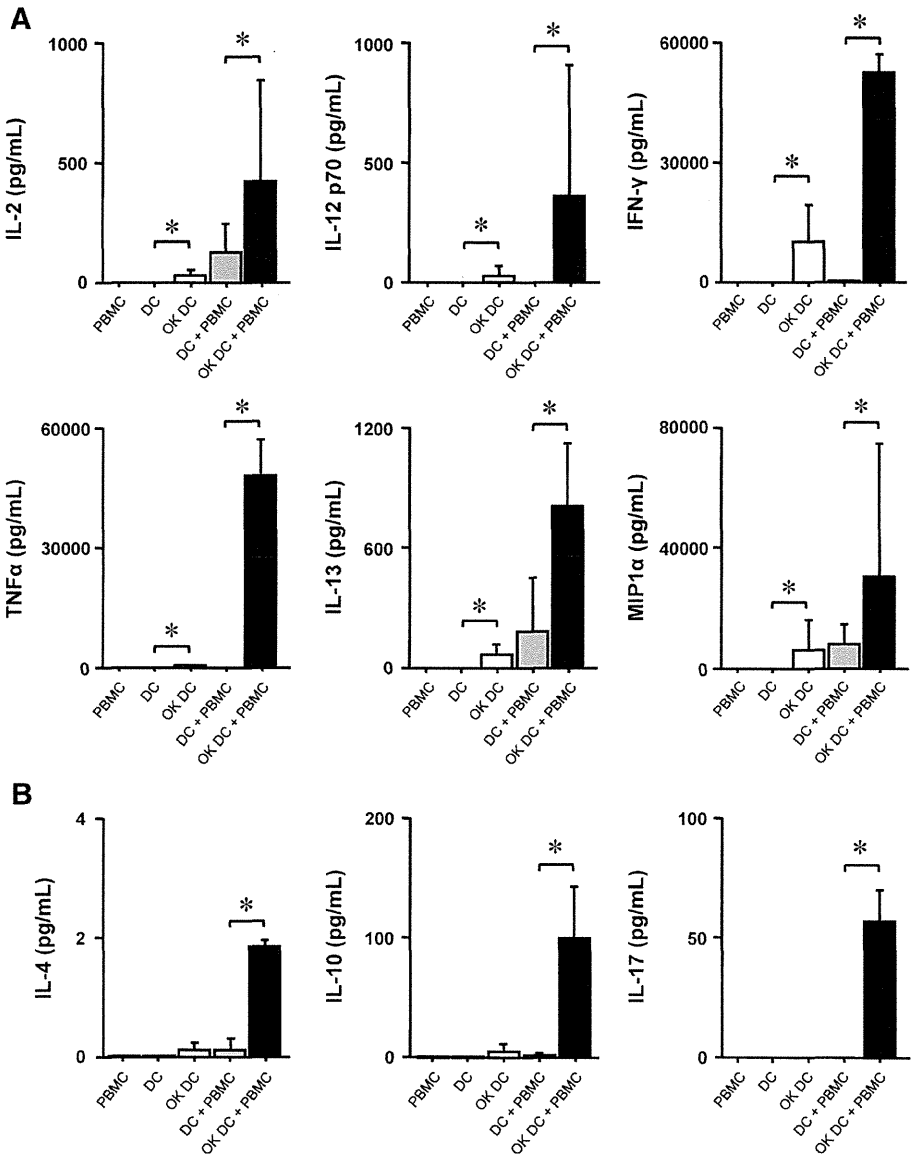

Fig. 6. Cytokine production of DCs induced from PBMCs of HCC patients with and without OK-432 stimulation in MLR. The cytokine levels of culture supernatants in allogeneic MLR were assessed by Bioplex assay. As controls, supernatants of medium cultured with PBMCs alone and DCs alone with and without 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.

Acknowledgments

The authors thank Mariko Katsuda, Kazumi Fushimi and Maki Kawamura for technical assistance. We also thank the patients for participating in this study. This work was supported in part by research

grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Cancer Immunol Immunother (2014) 63:479–489
DOI 10.1007/s00262-014-1529-8

ORIGINAL ARTICLE

P53, hTERT, WT-1, and VEGFR2 are the most suitable targets for cancer vaccine therapy in HLA-A24 positive pancreatic adenocarcinoma

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Received: 23 May 2013 / Accepted: 22 February 2014 / Published online: 16 March 2014

Abstract Cancer vaccine therapy is one of the most attractive therapies as a new treatment procedure for pancreatic adenocarcinoma. Recent technical advances have enabled the identification of cytotoxic T lymphocyte (CTL) epitopes in various tumor-associated antigens (TAAs). However, little is known about which TAA and its epitope are the most immunogenic and useful for a cancer vaccine for pancreatic adenocarcinoma. We examined the expression of 17 kinds of TAA in 9 pancreatic cancer cell lines and 12 pancreatic cancer tissues. CTL responses to 23 epitopes derived from these TAAs were analyzed using enzyme-linked immunospot (ELISPOT), CTL, and tetramer assays in 41 patients, and factors affecting the immune responses were investigated. All TAAs were frequently expressed in pancreatic adenocarcinoma cells, except for adenocarcinoma antigens recognized by T cells 1, melanoma-associated antigen (MAGE)-A1, and MAGE-A3. Among the epitopes recognized by CTLs in more than two patients in the ELISPOT assay, 6 epitopes derived from 5 TAAs, namely, MAGE-A3, p53, human telomerase reverse transcriptase (hTERT), Wilms tumor (WT)-1, and vascular endothelial growth factor receptor (VEGFR)2, could induce specific CTLs that showed cytotoxicity against pancreatic cancer cell lines. The frequency of lymphocyte subsets correlated well with TAA-specific immune response. Overall survival was significantly longer in patients with TAA-specific CTL responses than in those without. P53, hTERT, WT-1, and VEGFR2 were shown to be attractive targets for immunotherapy in patients with pancreatic adenocarcinoma, and the induction of TAA-specific CTLs may improve the prognosis of these patients.

Keywords Epitope · Immunotherapy · Cytotoxic T lymphocyte (CTL) · Enzyme-linked immunospot (ELISPOT)

Abbreviations

CTL	Cytotoxic T lymphocyte
TAA	Tumor-associated antigen
ELISPOT	Enzyme-linked immunospot
MAGE	Melanoma-associated antigen
hTERT	Human telomerase reverse transcriptase
WT-1	Wilms tumor-1
VEGFR	Vascular endothelial growth factor receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

Electronic supplementary material The online version of this article (doi:10.1007/s00262-014-1529-8) contains supplementary material, which is available to authorized users.

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Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death worldwide [1]. Despite recent advances in diagnostic techniques, pancreatic adenocarcinoma is diagnosed at an advanced stage in most patients and, consequently, the overall 5-year survival rate is <5 % [2]. Thus, the development of a new treatment option is needed to improve the prognosis of pancreatic cancer patients without toxicity.

Immunotherapy is one of the most attractive therapies as a new treatment procedure for melanoma and other solid tumors [3]. Recent technical advances have enabled the identification of various tumor-associated antigens (TAAs) [4–21]; however, few of their epitopes are inducers of cytotoxic T lymphocyte (CTL) responses against tumors [22]. Several kinds of epitope have also been identified in patients with pancreatic adenocarcinoma [23, 24]. However, previous studies focused on the identification and evaluation of a particular antigen, and different TAAs have not yet been compared simultaneously; therefore, little is known about which epitope is the most immunogenic and useful in eliciting clinical responses in pancreatic adenocarcinoma patients.

