

They underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter, and tumour recurrences were followed for up to 360 days. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors, as reported previously [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at 1.4×10^7 cells in 2 ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in serum-free media (GMP CellGro® DC Medium; CellGro, Manassas, VA, USA) with 50 ng/ml recombinant human IL-4 (GMP grade; CellGro®) and 100 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (GMP grade; CellGro®) for 5 days to generate immature DC, and matured for a further 2 days in 0.1 KE/ml OK432 (Chugai Pharmaceuticals, Tokyo, Japan) to generate OK-DC. On day 7, the cells were harvested for injection, 5×10^6 cells were suspended in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of CD14⁺ and human leucocyte antigen (HLA)-DR⁺.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lineage cocktail 1 (lin-1; CD3, CD14, CD16, CD19, CD20 and CD56)-fluorescein isothiocyanate (FITC), anti-HLA-DR-peridinin chlorophyll protein (PerCP) (L243), anti-CCR7-phycoerythrin (PE) (3D12) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a fluorescence activated cell sorter (FACS0Calibur™ flow cytometer. Data

analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

DC phagocytosis

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACS-Calibur™ cytometer. Control DCs (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

Enzyme-linked immunosorbent assay (ELISA)

DCs were seeded at 200 000 cells/ml, and supernatant collected after 48 h. IL-12p40 and IFN- γ were detected using matched paired antibodies (BD Pharmingen) following standard protocols.

Cytotoxicity assays

The ability of DCs to exert cytotoxicity was assessed in a standard ⁵¹Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 [American Type Culture Collection (ATCC), Manassas, VA, USA] and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labelled with ⁵¹Cr. In a 96-well plate, 2.5×10^3 target cells per well were incubated with DCs for 8 h at different effector/target (E/T) ratios in triplicate. Percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100. Spontaneous release was always < 20% of the total.

NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using ⁵¹Cr-release assay, according to previously published methods [26], with PBMCs obtained from the patients. All experiments were performed in triplicate. Percentage of cytotoxicity was calculated as follows: {[experimental counts per minute (cpm) – spontaneous cpm]/[total cpm – spontaneous cpm]} \times 100.

Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μ g/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% CO₂ for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μ g/ml. After addition of stimuli, the surface staining was performed with anti-CD4-PC5 (13B8-2), anti-CD8-PerCP (SK1) and anti-CD56-PC5 (N901) (Beckman

Coulter). Subsequently, the cells were permeabilized, stained for intracellular IFN- γ and IL-4 using the FastImmune™ system (BD Pharmingen), resuspended in phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA), and analysed on a flow cytometer ($\approx 10\,000$ gated events acquired per sample).

IFN- γ enzyme-linked immunospot (ELISPOT) assay

ELISPOT assays were performed as described previously with the following modifications [28–30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 (SART2)₈₉₉ (SYTRLFLIL), SART3₁₀₉ (VYDYNCHVDL), multi-drug resistance protein 3 (MRP3)₇₆₅ (VYSADIFL), MRP3₅₀₃ (LYAWEPSFL), MRP3₆₉₂ (AYVPQQAWI), alpha-fetoprotein (AFP)₄₀₃ (KYIQESQAL), AFP₄₃₄ (AYTKKAPQL), AFP₃₅₇ (EYSRRHPQL), human telomerase reverse transcriptase (hTERT)₁₆₇ (AYQVCGPPL) (unpublished), hTERT₄₆₁ (VYGFVRACL) and hTERT₃₂₄ (VYAETKHFL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide (CMVpp65₃₂₈). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen.

Cytokine and chemokine profiling

Serum cytokine and chemokine levels were measured using the Bioplex assay (Bio-Rad, Hercules, CA, USA). Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 μ l aliquots of diluted sample were added in duplicate to the wells of a 96-well microtitre plate containing the coated beads for a validated panel of 27 human cytokines and chemokines (cytokine 27-plex antibody bead kit) according to the manufacturer's instructions. These included IL-1 β , IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN- γ , interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1 α , MIP-1 β , platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T cell-expressed and secreted (RANTES), TNF- α and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32 000 pg/ml) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1.

Arginase activity

Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine [31] using a kit supplied by the manufacturer (BioAssay Systems, Hayward, CA, USA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA, USA) to remove urea, combined with the sample buffer in wells of a 96-well plate, and incubated at 37°C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The colour produced was read at 520 nm using a microtitre plate reader.

Statistical analysis

Results are expressed as means \pm standard deviation (s.d.). Differences between groups were analysed for statistical significance by the Mann–Whitney *U*-test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

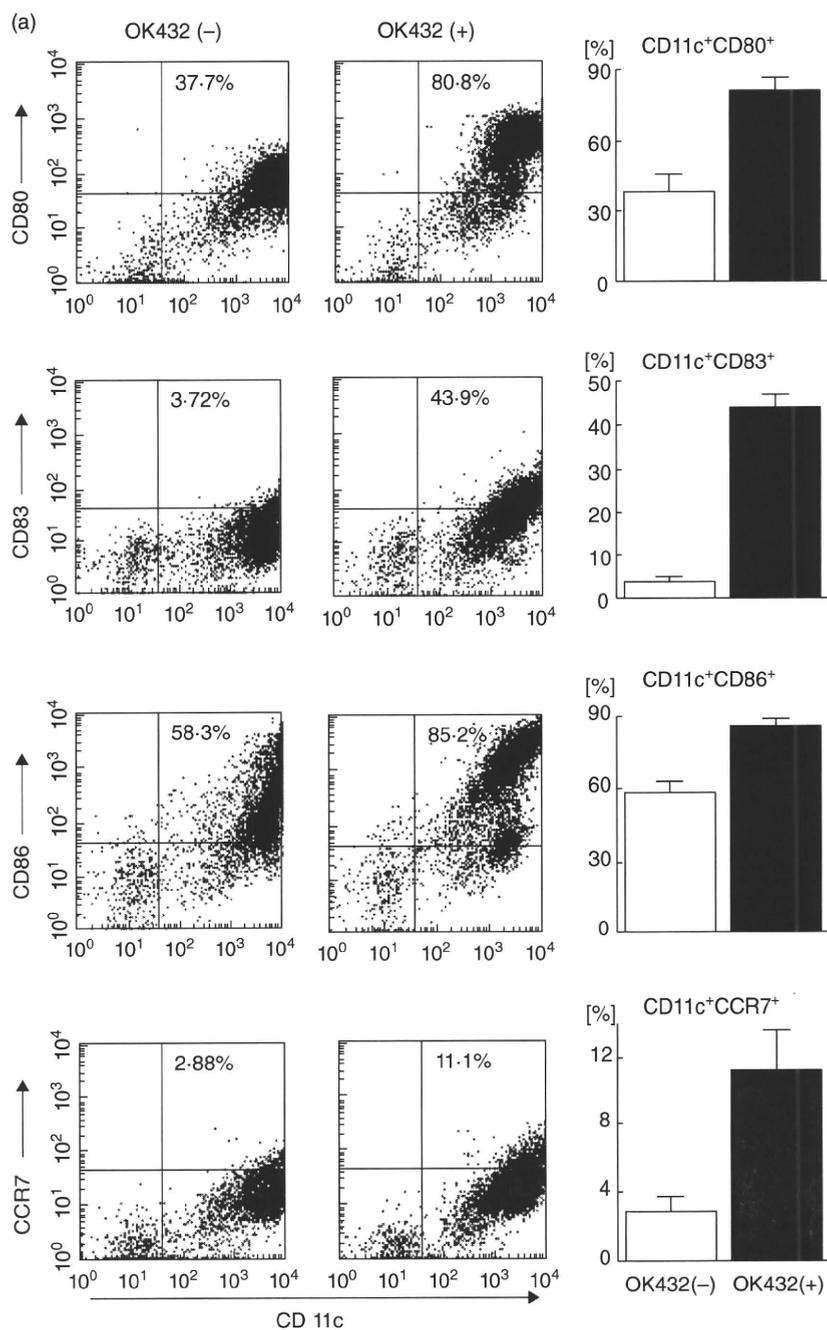
Results

Preparation of OK432-stimulated DCs

Adherent cells isolated from PBMCs of patients with cirrhosis and HCC (Table 1) were differentiated into DCs in the presence of IL-4 and GM-CSF. The cells were stimulated with 0.1 KE/ml OK432 for 3 days; 54.6 \pm 9.5% (mean \pm s.d.; *n* = 13) of OK432-stimulated cells showed high levels of MHC class II (HLA-DR) and the absence of lineage markers including CD3, CD14, CD16, CD19, CD20 and CD56, in which 30.9 \pm 14.2% were CD11c-positive (myeloid DC subset) and 14.8 \pm 11.2 were CD123-positive (plasmacytoid DC subset), consistent with our previous observations [20]. As reported [32,33], greater proportions of the cells developed high levels of expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) and an activation marker (CD83) compared to DCs prepared without OK432 stimulation (Fig. 1a). Furthermore, the chemokine receptor CCR7 which leads to homing to lymph nodes [13,34] was also induced following OK432 stimulation.

