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ORIGINAL ARTICLE

Prolonged Recurrence-Free Survival following OK432-Stimulated Dendritic Cell Transfer into Hepatocellular Carcinoma during Transarterial Embolization

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SUMMARY

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumor recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a Streptococcus-derived anticancer immunotherapeutic agent, into tumor tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0.1 KE/ml OK432 for two days. Thirteen patients were administered with 5 x 10^6 of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs 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. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan-Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls (P = 0.046, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumor necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial antitumor effects against liver cancer.

INTRODUCTION

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumor recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumor liver tissues causes de novo development of HCC [3,4]. One strategy to reduce tumor recurrence is to enhance antitumor immune responses that may induce sufficient inhibitory effects to prevent tumor cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumor cells.

To enhance tumor antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and costimulatory molecules, e.g., CD40, CD80 and CD86 [9,10], and loaded with tumor-associated antigens including tumor lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NKT) cell activation, DCs have been stimulated and modified to produce larger amounts of cytokines, e.g., interleukin (IL)-12, IL-18 and type I interferons [10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g., C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of Streptococcus pyrogenes, were recently suggested to produce large amounts of T helper-1 (Th1)-type cytokines including IL-12 and interferon-γ (IFN-γ)

and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and prostaglandin E2 (PGE2)] [16]. Furthermore, because OK432 modulates DC maturation through TLR4 and the β_2 integrin system [16,17] and TLR4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of antitumor immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have been shown themselves to mediate strong, specific cytotoxicity toward tumor cells via CD40 / CD40 ligand interactions [19].

Recently, we have reported that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumor tissues and contributed to the recruitment and activation of immune cells in situ. However, this approach by itself yielded limited antitumor effects probably due to insufficient stimulation of immature DCs [the preparation of which seems closely related to therapeutic outcome [21,22]]. The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumor tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles, and, most importantly, experienced prolonged recurrence-free survival.

PATIENTS AND METHODS

Patients.

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of ≥70 %, an age of ≥20 years, informed consent, and the following normal baseline hematological parameters (within 1 week before DC administration): hemoglobin ≥8.5 g/dl; white cell count ≥2,000 /μl; platelet count ≥50,000 /μl; creatinine <1.5mg/dl, and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, hematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within four weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or antihistamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (4 women and 9 men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled in the study, with an age range from 56 to 83 years (**Table 1**). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of twenty-two historical controls (9 women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects one week later [24]. They underwent ultrasound, CT scan or magnetic resonance imaging (MRI) of the abdomen about one month after treatment and at a minimum of once every three months thereafter, and tumor 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 one 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 previously reported [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in 6-well tissue culture dishes (Costar, Cambridge, MA) at 1.4 x 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) 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 x 10^6 cells were suspended in 5 ml normal saline containing 1 % autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ) 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 leukocyte antigen (HLA)-DR^+.

Flow cytometry analysis.

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lin 1 (lineage cocktail 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), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA). Cells were analyzed on a FACSCalibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA).

DC phagocytosis.

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACSCalibur™ 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 ^{51}Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 (ATCC, Manassas, VA) and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labeled with ^{51}Cr. In a 96-well plate, 2.5 x 10^3 target cells per well were incubated with DCs for 8 h at different effector / target (E / T) ratios in triplicates. Percent specific lysis was calculated as follows: (experimental release - spontaneous

release) / (maximum release - spontaneous release) x 100. Spontaneous release was always < 20 % of the total.

NK cell activity.

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

Intracellular cytokine expression.

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37 °C in humidified 7 % CO_2 for 4 h. To block cytokine secretion, brefeldin A (Sigma, St. Louis, MO) [27] was added to a final concentration of 10 μg/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^{TM} system (BD Pharmingen), resuspended in PBS containing 1 % paraformaldehyde (PFA), and analyzed on a flow cytometer (≈10,000 gated events acquired per sample).

IFN-γ enzyme-linked immunospot (ELISPOT) assay.

ELISPOT assays were performed as previously described with the following modifications [28-30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 $(SART2)_{899}$ (SYTRLFLIL), $SART3_{109}$ (VYDYNCHVDL), multidrug resistance protein 3 $(MRP3)_{765}$ (VYSDADIFL), $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), alpha-fetoprotein $(AFP)_{403}$ (KYIQESQAL), AFP_{434} (AYTKKAPQL), AFP_{357} (EYSRRHPQL), human telomerase reverse

transcriptase (hTERT)$_{167}$ (AYQVCGPPL) (unpublished), hTERT$_{461}$ (VYGFVRACL), hTERT$_{324}$ (VYAETKHFL), were used in this study. Negative controls consisted of an HIV envelope-derived peptide (HIVenv$_{584}$). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide (CMVpp65$_{328}$). The colored 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 two fold 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). Briefly, frozen serum samples were thawed at room temperature, diluted 1 : 4 in sample diluents, and 50 µl aliquots of diluted sample were added in duplicate to the wells of 96-well microtiter 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-1ß, 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 FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and 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 done 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 the manufacturer (BioAssay Systems, Hayward, CA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA) to remove urea, combined with the sample buffer in wells of 96-well plate, and incubated

at 37^{o}C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The color produced was read at 520 nm using a microtiter plate reader.