In the present study, we compared CTL responses with various TAA-derived epitopes in identical patients with pancreatic adenocarcinomas and examined the factors that affect immune responses. This approach provided information that is useful for selecting immunogenic TAAs and suitable patients and developing a new immunotherapy for pancreatic adenocarcinoma.

Materials and methods

Patients and clinical information

In this study, we examined 41 HLA-A24-positive patients with pancreatic adenocarcinoma and 14 healthy volunteers who were HLA-A24-positive, but did not have any cancers, as negative controls. Fine-needle biopsy, a surgical specimen, or autopsy was used for the pathological diagnosis of pancreatic adenocarcinoma in 18 patients. Diagnosis of the remaining 23 patients was achieved using the radiological findings of computed tomography and/or magnetic resonance imaging. We investigated patient background, treatment procedures, and outcomes.

Clinical information was obtained from the medical records of patients. We evaluated the tumor stage using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (7th version) (UICC stage). The frequency of lymphocyte subsets was calculated by dividing the absolute lymphocyte count by the absolute leukocyte count. HLA typing of peripheral blood mononuclear cells (PBMCs) from patients and healthy volunteers was performed by the reverse sequence-specific oligonucleotide with polymerase chain reaction (PCR-RSSO). This study was approved by the Ethics Committees of Kanazawa University (No. 1237) and Kanazawa Medical Center (No. 17), and all patients gave written informed consent to participate in accordance with the Helsinki Declaration.

Table 1 Peptides used in this study

Peptide No.	TAA	Amino acid sequence	Reference
1	$ART1_{188}$	EYCLKFTKL	[14]
2	$ART4_{161}$	AFLRHAAL	[11]
3	$ART4_{899}$	DYPSLSATDI	[11]
4	$Cyp\text{-}B_{109}$	KFHRVIKDF	[7]
5	$Cyp\text{-}B_{315}$	DFMIQGGDF	[7]
6	Lck_{208}	HYTNASDGL	[8]
7	Lck_{488}	DYLRSVLEDF	[8]
8	$MAGE\text{-}A1_{135}$	NYKHCFPEI	[6]
9	$MAGE\text{-}A3_{195}$	IMPKAGLLI	[16]
10	$SART1_{690}$	EYRGFTQDF	[12]
11	$SART2_{899}$	SYTRLFLIL	[13]
12	$SART3_{109}$	VYDYNCHVDL	[21]
13	$Her\text{-}2/neu_8$	RWGLLLALL	[17]
14	$p53_{161}$	AIYKQSQHM	[18]
15	$p53_{204}$	EYLDDRNTF	[5]
16	$MRP3_{765}$	VYSDADIFL	[20]
17	$MRP3_{503}$	LYAWEPSFL	[20]
18	$hTERT_{461}$	VYGFVRACL	[4]
19	$hTERT_{324}$	VYAETKHFL	[4]
20	$WT\text{-}1_{235}$	CMTWNQMNL	[15]
21	$VEGFR2_{169}$	RFVPDGNRI	[19]
22	$VEGFR1_{1084}$	SYGVLLWEI	[10]
23	$survivin2B_{80}$	AYACNTSTL	[9]
24	HIV env_{584}	RYLRDQQLL	[25]
25	CMV $pp65_{328}$	QYDPVAALF	[26]

Synthetic peptides and preparation of PBMCs

The 23 epitopes derived from 17 different TAAs used in the present study are listed in Table 1. We selected epitopes that had previously been identified as HLA-A24-restricted and suggested to have immunogenicity in various cancers not restricted to pancreatic cancer [4–21]. Epitopes derived from the HIV envelope protein (HIV env_{584}) [25] and cytomegalovirus (CMV) pp65 (CMV$pp65_{328}$) [26] were also used to assess T cell responses. Peptides were synthesized at Mimotope (Melbourne, Australia), Sumitomo Pharmaceuticals (Osaka, Japan), COSMO BIO Co. (Tokyo, Japan), and Scrum Inc. (Tokyo, Japan). Purities were determined to be >80 % by analytical high-performance

liquid chromatography (HPLC). PBMCs were separated as described below; heparinized venous blood was diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque (Sigma, St. Louis, MO) in 50-ml tubes. After centrifugation at 2,000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS, centrifuged at 1,400 rpm for 10 min, and finally resuspended in complete culture medium consisting of RPMI (GibcoBRL, Grand Island, NY), 10 % heat-inactivated FCS (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

Cell lines

The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10 % FCS and 500 μg/ml hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10 % FCS [27]. MiaPaca2, AsPC1, BxPC3, Panc-1, CAPAN1, and CAPAN2 were purchased from the American Type Culture Collection (VA, USA). YPK-1 and YPK-2 were kind gifts from Prof. Oka and Dr. Yoshimura (Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan). PK-1 was provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Human pancreatic cancer cell lines were cultured in DMEM (GibcoBRL) or RPMI 1640 medium containing 10 % fetal calf serum (FCS). All media contained 100 U/mL penicillin and 100 μg/mL streptomycin.

RNA preparation and real-time PCR

The expression of TAA messenger RNA (mRNA) in human pancreatic cancer cell lines and pancreatic adenocarcinoma tissues was analyzed by real-time polymerase chain reaction (PCR). Cell lines were harvested, centrifuged, and washed with PBS, and total RNA was then isolated using Quick-Gene (Fuji Film, Tokyo). Total RNA from frozen pancreatic adenocarcinoma samples was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol. cDNA was synthesized from 150 ng of total RNA using a high-capacity cDNA reverse transcription kit (PE Applied Biosystems, CA, USA) and was then mixed with TaqMan Universal Master Mix (PE Applied Biosystems) and each TaqMan probe. Primer pairs and probes for various TAAs and β-actin were obtained from the TaqMan assay reagents library. Thermal cycling conditions were 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 1 min. cDNA was subjected to quantitative real-time PCR analyses targeting various TAAs and β-actin. Analyses were performed using the StepOne Real-Time PCR system and StepOne v2.0 software. Relative gene expression values were determined. Data are presented as fold differences in TAA expression normalized to the housekeeping gene β-actin as an endogenous reference.

Enzyme-linked immunospot assay (ELISPOT assay)

Ninety-six-well plates (Millititer, Millipore, Bedford, MA) were coated with anti-human interferon-γ (IFN-γ) (Mabtech, Nacka, Sweden) at 4 °C overnight and then washed 4 times with sterile PBS. The plates were then blocked with RPMI 1640 medium containing 5 % FCS for 2 h at room temperature. A total of 300,000 unfractionated PBMCs were added in duplicate cultures of RPMI 1640 containing 5 % FCS together with the peptides at 10 μg/ml. After 24 h, the plates were washed 8 times with PBS and incubated overnight with 100 μl of the biotin-conjugated anti-human IFN-γ antibody. After another 4 washes with PBS, streptavidin-AP was added for 2 h. Finally, the plates were washed again 4 times with PBS and developed with freshly prepared NBT/BCIP solution (Biorad, Hercules, CA). The reaction was stopped by washing with distilled water and drying at room temperature. Colored spots with fuzzy borders, which indicated the presence of IFN-γ-secreting cells, were counted. The number of specific spots was determined by subtracting the number of spots in the absence of the antigen. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least two-fold than that in its absence.