To evaluate the endocytic and phagocytic ability of the OK432-stimulated cells, uptake of FITC-dextran was quantitated by flow cytometry (Fig. 1b). The cells showed lower levels of uptake due to maturation compared to DCs prepared without OK432 stimulation, while the OK432-stimulated cells derived from HCC patients preserved a moderate uptake capacity. As expected, the OK432-stimulated cells produced large amounts of cytokines IL-12 and IFN- γ (Fig. 1c). In addition, they displayed high cyto-

Fig. 1. Effects of OK432 stimulation on the properties of dendritic cells (DCs) generated from blood monocyte precursors in patients with cirrhosis and hepatocellular carcinoma (HCC) ($n = 13$). (a) Lineage cocktail 1 (lin¹) human leucocyte antigen D-related (HLA-DR⁻) subsets with [OK432(+)] and without [OK432(-)] stimulation were analysed for surface expression of CD80, CD83, CD86 and CCR7. Dot plots of a representative case are shown in the left-hand panel. Mean percentages [\pm standard deviation (s.d.)] of positive cells are indicated in the right-hand panel. OK432 stimulation resulted in the expression of high levels of CD80, CD83, CD86 and CCR7 in the lin¹ human leucocyte antigen D-related (HLA-DR⁻) DC subset. (b) DC subsets with and without OK432 stimulation were incubated with fluorescein isothiocyanate (FITC) dextran for 30 min and the uptake was determined by flow cytometry. A representative analysis is shown in the upper panel. Mean fluorescence intensities (MFIs) (\pm s.d.) of the positive cells are indicated in the lower panel. OK432-stimulated cells showed lower levels of uptake due to maturation. (c) DC supernatants were harvested and the concentrations of interleukin (IL)-12 and interferon (IFN)- γ measured by enzyme-linked immunosorbent assay (ELISA). OK432-stimulated cells produced large amounts of the cytokines. The data indicate means \pm s.d. of the groups with and without the stimulation. All comparisons in (a–c) [OK432(+)] versus [OK432(-)] were statistically significant by the Mann-Whitney U -test ($P < 0.005$). (d) Tumoricidal activity of DCs assessed by incubation with ⁵¹Cr-labelled Hep3B, PLC/PRF/5 and T2 targets for 8 h at the indicated effector/target (E/T) cell ratios. OK432-stimulated cells displayed high cytotoxic activity against the target cells. The results are representative of the cases studied.



toxic activity against HCC cell lines (Hep3B and PLC/PRF/5) and a lymphoblastoid cell line (T2) although DCs without OK432 stimulation lysed none of the target cells to any great degree (Fig. 1d). Taken together, these results demonstrate that OK432 stimulation of IL-4 and GM-CSF-induced immature DCs derived from HCC patients promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity, consistent with previous observations [16,19].

Safety of OK432-stimulated DC administration

Prior to the administration of OK432-stimulated DCs to patients, the cells were confirmed to be safe in athymic nude mice to which 100-fold cell numbers/weight were injected subcutaneously (data not shown). Subsequently, OK432-stimulated DC administration was performed during TAE therapy in humans, in which DCs were mixed together with absorbable gelatin sponge (Gelfoam) and infused through an arterial catheter following iodized oil (Lipiodol) injection, as reported previously [20]. Adverse events were

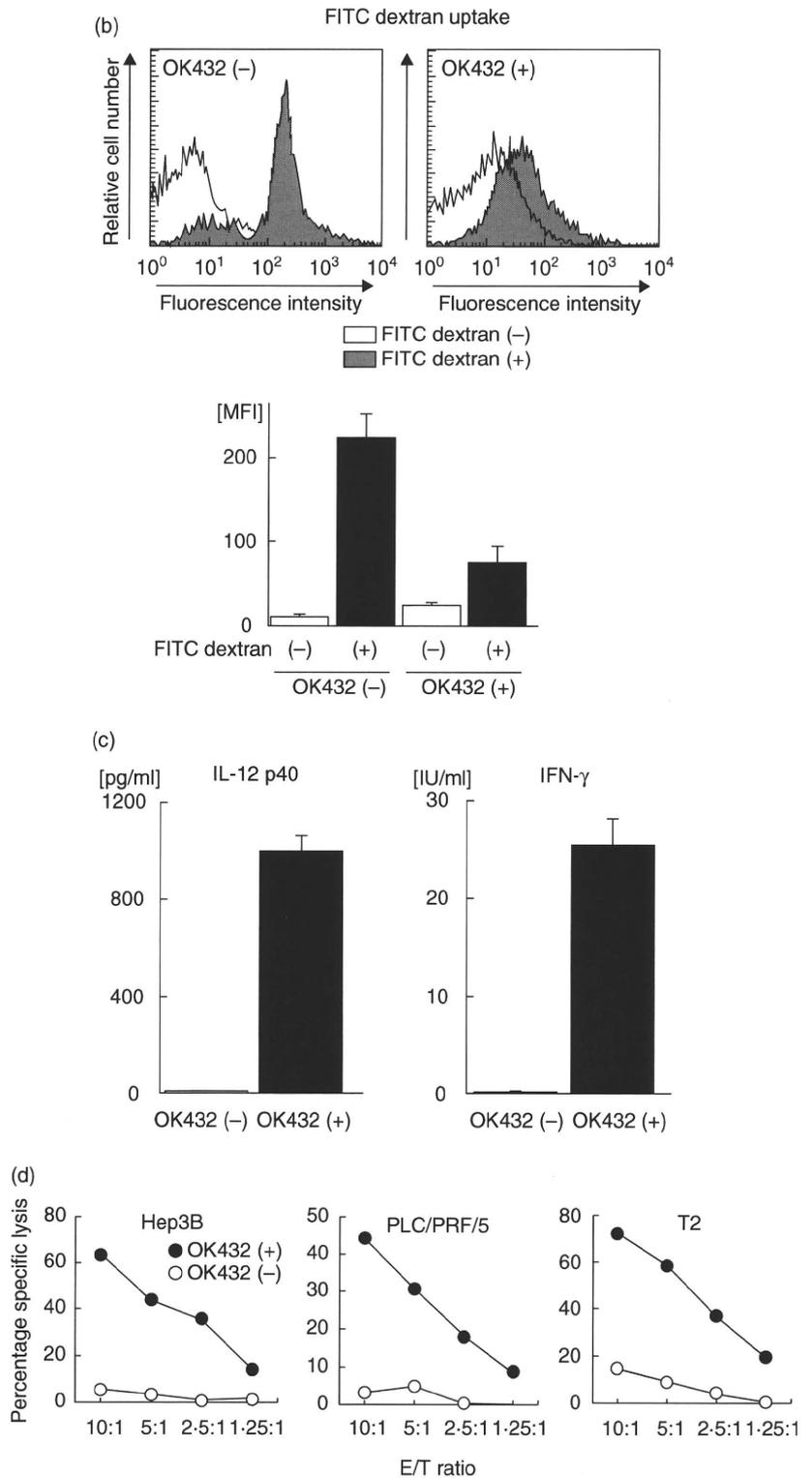


Fig. 1. *Continued*

monitored clinically and biochemically after DC infusion (Table 2). A larger proportion (12 of 13) of the patients were complicated with high fever compared to those treated previously with immature DCs (five of 10) [20], due probably to the proinflammatory responses induced by OK432-stimulated DCs. However, there were no grades III or IV

National Cancer Institute Common Toxicity Criteria adverse events, including vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorders, bleeding, hepatic abscess or autoimmune diseases associated with DC infusion and TAE in this study. There was also no clinical or serological evidence of hepatic failure or autoimmune

Table 2. Adverse events.

Patient no.	Fever (days)	Vomiting	Abdominal pain	Encephalopathy	Others [†]
1	2	No	No	No	No
2	2	No	No	No	No
3	1	No	No	No	No
4	3	No	No	No	No
5	3	No	No	No	No
6	4	No	No	No	No
7	10	No	No	No	No
8	No	No	No	No	No
9	2	No	No	No	No
10	1	No	No	No	No
11	2	No	No	No	No
12	2	No	No	No	No
13	1	No	No	No	No

[†]Other adverse events include myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

response in any patients. Thus, concurrent treatment with OK432-stimulated DC infusions can be performed safely at the same time as TAE in patients with cirrhosis and HCC.

Recurrence-free survival following DC infusion

A further objective of this study was to determine clinical response following DC infusion. A group of historical controls treated with TAE without DC administration was reviewed for this study (Table 3). The clinical characteristics including tumour burden and hepatic reserve were comparable between patients treated with TAE and OK432-stimulated DC transfer ($n = 13$) and those historical controls with TAE but without DC administration ($n = 22$). We com-

pared the recurrence-free survival between these patient groups. Kaplan–Meier analysis indicated that patients treated with TAE and OK432-stimulated DC transfer had prolonged recurrence-free survival compared with the historical controls that had been treated with TAE alone (recurrence rates 360 days after the treatments; two of 13 and 12 of 22, respectively; $P = 0.046$, log-rank test) (Fig. 2). The results demonstrated that OK432-stimulated DC transfer during TAE therapy reduces tumour recurrence in HCC patients.

NK cell activity and intracellular cytokine responses in PBMCs

To assess systemic immunomodulatory effects of OK432-stimulated DC transfer, PBMCs were isolated 1 and 3 months after treatment and NK cell cytotoxicity against K562 erythroleukaemia target cells measured using the ⁵¹Cr-release assay (Fig. 3). The level of NK cell was unaltered following treatment. In addition, cytokine production capacity of lymphocyte subsets was quantitated by measuring intracellular IFN- γ and IL-4 using flow cytometry. There were also no significant changes in terms of cytokine production capacity in the CD4⁺, CD8⁺ and CD56⁺ subsets in the patients treated with OK432-stimulated DCs.

Immune responses to peptide epitopes derived from tumour antigens

To assess the effects on T cell responses to tumour antigens, PBMCs were obtained 4 weeks after DC infusion, pulsed with peptides derived from AFP, MRP3, SART2, SART3 and hTERT. IFN- γ production was then quantitated in an

Table 3. Clinical characteristics of patients treated with TAE + OK-DC and TAE alone.