Statistical analysis.

Results are expressed as means ± SD. Differences between groups were analyzed for statistical significance by the Mann-Whitney U test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumor 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 three days. 54.6 ± 9.5 % (mean ± SD; 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 ± 14.2 % were CD11c-positive (myeloid DC subset) and 14.8 ± 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 costimulatory 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 the cytokines IL-12 and IFN-γ (**Fig. 1C**). In addition, they displayed high cytotoxic 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 monitored clinically and biochemically after DC infusion (**Table 2**). A larger proportion (12 / 13) of the patients were complicated with high fever compared to those previously treated with immature DCs (5 / 10) [20], probably due to the proinflammatory responses induced by OK432-stimulated DCs. However, there were no grade 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 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 tumor 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 compared 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; 2 / 13 and 12 / 22, respectively; P = 0.046, log-rank test) (**Fig. 2**). The results demonstrated that OK432-stimulated DC transfer during TAE therapy reduces tumor 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 one and three months after treatment and NK cell cytotoxicity against K562 erythroleukemia target cells measured using the ^{51}Cr-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 tumor antigens.

To assess the effects on T cell responses to tumor 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 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 tumor antigens MRP3 and hTERT were induced in 3 of 6 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 4 (numbers t8, t19, t20 and t22) of 6 HLA-A24-positive patients. These data indicate that T lymphocyte responses to HLA-A24 restricted peptide epitopes of tumor 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 concentrations 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-1β (CCL4) were markedly induced 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-1β.

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 6- or 7-fold in patients treated with TAE. However, this increase was independent of the presence or absence of OK432-stimulated DC transfer (**Fig. 5B**). Nonetheless, 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 level 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 alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) activities. We did not observed 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 DCs during TAE treatment may reduce the immunosuppressive activities of MDSCs, and assist in developing a favorable environment for the induction of antitumor immunity.

DISCUSSION

Although many novel strategies including immunotherapies have been developed in an attempt to suppress tumor 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-1β were markedly enhanced after DC transfer. In addition, serum levels of arginase activity were decreased following DC transfer. Collectively, this study demonstrated the feasibility, safety, and beneficial antitumor effects of OK432-stimulated DC infusion into tumor tissues for patients with cirrhosis and HCC, suggesting the ability of an active immunotherapeutic strategy to reduce tumor recurrence after locoregional treatment of HCC.

DCs were stimulated with OK432 prior to infusion into tumor 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 directly infused into tumor tissues. Since 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]. The further studies are needed to determine the killing mechanisms of DCs derived from HCC patients in a direct (TNF, TRAIL, Fas ligand, 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, costimulation 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 antitumor effects were not clearly defined. The current study shows for the first time that OK432-stimulated DCs induce beneficial antitumor responses when transferred into tumor tissues during TAE therapy. The antitumor 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 of these alterations could contribute to the further enhancement of antitumor effects compared to those in our previous study with immature DCs [20]. Furthermore, the tumor 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 tumor environment.

NK cell activity and intracellular cytokine responses in $CD4^+$ and $CD8^+$ T lymphocytes and $CD56^+$ NK cell subsets in PBMCs were not significantly changed in patients treated with OK432-stimulated DCs. Furthermore, we did not observe tumor antigen-specific T lymphocyte responses clearly associated with DC administration. The data therefore suggest that the immune responses induced by the therapy applied here were not systemically detectable. Because cytotoxic T lymphocyte responses were

enhanced in patients receiving > 3 X 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 antitumor 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], tumor-specific effector T lymphocytes coexist with the tumors. Consistent with these observations, the current results suggest that cytotoxic T lymphocyte responses in PBMCs are not reliable predictors of beneficial antitumor 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-1β 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 hematopoietic 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 antitumor immune responses [57]. Eotaxin is known to selectively recruit eosinophils also contributing to antitumor effects [58,59], and MIP-1β 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 antitumor effects, although the precise molecular and cellular mechanisms associated with the actions of these cytokines and chemokines were not clearly defined in the current

analysis.