Peptide-specific CTL induction and cytotoxicity assay

Synthetic peptide-specific T cells were expanded from PBMCs in 96-well round-bottom plates (NUNC, Naperville, IL). Four hundred thousand cells/well were stimulated with synthetic peptides at 10 μg/ml, 10 ng/ml rIL-7, and 100 pg/ml rIL-12 (Sigma) in RPMI 1640 supplemented with 10 % heat-inactivated human AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were restimulated with 10 μg/ml peptide, 20 U/ml rIL-2 (Sigma), and 10^5 mitomycin C-treated autologous PBMCs as feeder cells on days 7 and 14. One hundred microliters of RPMI medium with 10 % human Ab serum and rIL-2 at a final concentration of 10 U/ml were added to each well on days 4, 11, and 18. The cytotoxicity assay was conducted on day 22.

The C1R-A24 and human pancreatic cancer cell lines were used as target cells for the ^{51}Cr release assay. C1R-A24 cells were incubated overnight with 10 μg/ml synthetic peptides and labeled with 25 μCi of ^{51}Cr for 1 h. Pancreatic cancer cell lines were also labeled with 25 μCi of ^{51}Cr for 1 h without incubation with peptides. After three washes with PBS, target cells were plated at 3,000 cells/well in complete medium

in round-bottom 96-well plates. Unlabeled K562 (120,000 cells/well) was added to reduce non-specific lysis. Peptide-stimulated PBMCs were added at various effector-to-target ratios as indicated. Maximum release was determined by the lysis of ^{51}Cr-labeled targets with 5 % Triton X-100 (Sigma Chemical). Spontaneous release was <10 % of maximum release for all experiments, except for when it was <15 % when the target cells were human pancreatic cancer cell lines. Percent-specific cytotoxicity was determined using the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release), and specific cytotoxic activity was calculated as follows: (cytotoxic activity in the presence of the peptide) − (cytotoxic activity in the absence of the peptide). Specific cytotoxicity of more than 10 % was considered to be positive.

Tetramer staining and flow cytometry

TAA-specific tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). Tetramer staining was performed as described below. One million isolated PBMCs or peptide-specific CTLs pulsed with TAA-derived peptides were washed, resuspended in 200 μl of PBS without calcium or phosphate, and stained with 40 μg/ml tetrameric complexes and monoclonal antibodies against cell surface proteins for 30 min at room temperature. The following monoclonal antibodies were used: anti-CD8-APC (BD PharMingen, San Diego, CA), anti-CCR7-FITC, anti-CD45RA-PerCP, and tetramer-PE. Cells were washed, fixed with 0.5 % paraformaldehyde/PBS, and analyzed on a Becton–Dickinson FACSAria II system.

Statistical analysis

Fisher's exact test and unpaired Student's *t* test were used to analyze the effect of variables on immune responses in pancreatic cancer patients. Overall survival was calculated from the day of pancreatic cancer diagnosis until the date of death or the last day of the follow-up period. Cumulative survival proportions were calculated using the Kaplan–Meier method, and any differences were evaluated using the log-rank test. A p value of <0.05 was considered to be significant, and all the tests were two-sided. All statistical analyses were performed using the SPSS statistical software program package (SPSS version 11.0 for Windows).

Results

Patients

Patient characteristics are summarized in the Supplementary Table. The median age of patients was 72 years, and patients included 24 males (59 %). The main localization of the tumors was the pancreatic head in 39 % of patients and the pancreatic body or tail in 61 %. The majority of patients (93 %) had advanced-stage cancer, namely, UICC stage III or IV. Therapeutic procedures mainly involved chemotherapy consisting of protocols such as gemcitabine monotherapy, S-1 monotherapy, or a combination of both drugs. Only 11 patients received the best supportive therapy to relieve physical and spiritual pain. A total of 61 % of patients had died by the last day of the follow-up period, and the median overall survival time of patients was 7.2 months.

TAA expression in pancreatic cancer cell lines and human cancer tissues

We evaluated the expression of 17 different TAAs in 9 human pancreatic cancer cell lines using real-time PCR. Although differences were observed from cell to cell, TAAs were expressed in more than 40 % of pancreatic adenocarcinoma cell lines, except for adenocarcinoma antigens recognized by T cells (ART)1 (11 %) and ART4 (33 %) (Table 2). We then investigated TAA expression in 7 surgical and 5 autopsy specimens. The expression of most TAAs in pancreatic adenocarcinoma specimens was similar to or more frequent than that in human pancreatic cancer cell lines, except for melanoma-associated antigen (MAGE)-A1 and MAGE-A3 (Table 2).

Detection of TAA-specific T cells by IFN-γ ELISPOT analysis

IFN-γ ELISPOT responses were evaluated with PBMCs to determine how frequently T cells respond to TAA-derived peptides and control peptides in patients with pancreatic adenocarcinoma (Fig. 1a). Positive responses to at least one TAA-derived peptide were observed in 28 of 41 (68 %) patients. On the other hand, 14 of 23 (61 %) peptides were recognized by T cells obtained from at least one patient. $ART1_{188}$, $ART4_{161}$, $ART4_{899}$, lymphocyte-specific protein tyrosine kinase $(Lck)_{208}$, $MAGE\text{-}A3_{195}$, $p53_{161}$, human telomerase reverse transcriptase $(hTERT)_{461}$, $hTERT_{324}$, Wilms tumor $(WT)\text{-}1_{235}$, vascular endothelial growth factor receptor $(VEGFR)2_{169}$, and $VEGFR1_{1084}$ were recognized in more than two patients, which suggested that these peptides have the potential to be immunogenic. Peptides 24 ($HIVenv_{584}$) and 25 ($CMVpp65_{328}$) were recognized in 0 and 38 % of patients, respectively.

Peptides $ART4_{161}$, $ART4_{899}$, Cyclophilin B $(Cyp\text{-}B)_{315}$, Lck_{208}, $hTERT_{324}$, and $VEGFR1_{1084}$ were recognized in more than one healthy volunteer, and/or the percentage of positive responses was higher in healthy volunteers than in pancreatic adenocarcinoma patients, which indicated

Table 2 Expression of various TAAs mRNA in pancreatic cancer cell lines and pancreatic cancer tissues measured by real-time PCR

TAA	Primer	Positive cell lines/ cell lines tested *n* (%)	Positive specimens/ specimens tested *n* (%)
ART1	Hs00188841_m1	1/9 (11)	5/12 (42)
ART4	Hs00221465_m1	3/9 (33)	11/12 (92)
CypB	Hs00168719_m1	9/9 (100)	12/12 (100)
Lck	Hs00178427_m1	8/9 (89)	11/12 (92)
MAGEA1	Hs00607097_m1	4/9 (43)	1/12 (8)
MAGEA3	Hs00366532_m1	4/9 (43)	1/12 (8)
SART1	Hs00193002_m1	9/9 (100)	12/12 (100)
SART2	Hs00203441_m1	9/9 (100)	12/12 (100)
SART3	Hs00206829_m1	9/9 (100)	12/12 (100)
HER2/neu	Hs00170433_m1	9/9 (100)	12/12 (100)
p53	Hs00153340_m1	9/9 (100)	12/12 (100)
MRP3	Hs00358656_m1	9/9 (100)	12/12 (100)
hTERT	Hs00162669_m1	9/9 (100)	9/12 (75)
WT-1	Hs00240913_m1	5/9 (56)	9/12 (75)
VEGFR2	Hs00911700_m1	5/9 (56)	11/12 (92)
VEGFR1	Hs01052961_m1	6/9 (67)	12/12 (100)
Survivin	Hs00153353_m1	9/9 (100)	12/12 (100)

that the responses to these peptides were not specific to T cells from patients with pancreatic adenocarcinoma (Fig. 1b). In other words, peptides ART1$_{188}$, MAGE-A3$_{195}$, p53$_{161}$, hTERT$_{461}$, WT-1$_{235}$, and VEGFR2$_{169}$ have specific immunogenic potential in patients with pancreatic adenocarcinoma.