	TAE + OK-DC	TAE	<i>P</i>
No. of patients	13	22	
Age (years)	68.2 \pm 9.1	70.0 \pm 7.6	n.s. [†]
Gender (M/F)	9/4	13/9	n.s. [‡]
White cell count ($\times 10^2/\mu\text{l}$)	34.4 \pm 11.6	41.4 \pm 18.9	n.s. [†]
Lymphocytes ($\times 10^2/\mu\text{l}$)	10.4 \pm 3.6	12.4 \pm 4.7	n.s. [†]
Platelets ($\times 10^4/\mu\text{l}$)	11.5 \pm 10.2	10.3 \pm 5.8	n.s. [†]
Hepaplastin test (%)	64.6 \pm 11.6	75.5 \pm 24.3	n.s. [†]
ALT (IU/l)	56.7 \pm 38.9	67.9 \pm 44.6	n.s. [†]
Total bilirubin (mg/dl)	1.3 \pm 0.7	1.1 \pm 0.6	n.s. [†]
Albumin (g/dl)	3.4 \pm 0.6	3.6 \pm 0.4	n.s. [†]
Non-cancerous liver parenchyma (no.)			
Chronic hepatitis	0	8	
Cirrhosis (Child–Pugh A/B/C)	13 (5/8/0)	14 (6/8/0)	n.s. [‡]
TNM stages (I/II/III/IV-A/IV-B)	0/4/9/0/0	3/8/11/0/0	n.s. [‡]
No. of tumours	2.5 \pm 1.3	1.9 \pm 1.3	n.s. [†]
Largest tumour (mm)	30.2 \pm 9.4	32.6 \pm 15.2	n.s. [†]
AFP	204.8 \pm 404.1	201.8 \pm 544.2	n.s. [†]

Results are expressed as means \pm standard deviation. [†]Mann–Whitney *U*-test. [‡]Fisher's exact test. TAE, transcatheter arterial embolization; OK-DC, OK432-stimulated dendritic cells; ALT, alanine transaminase; TNM, tumour–node–metastasis; AFP, alpha-fetoprotein; Child–Pugh, Child–Pugh classification; n.s., not significant.

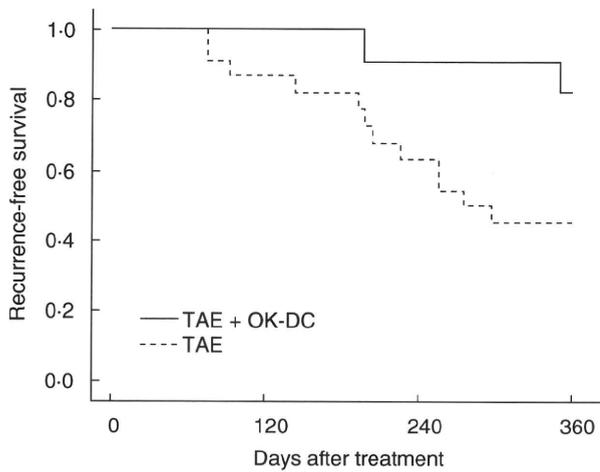


Fig. 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with [TAE + OK-stimulated dendritic cells (DC); $n = 13$] and without (TAE: historical controls; $n = 22$) OK432-stimulated DC administration. Time zero is the date of TAE. All patients underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter. Kaplan–Meier analysis indicated that TAE + OK-DC treatment prolonged recurrence-free survival compared with the TAE-alone group (recurrence rates 360 days after the treatments; two of 13 and 12 of 22, respectively; $P = 0.046$, log-rank test).

ELISPOT assay. Cells producing IFN- γ in response to stimulation with HLA-A24 [the most common HLA-A antigen (58.1%) in Japanese populations [35]]-restricted peptide epitopes derived from tumour antigens MRP3 and hTERT were induced in three of six HLA-A24-positive patients (numbers 2, 6 and 11) after treatment with TAE and OK432-stimulated DCs (Fig. 4). To understand the immunological and clinical significance of the T lymphocyte responses, PBMCs obtained from the historical control patients who had been treated with TAE without DC administration were also evaluated by ELISPOT. Similarly, positive reactions were observed in four (numbers t8, t19, t20 and t22) of six HLA-A24-positive patients. These data indicate that T lymphocyte responses to HLA-A24 restricted peptide epitopes of tumour antigens were induced following the TAE therapy, but no additional responses were observed as a result of OK432-stimulated DC transfer in the current study.

Serum levels of cytokines, chemokines and arginase activity

To screen for immunobiological responses induced following OK432-stimulated DC transfer, serum levels of cytokines and chemokines were measured simultaneously using the Bio-Plex multiplex suspension array system. The results were compared with the historical control patients treated with TAE without DC administration. Interestingly, serum con-

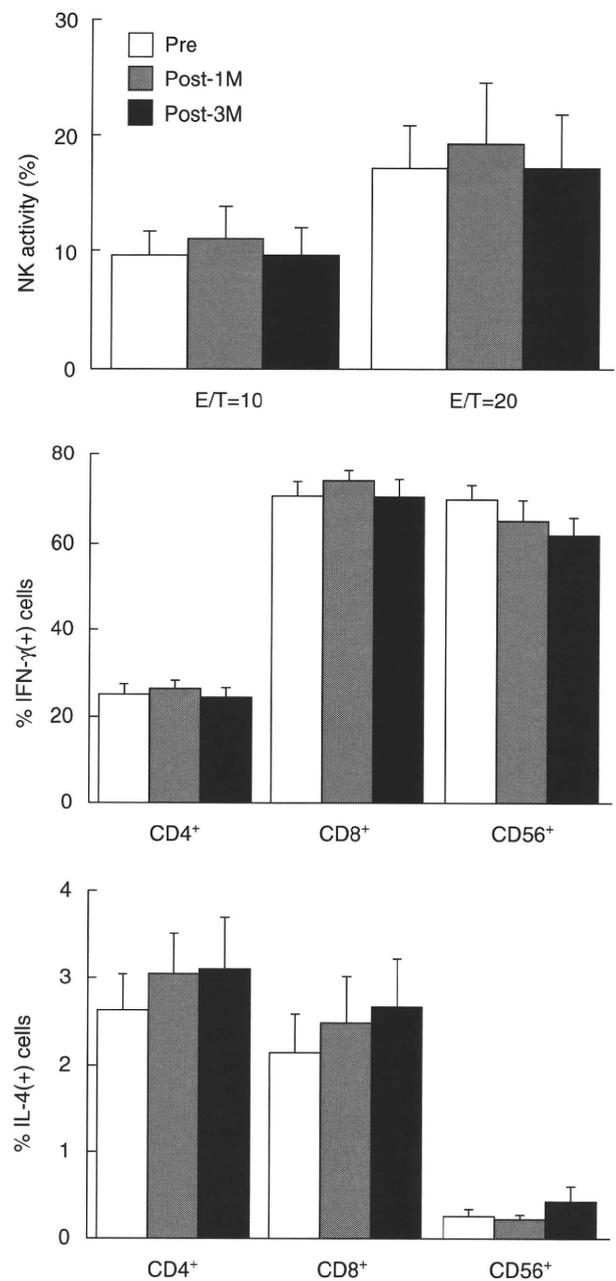
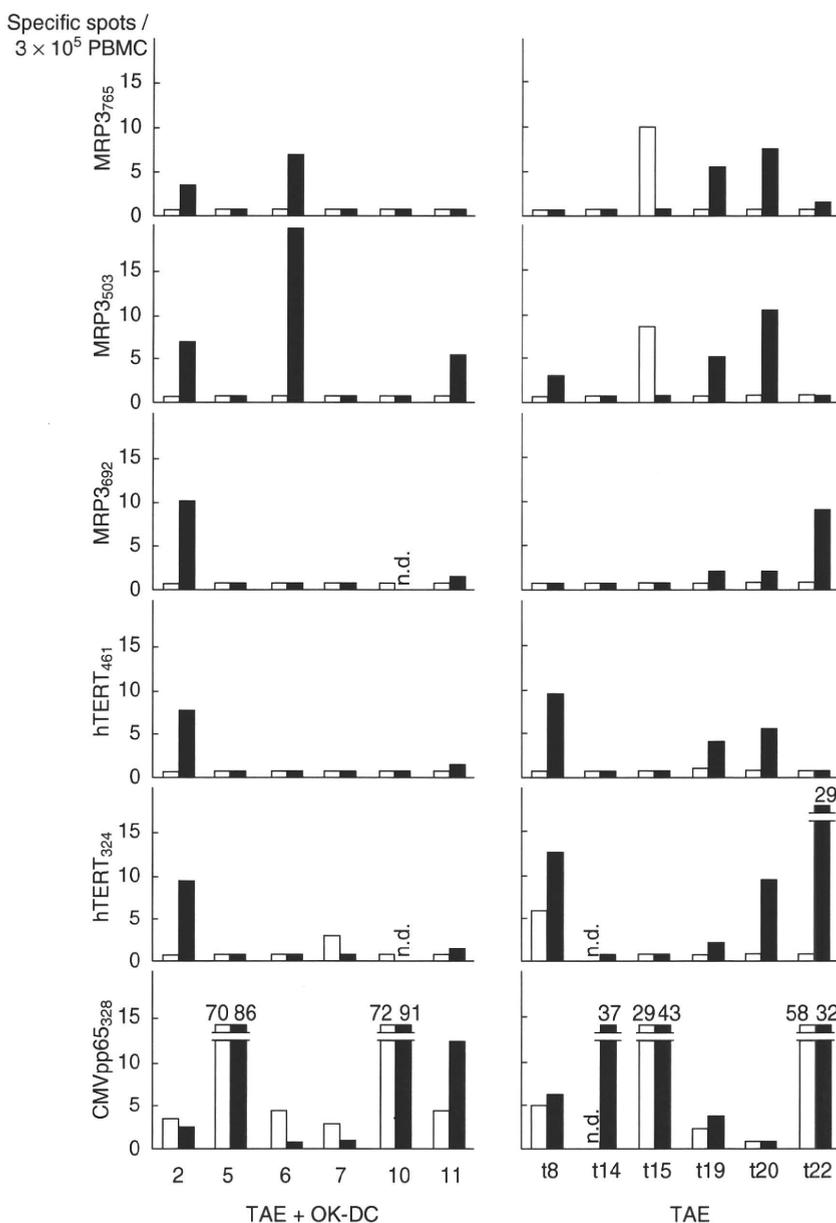


Fig. 3. Natural killer (NK) cell activity and intracellular cytokine production in peripheral blood mononuclear cells (PBMCs) of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy ($n = 13$). PBMCs were isolated before and 1 and 3 months after treatment and used for the analyses. Upper panel: NK cell cytotoxicity against K562 erythroleukaemia target cells was evaluated at the effector/target (E/T) cell ratios shown. NK cell activities were not changed following treatment. Middle and lower panels: PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, stained for CD4, CD8 and CD56 expression, permeabilized and stained for intracellular interferon (IFN)- γ and interleukin (IL)-4. Percentages of cytokine-positive cells were quantitated by flow cytometry. There were no significant changes in terms of cytokine production capacity in the CD4⁺, CD8⁺ and CD56⁺ subsets following the treatments. The data are given as means \pm standard deviation of the groups.