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DISCLOSURE

The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

Figure 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**) lin 1$^-$HLA-DR$^-$ subsets with [OK432(+)] and without [OK432(-)] stimulation were analyzed for surface expression of CD80, CD83, CD86 and CCR7. Dot plots of a representative case are shown in the left-hand panel. Mean percentages (± SD) 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 lin 1$^-$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) (± SD) 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 IL-12 and IFN-γ measured by ELISA. OK432-stimulated cells produced large amounts of the cytokines. The data indicate means ± SD of the groups with and without the stimulation. All comparisons in A - C [OK432(+) vs. OK432(-)] were statistically significant by the Mann-Whitney U test ($P < 0.005$). (**D**) Tumoricidal activity of DCs assessed by incubation with ^{51}Cr-labeled Hep3B, PLC/PRF/5 and T2 targets for 8 h at the indicated E / T (effector / target) cell ratios. OK432-stimulated cells displayed high cytotoxic activity against the target cells. The results are representative of the cases studied.

Figure 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with (TAE + OK-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, CT scan or 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; 2 / 13 and 12 / 22, respectively; $P = 0.046$, log-rank test).

Figure 3. Natural killer (NK) cell activity and intracellular cytokine production in PBMCs of patients treated with OK432-stimulated DCs during TAE therapy (n = 13). PBMCs were isolated before and one and three months after treatment and used for the analyses. Upper panel, NK cell cytotoxicity against K562 erythroleukemia target cells was evaluated at the 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 IFN-γ and IL-4. Percentages of cytokine-positive cells were quantitaed 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 ± SD of the groups.

Figure 4. Immune responses to HLA-A24-restricted peptide epitopes derived from tumor antigens in HLA-A24-positive patients treated with OK432-stimulated DCs during TAE therapy (numbers 2, 5, 6, 7, 10 and 11) and HLA-A24-positive historical controls treated with TAE without DC transfer (numbers t8, t14, t15, t19, t20 and t22). PBMCs were obtained before (open bars) and one month after the infusion (solid bars), pulsed with the peptides derived from SART2, SART3, MRP3, AFP and hTERT, and IFN-γ production was quantitated by ELISPOT. Negative controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml PMA or a CMV pp65-derived peptide ($CMVpp65_{328}$). 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.

Figure 5. Cytokine and chemokine profiling and arginase activity in **s**era of patients treated with OK432-stimulated DCs during 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 two weeks after the treatments were calculated as follows: [(posttreatment level – pretreatment level) / pretreatment level] X 100. The data are means ± SEM 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 four weeks after treatment, the difference did not reach statistical significance (*P* > 0.05). Percentage changes in serum levels two weeks after the treatments were calculated as follows: [(posttreatment level – pretreatment level) / pretreatment level] X 100. The data indicate means ± SEM of the groups.

Table 1. Patient Characteristics

Patient no.	Gender	Age (yrs)	HLA	TNM stages	No. of tumors	Largest tumor (mm)	Child-Pugh	KPS	Post-TAE Rx
1	M	60	A11 A33	Ⅲ	5	35	B	100	RFA
2	M	57	A11 A24	Ⅲ	1	21	B	100	RFA
3	M	57	A11 A31	Ⅲ	2	39	B	100	RFA
4	M	77	A2 A24	Ⅲ	2	35	A	100	RFA
5	F	83	A11 A24	Ⅲ	3	29	B	100	RFA
6	F	74	A2 A24	Ⅱ	1	35	A	100	RFA
7	F	72	A24 A33	Ⅲ	3	41	B	100	RFA
8	F	65	A2 A11	Ⅱ	4	12	B	100	RFA
9	M	71	A2 A11	Ⅱ	4	16	A	100	RFA
10	M	79	A11 A24	Ⅲ	2	40	A	100	RFA
11	M	71	A2 A24	Ⅱ	1	28	A	100	RFA
12	M	56	A2 A26	Ⅲ	2	25	B	100	RFA
13	M	64	A2 A33	Ⅲ	2	37	B	100	RFA

TNM, tumor-node-metastasis; Child-Pugh, Child-Pugh classification; KPS, Karnofsky performance scores; TAE, transcatheter arterial embolization; Rx, Treatment; HCC, hepatocellular carcinoma; RFA, percutaneous radiofrequency ablation.