The number of peptide-specific IFN-γ-producing T cells was counted to examine the frequency of T cells responsive to TAA-derived peptides. A range of 10–46 T cells per 300,000 PBMCs in patients with pancreatic adenocarcinoma produced IFN-γ (Fig. 1c).

TAA-specific CTL induction and cytotoxic activity

We attempted to induce peptides specific to CTLs from the PBMCs of pancreatic adenocarcinoma patients. Cytotoxicity assays were performed in more than five patients for each peptide. Of the 11 peptides recognized in more than two patients in the IFN-γ ELISPOT assay, 6 peptides (MAGE-A3$_{195}$, p53$_{161}$, hTERT$_{461}$, hTERT$_{324}$, WT-1$_{235}$, and VEGFR2$_{169}$) could induce their specific CTLs, which were confirmed to be able to respond to C1RA24 cells pulsed with corresponding peptides by the cytotoxicity assay, as shown in Fig. 2a.

We conducted a cytotoxicity assay to determine whether peptide-specific CTLs from healthy volunteers could show their cytotoxic activity against pancreatic carcinoma cell lines. P53$_{161}$-, hTERT$_{461}$-, and hTERT$_{324}$-specific CTLs showed cytotoxicity against YPK-2 (HLA-A24-, p53-, and hTERT-positive), but not against Panc-1 (HLA-A24-negative, p53- and hTERT-positive). MAGE-A3$_{195}$-, WT-1$_{235}$-, and VEGFR2$_{169}$-specific CTLs also showed cytotoxic activity against YPK-2 (HLA-A24-, MAGE-A3-, WT-1-, and VEGFR2-positive), but not against YPK-1 (HLA-A24-positive, MAGE-A3-, WT-1-, and VEGFR2-negative). Representative data are shown in Fig. 2b.

Phenotypic analysis of TAA-derived peptides specific to T cells

To analyze the characteristics of TAA-derived peptides specific to T cells and select the appropriate epitope for immunotherapy in patients with pancreatic adenocarcinoma, we performed phenotypic analysis by tetramer staining and FACS analysis. We first attempted to detect MAGE-A3$_{195}$-, hTERT$_{461}$-, and WT-1$_{235}$-specific tetramer-positive T cells in PBMCs and CTLs induced by the corresponding peptides in healthy volunteers. The ratio of tetramer-positive T cells was increased in CTLs and their frequencies were 1.481–2.930 % of CD8$^+$ T cells, suggesting that these tetramers work well (Fig. 3a). We also conducted similar assays in pancreatic adenocarcinoma patients and detected tetramer-positive T cells in CTLs (Fig. 3b).

We then examined the naïve/effector/memory phenotype of tetramer-positive cells in the PBMCs of patients. The memory phenotype was investigated by the criterion of CD45RA/CCR7 expression [28]. In tetramer analysis, the frequencies of MAGE-A3$_{195}$-, hTERT$_{461}$-, and WT-1$_{235}$-specific tetramer-positive T cells were 0.003–0.044,

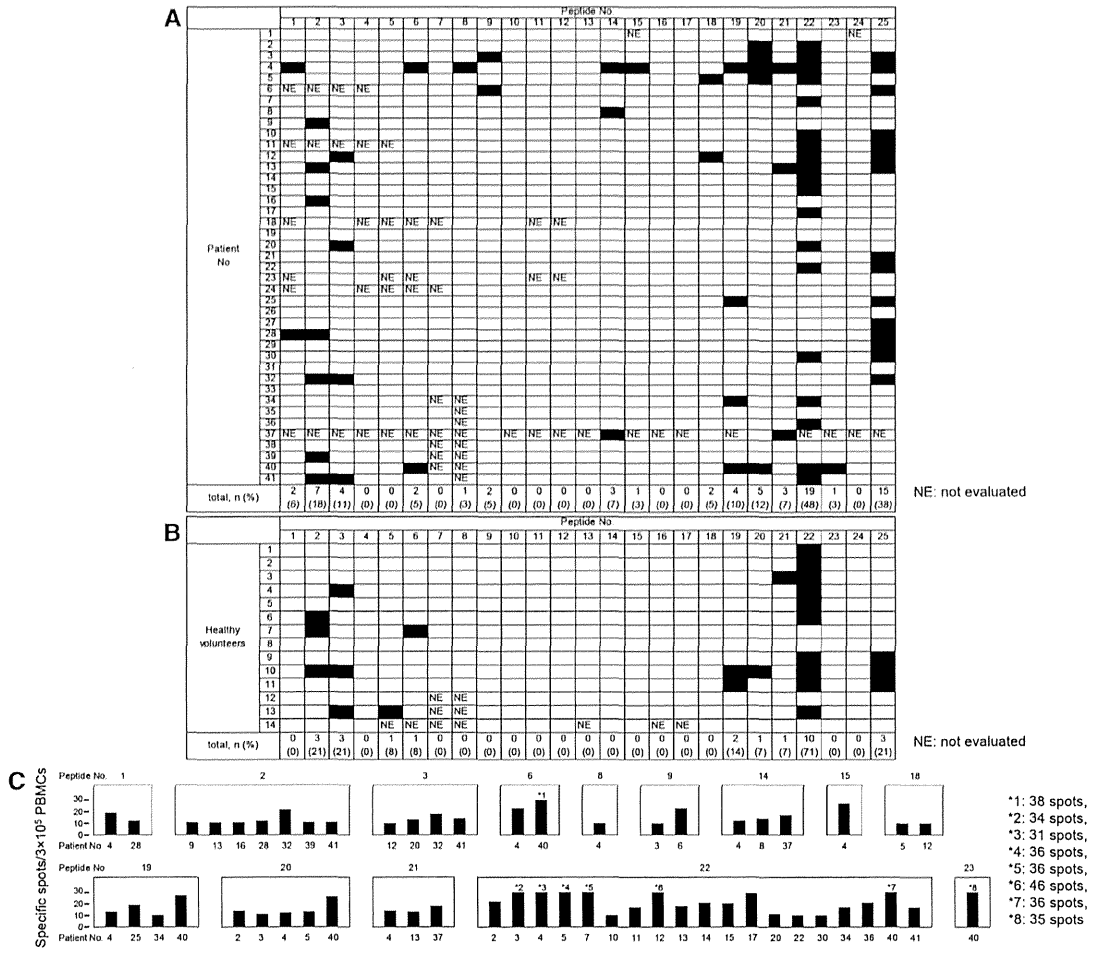

Fig. 1 T cell responses to TAA-derived peptides and control peptides in pancreatic adenocarcinoma patients **a** and healthy volunteers **b**. T cell responses were evaluated by the IFN-γ ELISPOT assay. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least twofold that in its absence. *Black boxes* indicate positive responses. **c** The frequency of TAA-specific IFN-γ-producing T cells evaluated by the ELISPOT assay. *Black bars* indicate the response of one patient

0.006–0.053, and 0.030–0.191 % of $CD8^+$ T cells, respectively. The frequency of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells in tetramer-positive cells depended on the patient and all phenotypes were observed in all patients, except for patients 1, 8, 28, 29, and 4 (Supplementary Fig. 1).