Fig. 4. Immune responses to human leucocyte antigen (HLA-DR⁻)-A24-restricted peptide epitopes derived from tumour antigens in HLA-A24-positive patients treated with OK432-stimulated DCs during transcatheter hepatic arterial embolization (TAE) therapy (numbers 2, 5, 6, 7, 10 and 11) and HLA-A24-positive historical controls treated with TAE without dendritic cell (DC) transfer (numbers t8, t14, t15, t19, t20 and t22). Peripheral blood mononuclear cells (PBMCs) were obtained before (open bars) and 1 month after the infusion (solid bars), pulsed with the peptides derived from squamous cell carcinoma antigen recognized by T cells 2 (SART2), SART3, multi-drug resistance protein 3 (MRP3), alpha-fetoprotein (AFP), human telomerase reverse transcriptase (hTERT) and interferon (IFN)- γ production was quantitated by enzyme-linked immunospot (ELISPOT). Negative controls consisted of a human immunodeficiency virus (HIV) envelope-derived peptide (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA) or a cytomegalovirus (CMV) pp65-derived peptide (CMVpp65₃₂₈). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. T lymphocyte responses to the peptide epitopes were induced following TAE therapy, but no additional responses were observed after DC transfer. Numbers denote specific spots beyond the upper limit of y-axis; n.d., not determined.



centrations of IL-9, IL-15 and TNF- α were greatly increased after OK432-stimulated DC infusion, in contrast to their reduction following TAE treatment alone (Fig. 5a). Furthermore, the chemokines eotaxin (CCL11) and MIP-1 β (CCL4) were induced markedly after DC transfer, although they were also decreased after TAE alone. These data indicate that transfer of OK432-stimulated DC during TAE therapy induced unique immune responses that may be mediated by the cytokines IL-9, IL-15 and TNF- α and the chemokines eotaxin and MIP-1 β .

In addition, serum arginase activity was reported to reflect numbers of myeloid-derived suppressor cells (MDSCs) that may inhibit T lymphocyte responses in cancer patients [36]. Therefore, serum arginase activity was measured after OK432-stimulated DC infusion, and it was found that it was

increased six- or sevenfold in patients treated with TAE. However, this increase was independent of the presence or absence of OK432-stimulated DC transfer (Fig. 5b). None the less, serum arginase activity was decreased again 4 weeks after treatment with both TAE and OK432-stimulated DC transfer but tended to be maintained at a high levels in patients treated with TAE without DC transfer. However, these differences did not reach statistical significance ($P > 0.05$). Because arginase activity is known to be relatively high in liver and HCC cells [37], the influence of tissue injury was assessed biochemically by measuring serum levels of ALT and LDH activities. We did not observe ALT or LDH elevation, indicating that the increase of arginase activity was not due to tissue damage following treatment. Collectively, these results demonstrate that infusion of OK432-stimulated

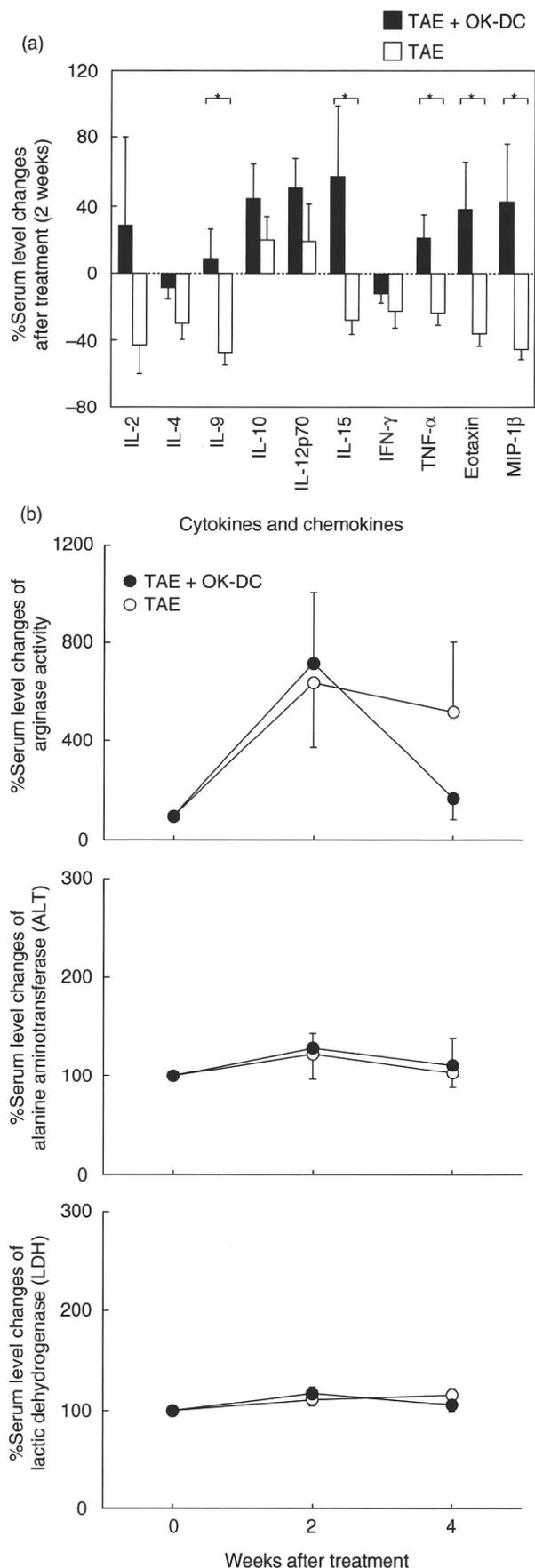


Fig. 5. Cytokine and chemokine profiling and arginase activity in sera of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy (TAE + OK-DC; $n = 13$) and the historical controls treated with TAE without DC transfer (TAE; $n = 22$). (a) Serum samples were examined for their content of a validated panel of cytokines and chemokines using the Bioplex assay. Percentage changes in serum levels 2 weeks after the treatments were calculated as follows: [(post-treatment level – pretreatment level)/pretreatment level] \times 100. The data are means \pm standard error of the mean (s.e.m.) of the groups. * $P < 0.05$ when compared by the Mann–Whitney U -test. (b) Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine, and for alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) activities. While there was a trend for the arginase activity in the TAE + OK-DC group to decrease 4 weeks after treatment, the difference did not reach statistical significance ($P > 0.05$). Percentage changes in serum levels 2 weeks after the treatments were calculated as follows: [(post-treatment level – pretreatment level)/pretreatment level] \times 100. The data indicate means \pm s.e.m. of the groups.

DCs during TAE treatment may reduce the immunosuppressive activities of MDSCs, and assist in developing a favourable environment for the induction of anti-tumour immunity.

Discussion

Although many novel strategies, including immunotherapies, have been developed in an attempt to suppress tumour recurrence after curative treatments for HCC, recurrence rates and survival times have not been improved significantly [38]. In the current study, we first established that OK432-stimulated DC administration during TAE therapy did not cause critical adverse events in patients with cirrhosis and HCC. Most importantly, DC transfer resulted in prolonged recurrence-free survival after combination therapy with TAE and OK432-stimulated DC administration. In terms of the immunomodulatory effects of DC transfer, although NK cell activity, intracellular cytokine production and T lymphocyte-mediated immune responses were not altered in PBMCs from treated patients, serum levels of IL-9, IL-15 and TNF- α and the chemokines eotaxin and MIP-1 β were enhanced markedly after DC transfer. In addition, serum levels of arginase activity were decreased following DC transfer. Collectively, this study demonstrated the feasibility, safety and beneficial anti-tumour effects of OK432-stimulated DC infusion into tumour tissues for patients with cirrhosis and HCC, suggesting the ability of an active immunotherapeutic strategy to reduce tumour recurrence after locoregional treatment of HCC.

DCs were stimulated with OK432 prior to infusion into tumour tissues through an arterial catheter. OK432 was reported to activate DCs through its binding to TLR-2 and -4 [16,39] that can be used for cancer therapy [33]. The current results indicate that OK432 stimulation of immature DCs

from HCC patients promoted their maturation processes while preserving antigen uptake capacity and enhancing tumoricidal activity, consistent with previous observations [16,19] and supporting the current strategy in which OK432-stimulated DCs were infused directly into tumour tissues. Because the tumoricidal activity of unstimulated DCs was not observed in *in vitro* experiments, OK432 stimulation obviously altered the cytotoxic properties of DCs. One of the mechanisms of DC killing was reported to be CD40/CD40 ligand interaction [19]. Further studies are needed to determine the killing mechanisms of DCs derived from HCC patients in a direct [TNE, TNF-related apoptosis inducing ligand (TRAIL), Fas ligand, nitric oxide (NO) and perforin/granzyme] and indirect (MHC-restricted) manner [40–43]. Although the main mechanism by which OK432-stimulated DCs prolonged the recurrence-free survival was not elucidated, the tumoricidal activity of mature DCs was implicated in *in vivo* enhancement of antigen presentation, co-stimulation and inflammatory cytokine production.