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

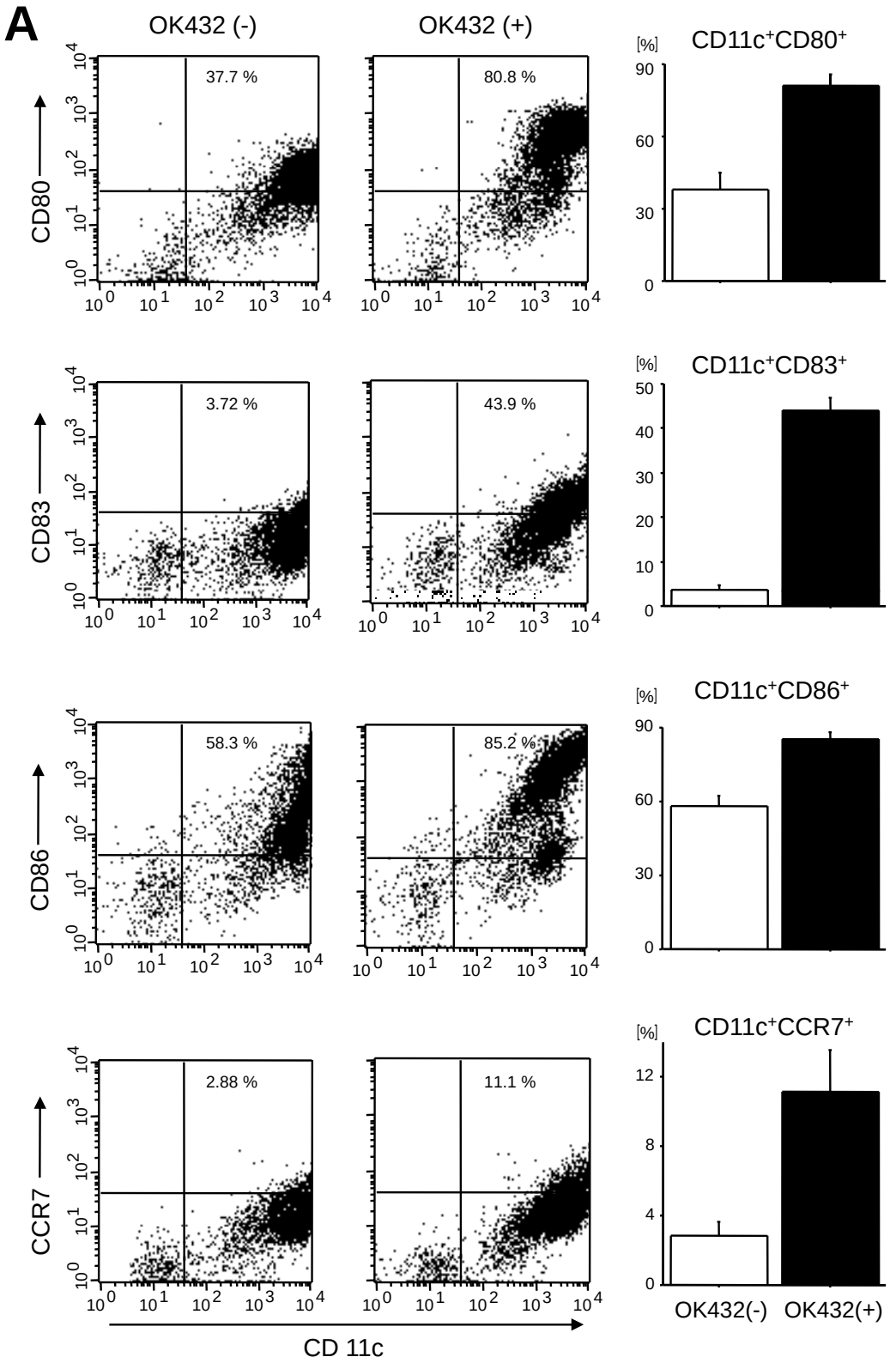
*Others of adverse events include myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