TAA-specific T cell responses and clinical features of pancreatic cancer patients

In the present study, we analyzed the clinical features that can affect TAA-specific immune responses. When we divided patients into two groups based on their frequencies of lymphocyte subsets in peripheral leukocytes (<24 %, the median value among all patients, or equal to or more than 24 %) and the strength of TAA-specific immune responses into three groups according to the frequency of TAA-specific T cells (<10 specific spots on ELISPOT assays, no response; 10–19 specific spots, weak response; equal to or more than 20 specific spots, strong response), the patients with more lymphocyte subsets in peripheral leukocytes showed stronger TAA-specific T cell responses (Supplementary Fig. 2). On the other hand, we could not find any relationship between TAA-specific immune responses and other clinical characteristics such as age, sex, tumor marker levels, UICC stage, or metastasis status.

We also analyzed the correlation between T cell responses and the prognosis of pancreatic cancer patients. The median overall survival time of patients with T cell responses to at least one TAA-derived peptide evaluated

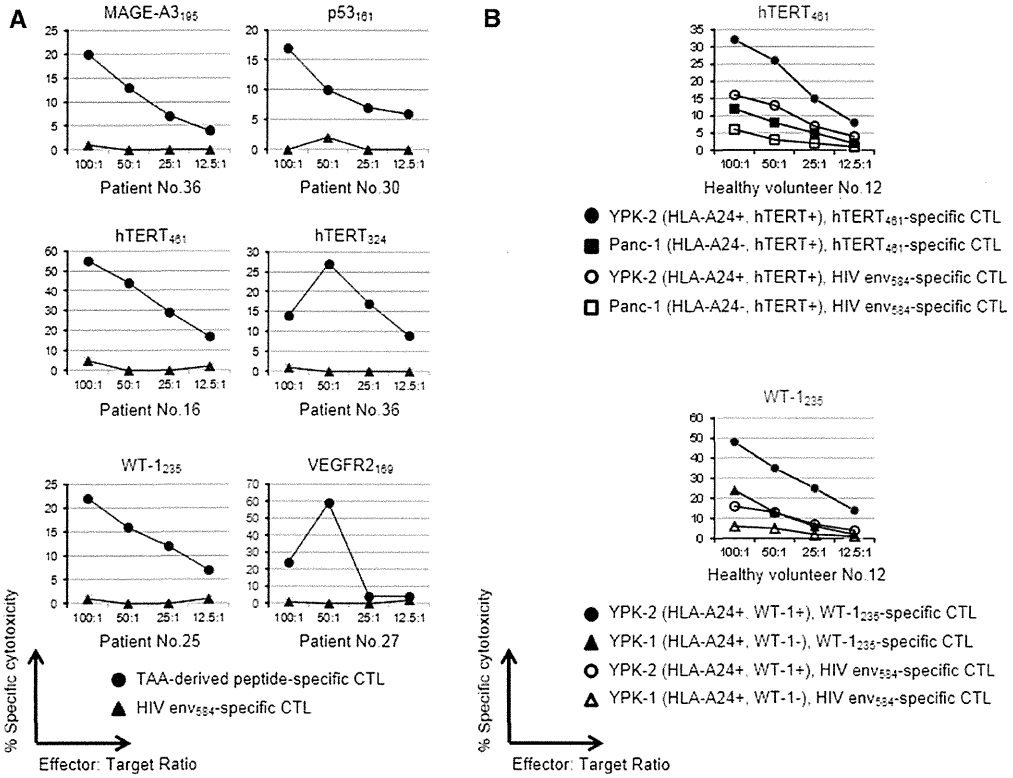

Fig. 2 **a** T cell responses to peptides evaluated by the cytotoxicity assay. Peptide-specific CTL induction and cytotoxicity assays were performed on the PBMCs from at least five patients, and representative data are shown when peptide-specific CTLs were induced in one or more patients. A percent-specific cytotoxicity of more than 10 % was considered to be positive. Six peptides: 9, 14, 18, 19, 20, and 21, could induce their specific CTLs, and these could respond to C1RA24 cells pulsed with the corresponding peptides in the cytotoxicity assay. **b** Cytotoxic activity against the pancreatic carcinoma cell lines of TAA-specific CTLs from healthy volunteers evaluated by the cytotoxicity assay. Cytotoxicity was stronger against pancreatic carcinoma cells that were HLA-A24-restricted and expressed corresponding TAAs than against those not HLA-A24-restricted or not expressing corresponding TAAs

by the ELISPOT assay was 12.2 months, which was significantly longer than that without T cell responses (4.3 months) ($p = 0.013$) (Fig. 4a). On the other hand, no correlation was observed between positive T cell responses and CMV-derived peptides and clinical outcomes (Fig. 4b), suggesting that TAA-specific T cell responses, but not the general immune response, is a prognostic factor in patients with pancreatic adenocarcinoma. The frequencies of regulatory T cells or the ratio of regulatory T cells to $CD8^+$ T cells had no impact on the outcomes of patients in this study.

Discussion

Immunotherapy is considered to be a fourth treatment procedure for cancer following surgical resection, radiotherapy, and chemotherapy [29]. Cancer vaccine therapy was previously shown to convey survival benefits to prostate cancer patients in a clinical phase III trial [30], and some candidates of other cancers have been identified and separately evaluated to determine whether a CTL response can be elicited, with the subsequent elimination of cancer cells and improvement in outcomes. Although a successful clinical response depends on how much tumor antigens elicit their specific CTLs, which are the most important effector cells for antitumor immune responses, to the best of our knowledge, no studies have attempted to identify which epitopes are optimal for peptide vaccine therapy in patients with pancreatic adenocarcinoma. Therefore, we simultaneously compared peptide-specific T cell responses among various TAAs in 41 identical patients with pancreatic adenocarcinoma under the same experimental conditions.

Therapeutic function is the most important factor to consider when determining the usefulness of cancer antigens for peptide vaccine therapy. However, it is very difficult to compare the efficacy of more than one epitope, especially in patients with pancreatic adenocarcinoma whose survival time is very short. Under such circumstances, immunogenicity, specificity, oncogenicity, expression levels, % of