Very recent reports document injection of OK432-stimulated DCs into patients with cancer of the gastrointestinal tract or pancreas [44,45], but their anti-tumour effects were not defined clearly. The current study shows for the first time that OK432-stimulated DCs induce beneficial anti-tumour responses when transferred into tumour tissues during TAE therapy. The anti-tumour responses may have been enhanced as a result of optimal activation of the DCs with OK432 or combining infusion of stimulated DCs with TAE therapy. Inappropriately activated DCs may be unable to generate sufficient numbers of properly activated effector T lymphocytes [46]. As shown in Fig. 1, all these alterations could contribute to the further enhancement of anti-tumour effects compared to those in our previous study with immature DCs [20]. Furthermore, the tumour cell death-promoting therapies, e.g. chemotherapy [47] and TAE [48], can be expected to enhance the effects of therapeutic cancer vaccines by redressing the immunosuppressive tumour environment.

NK cell activity and intracellular cytokine responses in CD4⁺ and CD8⁺ T lymphocytes and CD56⁺ NK cell subsets in PBMCs were not changed significantly in patients treated with OK432-stimulated DCs. Furthermore, we did not observe tumour antigen-specific T lymphocyte responses associated clearly with DC administration. The data suggest therefore that the immune responses induced by the therapy applied here were not detectable systemically. Because cytotoxic T lymphocyte responses were enhanced in patients receiving $> 3 \times 10^7$ cells [49,50], the numbers of transferred OK432-stimulated DCs were apparently not sufficient to induce responses detectable in the peripheral blood, but were enough to exert beneficial anti-tumour effects. In addition, many studies have concluded that cytotoxic T lymphocyte responses rarely predict clinical outcomes of DC-based immunotherapies [51,52] and that in many cases, also including our own studies

[28,30], tumour-specific effector T lymphocytes co-exist with the tumours. Consistent with these observations, the current results suggest that cytotoxic T lymphocyte responses in PBMCs are not reliable predictors of beneficial anti-tumour effects in patients treated with the current OK432-stimulated DC strategy.

Serum levels of the cytokines IL-9, IL-15 and TNF- α and the chemokines eotaxin and MIP-1 β were increased following OK432-stimulated DC transfer, but decreased after TAE therapy without DC administration. IL-9 and IL-15 belong to the cytokine receptor common gamma chain (γ_c ; CD132) family, a member of the type I cytokine receptor family expressed on most lymphocyte populations [53]. IL-9 exerts pleiotropic activities on T and B lymphocytes, mast cells, monocytes and haematopoietic progenitors [54,55]. IL-15 and TNF- α are known to prime T lymphocytes and NK cells when secreted by DCs [56] and to induce anti-tumour immune responses [57]. Eotaxin is known to selectively recruit eosinophils also contributing to anti-tumour effects [58,59], and MIP-1 β is a chemoattractant for NK cells, monocytes and a variety of other immune cells [60]. In addition, serum levels of arginase tended to decrease after DC transfer. Because serum arginase activity reflects the numbers of MDSCs that inhibit T lymphocyte responses in cancer patients [36], the patients treated with OK432-stimulated DCs might have developed lower levels of suppressor cells. Collectively, the results suggest that infusion of OK432-stimulated DCs may orchestrate the immune environment in the whole body that could enhance beneficial anti-tumour effects, although the precise molecular and cellular mechanisms associated with the actions of these cytokines and chemokines were not defined clearly in the current analysis.

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Disclosure

The authors have declared that no conflict of interest exists.

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Enhancement of tumor-specific T-cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma

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Transcatheter arterial embolization (TAE) destroys a tumor by the induction of necrosis and/or apoptosis and causes inflammation with cytokine production, which may favor immune activation and presentation of tumor-specific antigens. In the current study, we attempted to identify the effect of TAE on tumor-specific T-cell responses and the additional effect of dendritic cell (DC) infusion performed during TAE. The prevalence of tumor antigen-specific T cells was determined by interferon- γ enzyme-linked immunospot analysis using alpha-fetoprotein (AFP) and tumor antigen-derived peptides in 20 and 13 patients with hepatocellular carcinoma (HCC) who received TAE and TAE with DC infusion, respectively. The increased frequency of AFP-specific T cells was observed in 6 of 20 patients after TAE. It was observed more frequently in patients with DC infusion than in those with TAE alone. However, tumor recurrence was not completely prevented in patients albeit displayed enhanced immune responses. The evidence that the enhanced immune responses were transient and attenuated within 3 months was provided in time-course analysis. In conclusion, TAE with DC infusion enhances the tumor-specific immune responses more effectively than TAE alone. Although the effect is not sufficient to prevent HCC recurrence, these results may contribute to the development of novel immunotherapeutic approach for HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignancies and has gained major clinical interest because of its increasing incidence. Although current advances in therapeutic modalities have improved the prognosis of patients with HCC, the survival rate is still unsatisfactory.¹⁻⁴ One of the reasons for the poor prognosis is the high rate of recurrence after treatment.⁵ Therefore, the development of new antitumor therapies to protect against recurrence is important to improve the prognosis for HCC.

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Several recent studies of cancer treatment causing tumor necrosis or apoptosis have shown that they induce the activation of tumor-specific

immune responses.⁶⁻¹⁰ The mechanism to activate host immune responses against tumors is still unknown; however, several studies *in vitro* or *in vivo* suggest that cytokine production, attracting leukocyte infiltration, increase of tumor antigen uptake by macrophages or dendritic cells (DCs) and release of heat shock protein caused by inflammation at the tumor site are associated with the phenomenon.¹¹⁻¹⁷

Transcatheter arterial embolization (TAE) has been used extensively in the Western world and Asia to treat unresectable HCCs.¹⁸⁻²⁰ Although several previous randomized controlled trials have failed to show a survival benefit in patients treated with TAE compared to untreated patients,^{21,22} recent studies demonstrated a survival benefit for TAE *versus* conservative treatment in carefully selected patients.²³⁻²⁵

Histological assessment of resected HCC after TAE shows that the treatment induces necrotic and apoptotic changes in the tumor.²⁶⁻²⁹ Moreover, it is reported that the serum levels of macrophage-colony stimulating factor and the lipopolysaccharide-stimulated production of interleukin-1 beta, IL-6 and tumor necrosis factor-alpha in peripheral whole blood were increased after TAE.³⁰⁻³² Taken together with the previously described knowledge of immune responses after treatment to induce tumor necrosis or apoptosis, these observations support the hypothesis that the induction of apoptotic or necrotic cell death and inflammatory cytokines by TAE favors immune activation and induction of tumor-specific T-cell

Key words: immune response, AFP, CTL, immunotherapy, epitope
Abbreviations: HLA: human leukocyte antigens; IFN: interferon; HCV: hepatitis C virus; ELISPOT: enzyme-linked immunospot; TAE: transcatheter arterial embolization; MRP: multidrug resistance-associated protein; hTERT: human telomerase reverse transcriptase
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responses. In a previous study, we also made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.^{7,10} In addition, we have recently developed a new immunotherapeutic approach for HCC using DC infusion performed during TAE, showing the potential to enhance tumor-specific immune responses.⁷

In the current study, we first attempted to identify the effect of TAE for tumor-specific T-cell responses in patients with HCC. Next, we examined the additional effects of DC infusion to the tumor site after TAE. Finally, we analyzed the relationship between clinical characteristics of patients and T-cell responses after TAE and evaluated whether the activation of tumor-specific T-cell responses can prevent HCC recurrence.

Material and Methods

Patient population

The study examined 33 patients with HCC, consisting of 25 men and 8 women ranging from 48 to 83 years old with a mean age of 66 ± 9 years. Twenty patients were treated by TAE. Thirteen patients were treated by TAE with DC infusion as a part of clinical study, which was approved by ethical committee of Kanazawa University Graduate School of Medical Science and registered in September 2003. The patients who received TAE with DC infusion were selected according to the criteria we previously reported.⁷ All subjects were negative for Abs to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration.

Treatment of hepatocellular carcinoma

HCCs were detected by imaging modalities such as dynamic CT scan, MR imaging and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens, surgical resection or autopsy in 18 cases. For the remaining 15 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.³³ The tumor size was categorized as "small" (≤ 2 cm) or "large" (> 2 cm), and tumor multiplicity was categorized as "multiple" (≥ 2 nodules) or "solitary" (single nodule). The TNM stage was classified according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version).³⁴

Twenty patients were treated by TAE as previously described.^{19,35} In brief, after evaluation of the feeding arteries and surrounding vascular anatomy, a microcatheter (Microferret, Cook, Bloomington, IN) was inserted into the segmental or subsegmental artery with a coaxial method using a 0.016-inch guidewire (Radifocus GT wire, Terumo, Tokyo, Japan). A mixture of the anticancer drug and iodized oil was administered, and the feeding artery was embolized with gelatin sponge particles (Gelfoam; Pharmacia Upjohn, Kalamazoo, MI).

The mixture of anticancer drug and iodized oil contained 10–30 mg of Epirubicin (Farmorubicin; Kyowa Hakko Kogyo, Tokyo, Japan), 1–3 ml of iodized oil (Lipiodol Ultra Fluide) and 0.5–1.0 ml of iohexol (Omnipaque 300).