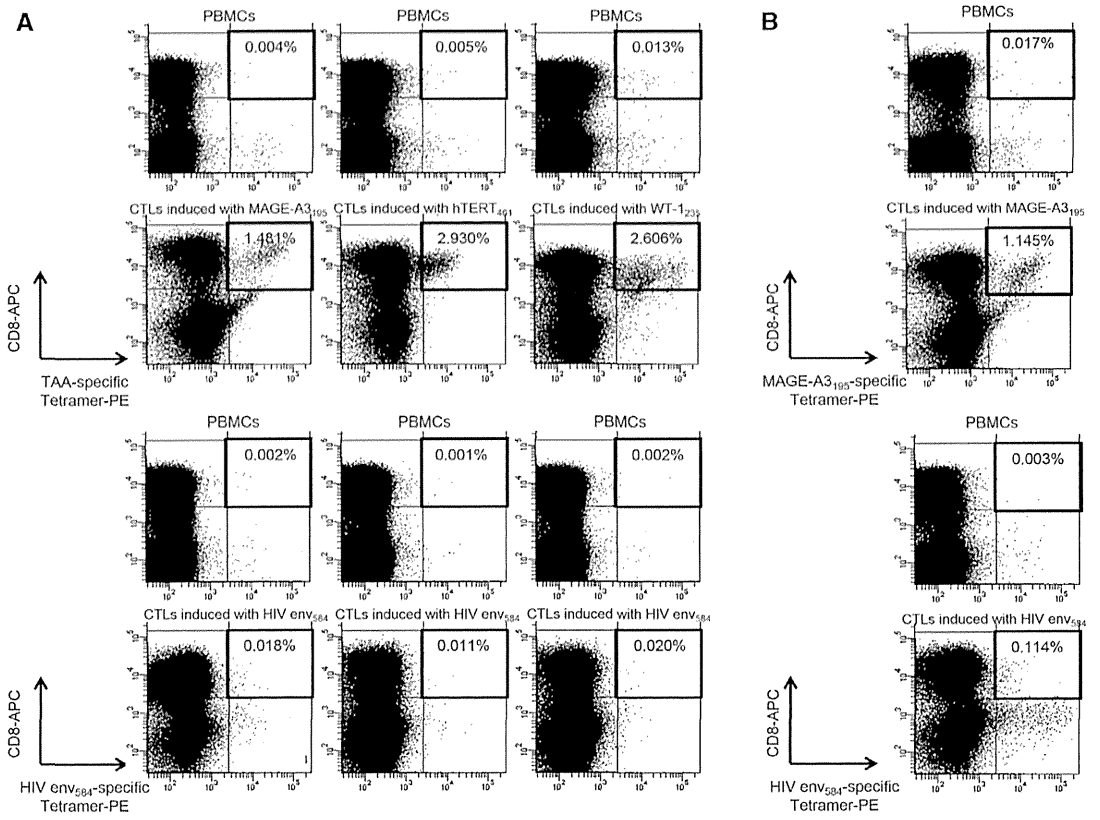

Fig. 3 Detection of TAA-specific, HLA-A24-tetramer$^+$, and CD8$^+$ lymphocytes in PBMCs from healthy volunteers and pancreatic adenocarcinoma patients. **a** Tetramer analyses were performed on eight healthy volunteers for each peptide (MAGE-A3$_{195}$, hTERT$_{461}$, and WT-1$_{235}$). Tetramer$^+$ and CD8$^+$ T cells were detectable in both PBMCs and CTLs induced by their corresponding peptides in at least one healthy volunteer, and representative data are shown in cases in which the ratio of tetramer$^+$ and CD8$^+$ T cells to CD8$^+$ T cells was higher in CTLs induced with each TAA-derived peptide than in PBMCs. **b** Tetramer analyses were performed on pancreatic adenocarcinoma patients using PBMCs and CTLs, which were induced with TAA-derived peptides and showed cytotoxicity against pancreatic cancer cell lines in cytotoxicity assay. Levels of tetramer$^+$ and CD8$^+$ T cells were higher in CTLs induced with TAA-derived peptides than in PBMCs. Representative data are shown in cases in which the ratio of tetramer$^+$ and CD8$^+$ T cells to CD8$^+$ was 0.017 % in PBMCs and 1.145 % in MAGE-A3$_{195}$-specific CTLs

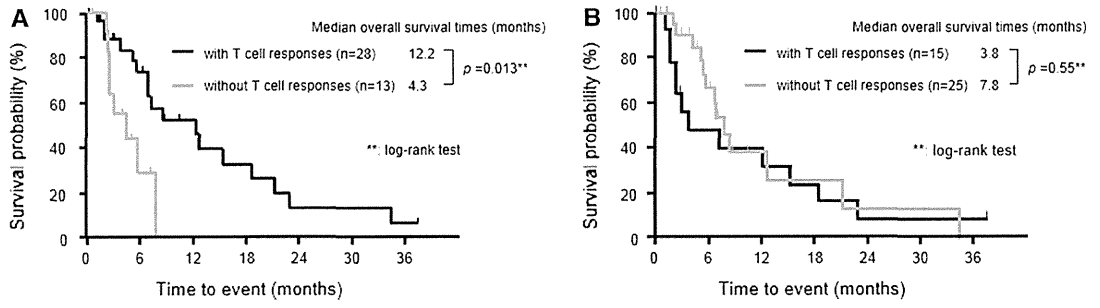

Fig. 4 Kaplan-Meier plot of the overall survival of pancreatic cancer patients according to **a** TAA-specific T cell responses and **b** T cell responses to CMV-derived peptides. **a** TAA-specific T cell responses were defined as positive if 10 or more specific spots to at least one TAA-derived peptide were detected on the ELISPOT assay. The overall survival time of patients with TAA-specific T cell responses was significantly longer than that of patients without TAA-specific T cell responses. **b** T cell responses to CMV-derived peptides were defined as positive if 10 or more specific spots to CMV-derived peptides were detected on ELISPOT assays. No correlation was observed between positive T cell responses to CMV-derived peptides and the clinical outcomes of patients

positive cells, and the number of patients with antigen-positive cancer are considered to be alternative criteria [31]. On the basis of our results, $p53_{161}$, $hTERT_{461}$, $WT\text{-}1_{235}$, and $VEGFR2_{169}$ are considered the most optimal epitopes that satisfy all of the above criteria for peptide vaccine therapy in pancreatic adenocarcinoma patients. Although $MAGE\text{-}A3_{195}$ showed immunogenicity, its expression did not appear to be high in pancreatic adenocarcinoma tissue [32]. Therefore, it may be a candidate for cancer vaccine therapy when MAGE-A3 is confirmed to be overexpressed in pancreatic cancer tissue.

A mutation in the p53 gene and overexpression of the p53 protein have been reported previously in pancreatic adenocarcinoma [33], and all pancreatic cancer cell lines and specimens used in our study expressed p53. Some strategies targeting p53 have been proposed over the last decade [34]. As peptide vaccine therapy, the wild-type p53 peptide is well preserved in mutant p53 because most mutations in the p53 gene are missense mutations, and are considered to be one of the attractive targets as a cancer antigen. The frequencies of the CTL response against HLA-A24-restricted $p53_{161}$ investigated by the ELISPOT assay in head and neck carcinoma and hepatocellular carcinoma were shown to be 35 and 10 %, respectively [35, 36]. Although the frequency of 7 % in our study is lower, given the difference according to the primary tumor site or balance between sensitivity and specificity, induced CTLs showed cytotoxic activity against pancreatic adenocarcinoma cell lines, which suggested that p53 may be an attractive target in patients with pancreatic cancer.

hTERT is widely overexpressed in various cancer cells including pancreatic cancer [37], which is consistent with our results. A clinical trial demonstrated that GV1001, a HLA class II epitope corresponding to the hTERT (611–626) fragment, was immunogenic in pancreatic cancer patients [38]. Another previous study evaluating T cell responses to several hTERT epitopes in patients with hepatocellular carcinoma [39] demonstrated that $hTERT_{461}$- and $hTERT_{324}$-specific CTLs were induced in 5 (6.9 %) and 9 (12.5 %) of 72 patients, respectively. In the current study, these frequencies were equivalent and the killing of pancreatic cancer cell lines was demonstrated, which suggested that these epitopes also had immunogenicity in pancreatic cancer patients.