Preparation and injection of autologous DCs

DCs were generated as previously described.⁷ In 6 patients, DCs were pulsed with 0.1 KE/ml OK-432 (Chugai Pharmaceutical, Tokyo, Japan), which is a biological response modifier derived from the weakly virulent Su strain of *Streptococcus pyogenes*,^{36,37} for 3 days before injection. The cells were harvested for injection; 5×10^6 cells were reconstituted in 5-ml normal saline containing 1% autologous plasma, mixed with gelatin sponge particles and infused through an arterial catheter following iodized oil injection during TAE.

After TAE or TAE with DC infusion, 26 patients received percutaneous tumor ablation by ethanol injection (PEIT), microwave coagulation (MCT) or radiofrequency (RF). Twenty-one patients were diagnosed with complete necrosis of the tumor lesion using dynamic CT after the completion of treatment. Follow-ups were conducted at outpatient clinics using blood tests and dynamic CT every 3 months for 1 year.

Laboratory and virologic testing

Blood samples were tested for HBsAg and HCVAb by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of PBMC from patients was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda. The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.³⁸ The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.*³⁹

Interferon- γ enzyme-linked immunospot assay

The prevalence of tumor antigen-specific T cells was determined by interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) analysis (Mabtech, Nacka, Sweden) as previously described.^{10,40} HLA-A24-restricted AFP-derived peptides (10 μ g/ml), which were AFP₃₅₇ (EYSRRHPQL), AFP₄₀₃ (KYIQESQAL) and AFP₄₃₄ (AYTKKAPQL),¹⁰ and 20 μ g/ml AFP derived from human placenta (Morinaga Institute of Biological Science, Yokohama, Japan, purity $> 98\%$) were added directly to the wells. These 3 AFP-derived peptides could induce CTLs showing cytotoxicity against hepatoma cells and were frequently recognized by PBMCs of patients with HCC as we previously reported,¹⁰ and therefore, we selected them as an immunogenic peptide. The HLA-A24-restricted AFP and CMV-derived peptides were used only for HLA-A24 or A23 positive patients. Other tumor antigen-derived peptides consisted of MRP₃₅₀₃ (LYAWEPSFL), MRP₃₆₉₂ (AYVPQQAWI), MRP₃₇₆₅ (VYSDADIFL), hTERT₁₆₇ (AYQVCGPPL), hTERT₃₂₄

(VYAETKHFL) and hTERT₄₆₁ (VYGFVRACL), which we previously reported that they were useful for analyzing host immune responses to HCC.^{40,41}

PBMCs were added to the wells at 3×10^5 cells/well. In the assay using PBMC depleted CD4⁺ or CD8⁺ cells, the number of cells was adjusted to 3×10^5 cells/well after the depletion. Depletion of CD4⁺ or CD8⁺ cells was performed by MACS separation system using CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. After the depletion, 1×10^6 cells were stained with CD4 and CD8 antibodies (Becton Dickinson, Tokyo, Japan) and analyzed by FACSCalibur (Becton Dickinson, Tokyo, Japan) to confirm the ratio of CD4⁺ and CD8⁺ cells. Data analysis was undertaken with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Plates were analyzed with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least 2-fold greater than the number of spots in the absence of antigen. Negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24-restricted epitope derived from HIV envelope protein (HIVenv₅₈₄) and were always <5 spots per 3×10^5 cells.⁴² The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide (CMVpp65₃₂₈).⁴³ All peptides used in this study were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). ELISPOT analysis was performed before and 2–4 weeks after TAE. In patients receiving additional treatment for complete ablation of tumor, analysis was performed just before the additional treatment. An increase of antigen-specific T cells was defined as significant when T-cell responses changed to positive or if the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment.

Statistical analysis

Unpaired Student's *t*-test was used to analyze the effect of variables on immune responses in patients with HCC. Fisher's exact test (2-sided *p*-value) was used to analyze the frequency of positive immune responses in patients between with TAE and TAE with DC infusion.

Results

T-cell responses to AFP in the patients who received TAE

The frequency of AFP-specific T cells before and after TAE was tested *ex vivo* in an IFN- γ ELISPOT assay. The serum AFP level and number of peripheral lymphocytes and antigen-specific T cells are shown in Table 1. Before treatment, 2 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein in 20 patients (Patients 1–20). After treatment, a T-cell response to AFP-derived pep-

tides and protein was detected in 4 and 3 patients, respectively.

When an increase of antigen-specific T cells was defined as significant if T-cell responses changed to positive or the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment, 6 of 20 (30%) patients (Patients 4, 6, 7, 11, 18 and 20) showed a significant increasing of AFP-specific T-cell frequency after treatment. It was observed even in the patient (Patients 6, 7 and 18) who had no T cells specific to corresponding AFP-derived peptides before treatment. When a decrease of antigen-specific T cells was defined as significant if T-cell responses changed from positive to negative or the number of spots detected after TAE was less than half of the number of spots detected before treatment, 4 of 20 (20%) patients (Patients 5, 14, 15 and 16) showed a significant decreasing of AFP-specific T-cell frequency after treatment.

AFP-specific IFN- γ -producing T cells were also analyzed by ELISPOT assay using PBMC depleted CD4⁺ or CD8⁺ cells to determine what kind of T cells is responsive to whole AFP. Depletion of CD4⁺ or CD8⁺ cells was performed by MACS separation system, and the results were confirmed by flow cytometric analysis (Fig. 1a). After depletion of CD4⁺ or CD8⁺ cells, the ratio of each cell population was decreased to less than 0.1% of PBMCs. The IFN- γ ELISPOT assay showed that IFN- γ -producing T cells against AFP consisted of both CD8⁺ and CD4⁺ cells (Fig. 1b).

To confirm the effect of TAE for host immune responses to HCC, we also examined the frequency of tumor antigen-specific T cells in 4 patients (Patients 5, 8, 10 and 14) using MRP3- or hTERT-derived peptides that we previously identified as useful for analyzing host immune responses to HCC.^{40,41} A significant increasing of MRP3- or hTERT-specific T-cell frequency was observed in all patients after TAE (Table 2).

T-cell responses to AFP in the patients who received TAE with DC infusion

In 13 patients receiving TAE with DC infusion (Patients 21–33), 2 patients showed a specific T-cell response with AFP-derived peptides and 2 patients with protein before treatment (Table 3). After treatment, 8 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein.

Next, we compared TAE with DC infusion with TAE alone regarding the effect to AFP-specific immune response. Table 4 shows the clinical features of patients with HCC who received TAE and TAE with DC infusion and they were not statistically different except liver function.

The frequency of patients who showed both positive and increasing T-cell response with AFP-derived peptides or protein after treatment was significantly higher in patients receiving TAE with DC infusion than in those receiving TAE alone (*p* = 0.04) (Fig. 2a). On the other hand, the frequency of patients who showed both positive and increasing T-cell

Table 1. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE

Patient	HLA	Additional treatment	Complete ablation	Before treatment						After treatment								
				AFP (ng/ml)	Lymph. (μl^{-1})	AFP ₃₅₇	AFP ₄₀₃	AFP ₄₃₄	AFP	CMVpp65 ₃₂₈	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP ₃₅₇	AFP ₄₀₃	AFP ₄₃₄	AFP	CMVpp65 ₃₂₈
1	A2	RF	C	<10	1,600	ND	ND	ND	1	ND	0	<10	1,400	ND	ND	0	ND	1
2	A26,A31	RF	C	61	1,700	ND	ND	0	ND	0	13	23	900	ND	ND	0	ND	0
3	A11,A26	No	-	100	1,700	ND	ND	5	ND	1	1	50	1,500	ND	ND	0	ND	0
4	A24	RF	C	18	700	0	7	0	6	0	25	16	500	1	10	1	1	2
5	A24,A33	RF	C	2,357	1,200	13	2	6	0	13	0	700	1,100	2	1	1	0	9
6	A24	RF	C	14	1,800	0	0	0	0	0	42	<10	1,400	53	27	38	14	36
7	A23,A33	No	-	96	500	0	0	5	291	0	0	138	800	46	0	0	3	484
8	A24,A26	No	-	142	600	1	0	0	0	0	0	126	500	2	0	0	0	166
9	A2,A24	RF	C	<10	700	6	1	0	0	9	0	<10	700	0	0	0	0	32
10	A24	PEIT	C	<10	1,300	8	4	8	8	146	5	<10	1,300	0	1	1	0	1
11	A24,A26	PEIT	N	18	1,100	0	0	1	ND	0	0	13	400	0	0	0	15	10
12	A24,A33	RF	N	11	800	3	2	0	4	94	10	11	700	0	0	0	0	24
13	A11,A24	PEIT	C	52	1,300	0	2	5	1	2	0	24	1,200	0	0	0	0	0
14	A24	RF	C	54	2,400	25	5	4	8	12	0	67	1,700	0	0	0	0	0
15	A2,A24	RF	N	62	1,200	0	3	0	25	2	3	14	800	0	0	0	8	0
16	A3,A24	RF	C	2,876	900	0	1	0	13	0	5	3,285	700	0	0	0	0	0
17	A24,A33	No	-	205	400	4	2	3	2	26	9	220	100	2	1	0	1	39
18	A24,A30	RF	C	18	1,100	4	0	3	8	14	7	13	900	1	16	1	5	12
19	A2,A24	RF	C	330	1,500	2	0	0	0	18	1	36	1,100	0	4	0	3	8
20	A2,A33	RF	C	10	1,400	ND	ND	ND	10	ND	68	<10	800	ND	ND	31	ND	101

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; No, not completed; C, completed; N, not completed; ND, not done. The bold letters show the positive responses in ELISPOT assays.

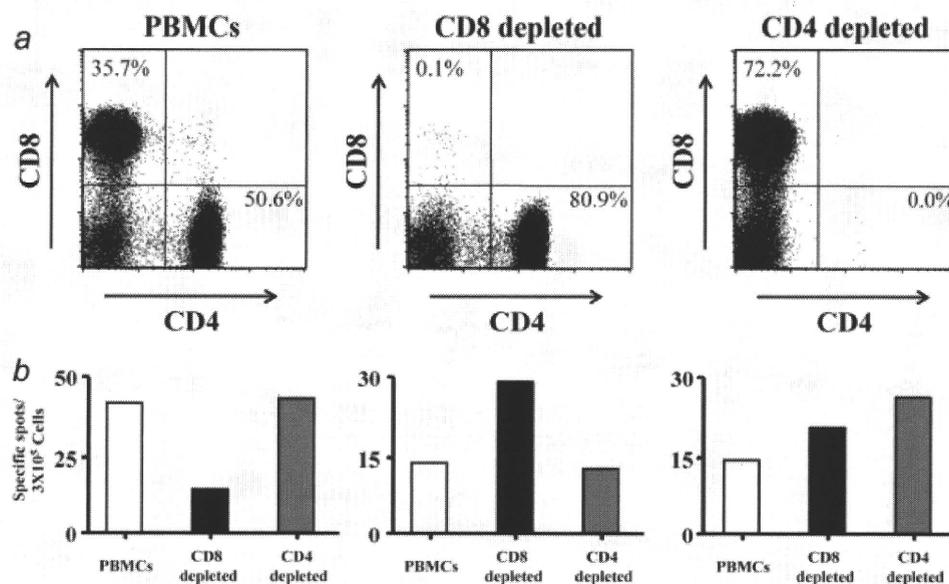


Figure 1. IFN- γ production of CD4⁻ or CD8⁻ depleted T cells against whole AFP. AFP-specific IFN- γ -producing T cells were analyzed by ELISPOT assay using PBMC depleted CD4⁺ or CD8⁺ cells to determine what kind of T cells is responsive to whole AFP. Depletion of CD4⁺ or CD8⁺ cells was performed by MACS separation system and the results were confirmed by flow cytometric analysis (a). IFN- γ ELISPOT assay using nontreated PBMCs and PBMC depleted CD4⁺ or CD8⁺ cells showed that T cells producing IFN- γ against whole AFP consisted of both CD8⁺ and CD4⁺ cells (b). Assays were performed in 5 patients and the representative result is shown.

Table 2. T cell response to other tumor antigen-derived peptides by ELISPOT assay before and after TAE

Patient	Before treatment						After treatment					
	MRP3 ₅₀₃	MRP3 ₆₉₂	MRP3 ₇₆₅	hTERT ₁₆₇	hTERT ₃₂₄	hTERT ₄₆₁	MRP3 ₅₀₃	MRP3 ₆₉₂	MRP3 ₇₆₅	hTERT ₁₆₇	hTERT ₃₂₄	hTERT ₄₆₁
5	2	7	8	0	3.5	7.5	0	0	0	7	3	35
8	6	6	1	3	ND	ND	17	18	22	18	14	9
10	0	1	3	0	5	7	0	4	7	6	11	4
14	6	5	0	9	5	13	6	14	22	8	10	7

Abbreviation: ND, not done. The bold letters show the positive responses in ELISPOT assays.

response with CMV-derived peptide or tetanus toxoid was not different between the 2 groups (Figs. 2b and 2c).

In the comparison of the mean values of spots generated with AFP-derived peptides, protein, CMV-derived peptides or tetanus toxoid, no significant difference was observed between patients with TAE alone before and after treatment (Figs. 3a–3d). In contrast, the mean values of spots generated with AFP-derived peptides were significantly higher in patients after TAE with DC infusion than in those before treatment (Fig. 3e). The mean values of spots generated with protein, CMV-derived peptides or tetanus toxoid were not significantly different between patients before and after TAE with DC infusion (Figs. 3f–3h). Based on the above results, we considered that the main difference between TAE alone and TAE with DC infusion was the response to HLA-A24-restricted AFP-derived epitopes. Therefore, to analyze the difference between TAE alone and TAE with DC infusion more precisely, we selected the patients with HLA-A24 or A23 and

compared the clinical parameters of both groups. However, there were no statistical differences except liver function in the 2 groups (Table 5).

Enhancement of AFP-specific T-cell responses and treatment outcome

To evaluate the effect of immune enhancement by TAE or TAE with DC infusion for the treatment outcome, we analyzed the clinical course of 17 patients who received complete ablation by additional RFA, PEIT or MCT after these treatments and could be followed up using dynamic CT every 3 months (Table 6). Seven patients showed increasing specific spots for AFP or AFP-derived peptides in ELISPOT assay after TAE. HCC recurrence within 3 months after complete ablation was observed in 3 patients who showed increasing AFP-specific T-cell responses after TAE. Furthermore, recurrence within 6 months after complete ablation was observed

Table 3. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE with DC infusion

Patient	HLA	Additional treatment	Complete ablation	Before treatment						After treatment									
				AFP (ng/ml)	Lymph. (μL^{-1})	AFP ₃₅₇	AFP ₄₀₃	AFP ₄₃₄	AFP	CMVpp65 ₃₂₈	TT	AFP (ng/ml)	Lymph. (μL^{-1})	AFP ₃₅₇	AFP ₄₀₃	AFP ₄₃₄	AFP	CMVpp65 ₃₂₈	TT
21	A24	No	-	332	1,100	7	1	4	ND	10	ND	819	800	11	0	10	ND	188	ND
22	A24,A26	RF	N	341	700	0	26	5	ND	68	ND	237	500	ND	59	ND	ND	81	ND
23	A11,A24	No	-	41	600	0	2	5	1	2	0	43	400	0	0	0	0	0	3
24	A2,A24	MCT	C	1,260	800	3	8	7	ND	19	ND	614	1,300	26	4	7	ND	12	ND
25	A24,A33	RF	C	11	1,500	0	1	0	31	5	15	19	900	1	4	15	26	3	4
26	A24,A33	RF	C	<10	2,000	0	0	0	0	0	0	<10	1,700	0	16	0	0	0	0
27	A24,A26	RF	C	16	700	0	0	0	1	1	0	16	700	2	1	15	9	0	1
28	A11,A31	RF	N	31	800	ND	ND	ND	3	ND	0	33	700	ND	ND	ND	0	ND	0
29	A11,A33	No	-	<10	1,100	ND	ND	ND	0	ND	0	<10	700	ND	ND	ND	0	ND	1
30	A2,A11	RF	C	13	1,300	ND	ND	ND	8	ND	1	14	1,500	ND	ND	ND	12	ND	7
31	A24,A33	RF	C	1,014	800	0	0	0	0	1	0	15	300	0	0	20	0	0	0
32	A11,A24	RF	C	<10	1,000	3	3	11	48	97	0	10	1,200	23	20	20	45	91	23
33	A2,A26	RF	C	29	1,300	ND	ND	ND	0	ND	0	27	1,300	ND	ND	ND	0	ND	0

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; MCT, microwave coagulation therapy; C, completed; N, not completed; -, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

Table 4. Patient characteristics

	Patients treated by TAE (n = 20)	Patients treated by TAE with DC (n = 13)	p-value ¹
Age (years) ²	66.6 ± 7.8	65.7 ± 10.0	NS
Sex (M/F)	14/6	11/2	NS
HLA (A23 or 24/others)	16/4	9/4	NS
ALT (IU/l)	51.0 ± 47.4	86.9 ± 62.8	NS
Total bilirubin (g/dl)	1.3 ± 0.9	1.5 ± 0.9	NS
Albumin (g/dl)	3.7 ± 0.7	3.2 ± 0.6	NS
AFP level (ng/ml)	322.7 ± 793.0	239.8 ± 418.2	NS
Diff. degrees of HCC (well/moderate or poor/ND ³)	2/6/12	4/4/5	NS
Tumor size (small/large ³)	4/16	1/12	NS
Tumor multiplicity (multiple/solitary)	18/2	12/1	NS
TNM stage (I, II/III, IV)	19/1	11/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	15/5	10/3	NS
Liver function (Child A/B or C)	14/6	3/10	0.02
Etiology (HCV/HBV/others)	12/2/6	13/0/0	NS

¹Abbreviations: NS, no statistical significance; ND, not determined. ²Data are expressed as the mean ± SD. ³Small: ≤2 cm, large: >2 cm.

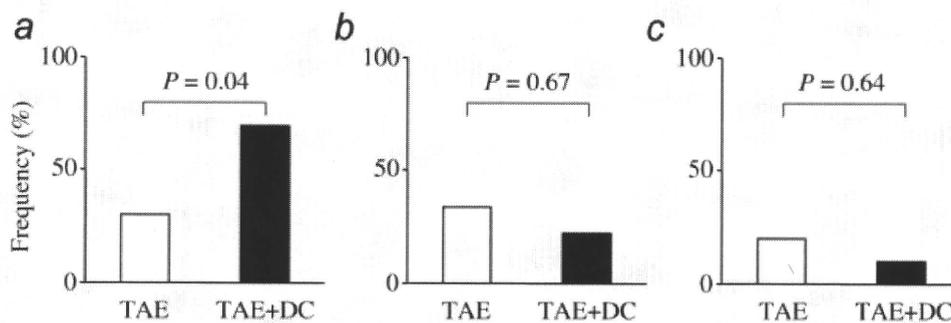


Figure 2. Frequency of the patients who showed enhancement of T-cell responses after treatment. The prevalence of antigen-specific T cells was determined by IFN- γ ELISPOT analysis using alpha-fetoprotein (AFP) and AFP-derived peptides (a), CMV pp65-derived peptide (b) or tetanus toxoid protein (c) in 20 and 13 patients with HCC who received TAE and TAE with DC infusion, respectively.

in 4 and 6 patients who did and did not show increasing AFP-specific T-cell responses, respectively.