Peptide vaccine therapies using $WT\text{-}1_{235}$ and $VEGFR2_{169}$ combined with gemcitabine have already been conducted in pancreatic adenocarcinoma patients [23, 24]. We clarified that $WT\text{-}1_{235}$- and $VEGFR2_{169}$-specific CTLs induced from PBMCs showed cytotoxicity for human pancreatic cancer cell lines, and the results of further investigations are anticipated.

We performed phenotypic analysis of TAA-derived epitope-specific T cells to determine the most appropriate epitope for immunotherapy in patients with pancreatic adenocarcinoma. Epitope-specific tetramer$^+$ cells in PBMCs were also found in patients without IFN-γ ELISPOT responses, which was consistent with the findings of previous studies [39, 40] and suggested the existence of dysfunctional epitope-specific T cells. Epitope-specific tetramer$^+$ cells were also identified at a very low frequency in PBMCs from healthy volunteers and increased in CTLs induced with TAA-derived peptides, which was also consistent with previous studies in which TAA-specific tetramer$^+$ T cells were detectable in samples from healthy donors [41] or the in vitro stimulation of PBMCs with the epitopes derived from TAA could induce TAA-specific CTLs in healthy volunteers [42], even though the precise mechanism has not yet been clarified. Phenotypic analysis showed that the frequency of T cells with each memory and effector phenotype depended on the patient and also that peptide-specific memory T cells existed in PBMCs of patients with pancreatic adenocarcinoma. Because T cells with the memory phenotype exert stronger antitumor effects by secondary stimulation with the antigen, our results suggest that an additional immunological approach such as that consisting of a TAA-derived protein or peptide, recombinant virus, and engineered tumor cells to boost T cell function may be useful to enhance host antitumor immune responses.

Another purpose of this study was to identify the factors influencing immune responses. Our results suggested that the frequencies of the lymphocyte subsets in peripheral leukocytes were very important in the induction of TAA-specific CTLs. Although the relationship between cancer, inflammation, and immunity has already been documented [43], the precise mechanism has yet to be fully understood. One of the speculated reasons why PBMC from patients with lymphocytopenia could not induce a good immune response in our study is that the release of inhibitory immunological cytokines such as transforming growth factor β or IL-10 from pancreatic adenocarcinoma tissue decreases lymphocyte counts and impairs the function of lymphocytes both systemically and in the microenvironment [44]. It was also reported that lymphocyte counts and CTL responses were prognostic markers in advanced cancer cases receiving peptide vaccine therapy [45, 46]. Our results showing a correlation between the T cell response and outcomes in pancreatic adenocarcinoma patients corresponded to these previous findings, which indicate that restricting the objective to those with an adequate lymphocyte subset could lead to a clinical trial with favorable outcomes.

A limitation of this study was the lack of data for the clinical response. Tumor shrinkage or survival benefits are not always observed in all patients who exhibit immune responses. Further, clinical studies using peptides that could induce TAA-specific CTLs are needed to confirm our findings.

In conclusion, we simultaneously compared T cell responses to various TAA-derived epitopes in patients with pancreatic adenocarcinomas; our results suggested that $p53_{161}$, $hTERT_{461}$, $WT\text{-}1_{235}$, and $VEGFR2_{169}$ were the most suitable epitopes for cancer vaccine therapy.

Acknowledgments The authors thank Kazumi Fushimi, Maki Kawamura, Nami Nishiyama, and Mikiko Nakamura for their technical assistance.

Conflict of interest The authors do not have any conflict of interest.

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Cancer Immunol Immunother (2014) 63:347–356
DOI 10.1007/s00262-013-1514-7

ORIGINAL ARTICLE

In vivo immunological antitumor effect of OK-432-stimulated dendritic cell transfer after radiofrequency ablation

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Received: 1 June 2013 / Accepted: 17 December 2013 / Published online: 3 January 2014

Abstract Radiofrequency ablation therapy (RFA) is a radical treatment for liver cancers and induces tumor antigen-specific immune responses. In the present study, we examined the antitumor effects of focal OK-432-stimulated dendritic cell (DC) transfer combined with RFA and analyzed the functional mechanisms involved using a murine model. C57BL/6 mice were injected subcutaneously with colon cancer cells (MC38) in their bilateral flanks. After the establishment of tumors, the subcutaneous tumor on one flank was treated using RFA, and then OK-432-stimulated DCs were injected locally. The antitumor effect of the treatment was evaluated by measuring the size of the tumor on the opposite flank, and the immunological responses were assessed using tumor-infiltrating lymphocytes, splenocytes and draining lymph nodes. Tumor growth was strongly inhibited in mice that exhibited efficient DC migration after RFA and OK-432-stimulated DC transfer, as compared to mice treated with RFA alone or treatment involving immature DC transfer. We also demonstrated that the antitumor effect of this treatment depended on both CD8-positive and CD4-positive cells. On the basis of our findings, we believe that combination therapy for metastatic liver cancer consisting of OK-432-stimulated DCs in combination with RFA can proceed to clinical trials, and it is anticipated to be markedly superior to RFA single therapy.

Keywords Metastatic liver cancer · MC38 · Immunotherapy · Intratumoral injection · Tumor-infiltrating lymphocyte

Abbreviations

RFA	Radiofrequency ablation
DC	Dendritic cell
HCC	Hepatocellular carcinoma
TAE	Transcatheter hepatic arterial embolization
TLR	Toll-like receptor
GFP	Green fluorescent protein
ELISPOT	Enzyme-linked immunospot
Treg	Regulatory T cell
MDSC	Myeloid-derived suppressor cell
IFN-γ	Interferon-γ

Introduction

Liver is one of the most common organs to which various cancers spread from their site of origin. In some types of cancer, the liver metastasis lesion is a target of surgical treatment. For instance, surgical resection of hepatic metastasis achieves longer median survival in colorectal and breast cancer patients [1, 2]. However, even if the hepatic lesions are surgically treated, the prognosis of the

Electronic supplementary material The online version of this article (doi:10.1007/s00262-013-1514-7) contains supplementary material, which is available to authorized users.

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patients is not satisfactory. As for colorectal cancers, the recurrence rate is over 50 % after radical resection of metastatic lesions [3]. Moreover, at the time of initial diagnosis, only a few patients meet the criteria for hepatic resection because of unresectability, low hepatic functional reserve or poor performance status [4].

Radiofrequency ablation therapy (RFA) has been developed as a radical and minimally invasive treatment method for metastatic liver cancers. Recently, RFA has been used as an adjunct to hepatic resection or as an alternative method to resection when surgical treatment is not feasible [5]. Additionally, it has been revealed that RFA for metastatic liver cancers generates tumor antigen-specific T-cell responses in man [6, 7]. We have previously reported that RFA could also control distant tumor growth in a murine hepatocellular carcinoma (HCC) model [8].

Dendritic cells (DCs) are potent antigen-presenting cells [9]. Recently, we have established new treatments using local DC injection with transcatheter hepatic arterial embolization (TAE) and have shown that this combination therapy could induce tumor antigen-specific T-cell responses in HCC patients [10].

OK-432 is derived from the Su strain of Group A *Streptococcus pyogenes* by means of treatment with benzylpenicillin and heat [11]. OK-432 can stimulate DCs via Toll-like receptor (TLR) 3, TLR4 and β2 integrin and subsequently induce antigen-specific cytotoxic lymphocytes [12–14].

On the basis of these results, we hypothesized that OK-432-stimulated DC transfer is a promising candidate for an enhancer that can strongly increase the antitumor effect of RFA. We have previously demonstrated in a clinical trial that the local infusion of OK-432-stimulated DC after TAE could prolong recurrence-free survival in HCC patients [15]. However, it remains unknown as to how the transferred DCs work in combination with RFA. In the present study, we examined the antitumor effects of OK-432-stimulated DCs when combined with RFA and analyzed the functional mechanisms involved using a murine subcutaneous colon cancer model.

Materials and methods

Animals

Wild-type 8–12-week-old female C57BL/6 J mice were obtained from Charles River Japan (Yokohama, Japan). Female C57BL/6-Tg (UBC-GFP) 30Scha/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were approved and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, which strictly conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell lines and bone marrow-derived dendritic cells

A murine colorectal cancer cell line, MC38 and hybridomas, clone GK1.5 and clone 2.43 were cultured in RPMI-1640 containing 10 % fetal bovine serum (Life Technologies, Co., Carlsbad, CA, USA) supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin (Wako Pure Chemical Industries Ltd., Osaka, Japan). Bone marrow-derived dendritic cells (BMDCs) were generated using 20 ng/ml of recombinant granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) as previously described [16]. OK-432 (Picibanil; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) was loaded into the supernatant from days 6–7 of the BMDC generation period at a concentration of 5 μg/ml.

In vitro evaluation of phagocytic activity by dendritic cells

MC38 cells were labeled with DiD dye (Life Technologies) according to the manufacturer's instructions followed by heat treatment at 80 °C for 90 s. OK-432-stimulated or immature DCs were co-incubated with the treated MC38 cells for 3 h at a ratio of 1:1. After incubation, the cell suspensions were observed using a fluorescence microscope (BZ9000: Keyence, Osaka, Japan) and analyzed by means of FACSCalibur (BD Immuno-Cytometry System, San Jose, CA, USA).

Animal model

Bilateral flanks of C57BL/6 mice were each injected subcutaneously with 1×10^6 MC38 cells. Seven days after injection, after they had grown to 5–6 mm in diameter, the subcutaneous tumors on one flank were treated using RFA, and 1×10^7 immature DCs or 1×10^7 OK-432-stimulated DCs were injected into the treated tumors at 24 h after RFA. After this, the volume of the untreated tumor on the contralateral flank was evaluated over a period of 10 days. Tumor volumes were calculated using the following formula: tumor volume (mm^3) = (longest diameter) × (shortest diameter)^2/2.

Radiofrequency ablation

Mice bearing tumors were anesthetized with an intraperitoneal injection of pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and the skin on the tumor was cut. Subsequently, an expandable RFA needle was inserted into the tumor, which was treated using a radiofrequency generator (RITA

500PA; RITA Medical Systems, Inc., Fremont, CA, USA). During the use of this system, the intratumor temperature was maintained at 70–90 °C, and the current was turned off when the tumor exhibited heat denaturation.

Flow cytometry

The DCs were detected by means of staining with anti-CD11c antibodies (Life technologies). The lymphocytes in the draining lymph node were stained with anti-CD4 antibodies, anti-CD8 antibodies, anti-CD11c antibodies and anti-CD69 antibodies (BD Bioscience, San Diego, CA, USA). The splenocytes were stained with anti-CD4 antibodies, anti-CD8 antibodies, CD11c antibodies, anti-NK1.1 antibodies, CD45 antibodies (BD Bioscience), anti-Gr-1 antibodies, and anti-CD11b antibodies and mouse regulatory T-cell staining solution (BioLegend, San Diego, CA, USA). The stained samples were analyzed using FACSAria II (BD Immuno-Cytometry System).

Immunohistochemical assay

The draining lymph nodes and the observed tumors were embedded in Sakura Tissue-Tek optimum cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for frozen sectioning. Tissue sections were fixed at −20 °C in methanol for 10 min. The draining lymph nodes were stained using rabbit anti-GFP antibody (Abcam, Cambridge, UK) that were detected using an EnVision+/HRP kit (Dako, Glostrup, Denmark). The observed tumors were stained with anti-CD4 and anti-CD8a (BD Bioscience), which were detected using the Nichirei Histofine Simple Stain Mouse Max PO (Rat) system (Nichirei Co., Tokyo, Japan) or the Vectastain ABC kit (Vector Laboratory, Inc., Burlingame, CA, USA).

Interferon gamma enzyme-linked immunospot assay

The splenocytes, the tumor-infiltrating lymphocytes (TILs) in the untreated tumors that were isolated by mechanical homogenizations and density gradient centrifugations, and the lymphocytes in the draining lymph nodes were loaded into the interferon gamma enzyme-linked immunospot assay to estimate the tumor-specific immune reactions, as previously described [8, 17]. Briefly, 3×10^5 lymphocytes or 1×10^5 TILs were incubated for 24 h with or without 6×10^5 MC38 lysates, which were prepared through five cycles of rapid freezing in liquid nitrogen, thawing at 55 °C and centrifugation. The number of MC38-specific IFN-γ spots was determined by subtracting the number of spots incubated without MC38 lysates from the number of spots incubated with MC38 lysates. For CD4 or CD8 depletion, we used magnetic CD4 beads or CD8 beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

In vivo CD4/CD8 depletion

For in vivo CD4 or CD8 depletion, B6 mice were injected intraperitoneally with 200 μg of purified monoclonal antibodies specific to CD4 or CD8 at 1 day before and 3 days after RFA treatment; the monoclonal antibodies were prepared from GK1.5 hybridoma and 2.43 hybridoma, respectively [18]. The depletion was confirmed by flow cytometry using peripheral blood lymphocytes stained with anti-CD4 and anti-CD8 antibodies.

Statistical analysis

The data obtained were analyzed statistically using the t test or one-way analysis of variance followed by Tukey's multiple-comparison test. A P value <0.05 was considered as being statistically significant.

Results

Migration efficacy and phagocytic ability of OK-432-stimulated DCs

We employed OK-432 as a modifying agent for DCs, because we have previously shown in clinical studies that OK-432 prolonged recurrence-free survival after combination therapy involving DC injection with TAE for HCC patients [10, 15]. We first confirmed that the OK-432-stimulated murine DCs showed higher expression of maturation markers such as CD40, CD80, CD86, MHC class II and CCR7 (Supplementary Fig. 1), as previously reported [19, 20].

To evaluate their phagocytic abilities, we incubated the immature DCs and the OK-432-stimulated DCs with MC38 tumor cells. Heat-treated MC38 cells were taken up well by both immature DCs and OK-432-stimulated DCs, as compared to nontreated MC38 cells (Fig. 1a–c). In addition, the phagocytic ability of OK-432-stimulated DCs was not inferior to that of immature DCs. These results were consistent with the dextran uptake assay (Supplementary Fig. 2) and our previous data on human monocyte-derived DCs [15]. Since heat-treated MC38 cells were thought to be in a similar condition to those in the MC38 tumor in mice treated with RFA, OK-432-stimulated DCs were expected to effectively phagocytose RFA-treated MC38 tumor cells in vivo.

We next estimated the kinetics of the transferred DCs in mice bearing subcutaneous MC38 tumors treated with RFA. Immature DCs or OK-432-stimulated DCs that were derived from GFP-Tg mice were injected intratumorally