Kinetics of AFP-specific T-cell responses before and after TAE

Next, we examined the kinetics of AFP-specific T cells in 8 patients who showed increasing frequency of IFN- γ -producing T cells against AFP or AFP-derived peptides after TAE. The frequency was examined by ELISPOT assay before and 2–4 weeks and 3 months after TAE. Thirteen kinds of AFP-specific T cells showed increasing frequency 2–4 weeks after TAE (Fig. 4); however, the increase was transient and most cell types decreased 3 months after TAE. Three patients showed more than 10 specific spots for AFP or AFP-derived peptides 3 months after TAE (Patients 6, 11 and 30). In analysis of the correlation between the maintenance of AFP-specific T-cell responses and HCC recurrence, 1 patient (Patient

6) had HCC recurrence after 6 months and 1 patient (Patient 30) did not show recurrence. Another patient (Patient 11) did not receive curative ablation and was not analyzed. There was no difference in the kinetics of AFP-specific T cells between patients who received TAE with and without DC infusion.

Discussion

In a previous study, we made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.^{7,10} Similarly, as in our previous or other group's results,⁸ we observed enhancement of AFP-specific immune responses in 6 of 20 patients with TAE alone in this study. The enhancement of tumor antigen-specific immune responses was also observed in the cases using MRP3- or hTERT-derived peptides.

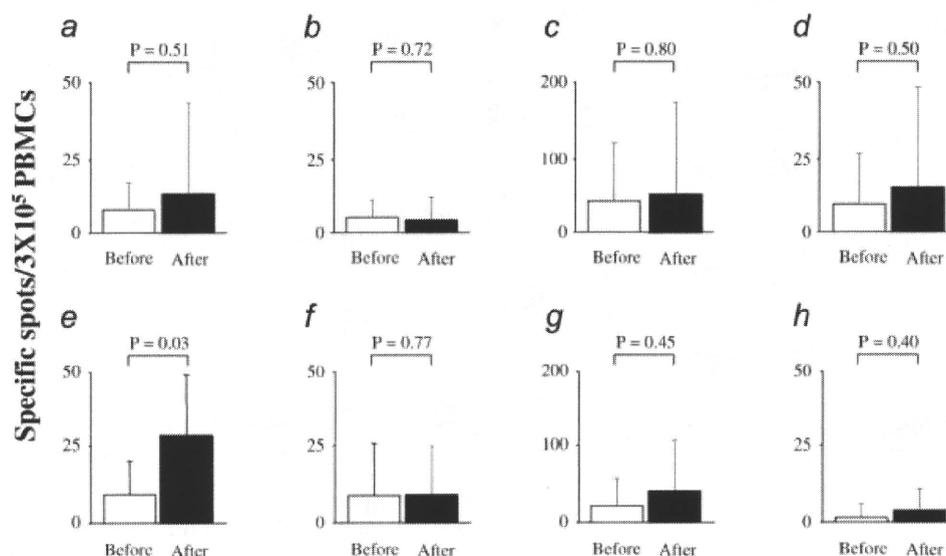


Figure 3. Comparison of direct *ex vivo* analysis (IFN- γ ELISPOT assay) before and after treatment of HCC. The assay was performed using PBMCs of patients who received TAE for AFP-derived peptides (a), AFP (b), CMV pp65-derived peptide (c) or tetanus toxoid protein (d). The same assay was performed using PBMCs of patients who received TAE with DC infusion for AFP-derived peptides (e), AFP (f), CMV pp65-derived peptide (g) or tetanus toxoid protein (h). AFP and CMV pp65-derived peptides were tested in only HLA-A24 or A23 positive patients. Data are expressed as the mean + SD of specific spots.

Table 5. Characteristics of the patients with HLA-A24 or A23

	Patients treated by TAE (n = 16)	Patients treated by TAE with DC (n = 9)	p-value ¹
Age (years) ²	65.7 \pm 7.8	67.8 \pm 10.8	NS
Sex (M/F)	10/6	7/2	NS
ALT (IU/l)	55.9 \pm 51.9	75.4 \pm 53.0	NS
Total bilirubin (g/dl)	1.4 \pm 0.8	1.4 \pm 1.1	NS
Albumin (g/dl)	3.6 \pm 0.7	3.1 \pm 0.6	NS
AFP level (ng/ml)	392.1 \pm 877.8	337.2 \pm 477.1	NS
Diff. degree of HCC (well/moderate or poor/ND ¹)	2/5/9	3/3/3	NS
Tumor size (small/large ³)	3/13	0/9	NS
Tumor multiplicity (multiple/solitary)	15/1	8/1	NS
TNM stage (I, II/III, IV)	15/1	7/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	13/3	8/1	NS
Liver function (Child A/B or C)	10/6	0/9	0.003
Etiology (HCV/HBV/others)	11/1/4	9/0/0	NS

¹Abbreviations: NS, no statistical significance; ND, not determined. ²Data are expressed as the mean \pm SD. ³Small: \leq 2 cm, large: $>$ 2 cm.

The precise mechanism of this phenomenon is still unknown; however, in recent studies, several treatments to destroy tumor cells by necrosis and/or apoptosis have induced antitumor immune responses in animal models^{14,44} and even in humans.⁶⁻¹⁰ In the study of *in situ* tumor ablation, it is reported that tumor ablation creates a tumor antigen source for the induction of antitumor immunity.^{9,44} In another study regarding photodynamic therapy (PDT),⁴⁵ it is

reported that acute inflammation, expression of heat-shock proteins and providing tumor antigens to DCs caused by PDT induce tumor-specific immune responses.

Based on these results, we hypothesize that DC infusion with TAE can induce antitumor immune responses more effectively than TAE alone. According to DC research in recent years, successful enhancement of the antitumor immune response has been reported by intratumoral

Table 6. Enhancement of AFP-specific T cell response and treatment outcome

	Enhancement of AFP-specific T cell response	Recurrence, 3 months	Recurrence, 6 months
Patient 1	-	N	U
Patient 2	-	N	M
Patient 4	+	M	ND
Patient 5	-	N	M
Patient 6	+	N	U
Patient 9	-	N	M
Patient 10	-	N	N
Patient 13	-	N	N
Patient 14	-	N	N
Patient 16	-	N	M
Patient 19	-	N	U
Patient 24	+	U	ND
Patient 25	+	M	ND
Patient 26	+	N	N
Patient 30	+	N	N
Patient 31	+	N	N
Patient 33	-	N	N

Abbreviations: N, no recurrence; U, uninodular recurrence; M, multinodular recurrence; ND, not determined.

administration of DC in combination with tumor ablation.^{46,47} Furthermore, immunotherapies using DC have been performed in patients with HCC and their antitumor effects are reported.⁴⁸⁻⁵⁰ These results support our hypothesis and therefore, in the next step, we examined the immunological effects of DC infusion with TAE.

The comparison of frequency in patients who showed enhancement of AFP-specific immune responses revealed more frequency in patients with DC infusion than in those with TAE alone. On the other hand, there were no differences in the 2 groups in the comparison of frequency for patients who showed enhancement of CMV or TT-specific immune responses. These results suggest that DC infusion with TAE affects tumor-specific immune responses and that the effects are limited to the tumor area.

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific

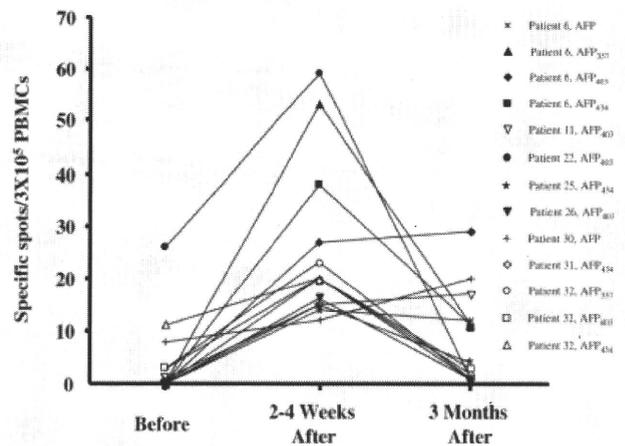


Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN- γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2-4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments as indicated in Tables 1 and 3 for a curative treatment after the measurement of T-cell responses at 2-4 weeks after TAE.

T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

In analysis of the association between the enhancement of AFP-specific T cells and clinical responses, no correlation could be shown, suggesting that enhancement of T-cell response associated with TAE or TAE with DC infusion may not have protective effect against HCC recurrence. To clarify the mechanism in more detail, we examined the kinetics of AFP-specific T-cell response. Increased frequency of AFP-specific T cells was transient and fell in 4 of 8 patients 3 months after treatment (Fig. 4). Similar to our results, Ayearu *et al.* also reported that the frequency of AFP-specific CD4⁺ T cells fell in all patients by 1-3 months after TAE.⁸ In addition, our results suggest that DC infusion with TAE is not effective to maintain the increased frequency of AFP-specific T cells.

Recent genome profiling studies of HCC show that HCC is a very heterogenous tumor.⁵¹ Furthermore, HCC has multicentric carcinogenesis and develops at different time points. These characters of HCC may also be another reason for no correlation between the enhancement of AFP-specific T cells and clinical responses. The identification of many more tumor antigens and their T-cell epitopes is necessary for more precise analysis of the relationship between anti-tumor immune response and clinical response, and for immunotherapy.

In the recent study, it is reported that CD8⁺ T-cell response to AFP is multispecific and AFP-specific IFN- γ -producing CD8⁺ T cells are directed against different epitopes spreading over the entire AFP sequence with no single