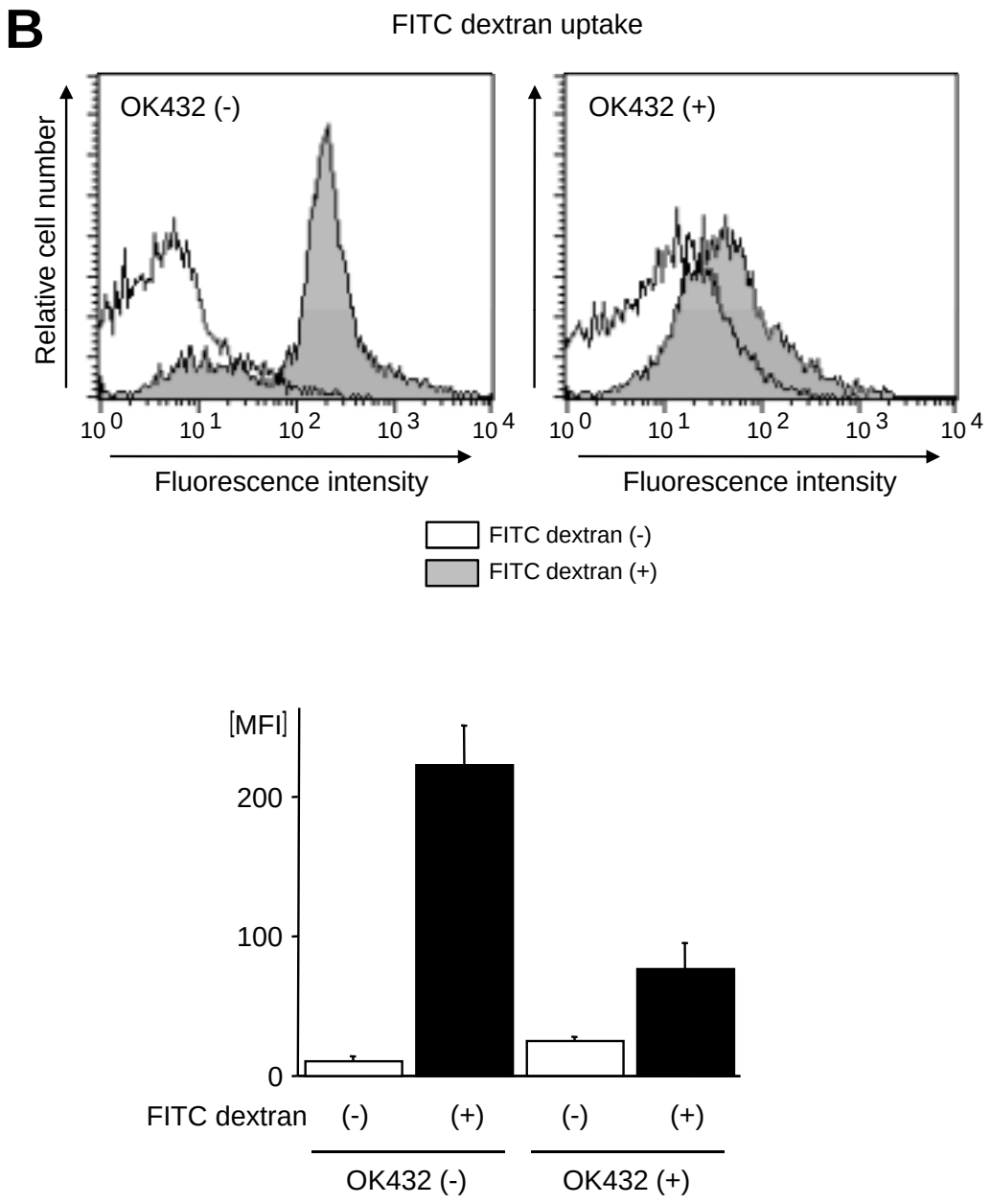
Table 3. Clinical Characteristics of Patients Treated with TAE + OK-DC and TAE alone

	TAE + OK-DC	TAE	*P*
No. of patients	13	22	
Age (yrs)	68.2 ± 9.1	70.0 ± 7.6	NS*
Gender (M/F)	9 / 4	13 / 9	NS†
White cell count ($\times 10^2/\mu L$)	34.4 ± 11.6	41.4 ± 18.9	NS*
Lymphocytes ($\times 10^2/\mu L$)	10.4 ± 3.6	12.4 ± 4.7	NS*
Platelets ($\times 10^4/\mu L$)	11.5 ± 10.2	10.3 ± 5.8	NS*
Hepaplastin test (%)	64.6 ± 11.6	75.5 ± 24.3	NS*
ALT (IU/L)	56.7 ± 38.9	67.9 ± 44.6	NS*
Total bilirubin (mg/dL)	1.3 ± 0.7	1.1 ± 0.6	NS*
Albumin (g/dL)	3.4 ± 0.6	3.6 ± 0.4	NS*
Noncancerous liver parenchyma (no.)			
Chronic hepatitis	0	8	
Cirrhosis (Child-Pugh A/B/C)	13 (5/8/0)	14 (6/8/0)	NS†
TNM stages (I/II/III/IV-A/IV-B)	0/4/9/0/0	3/8/11/0/0	NS†
No. of tumors	2.5 ± 1.3	1.9 ± 1.3	NS*
Largest tumor (mm)	30.2 ± 9.4	32.6 ± 15.2	NS*
AFP	204.8 ± 404.1	201.8 ± 544.2	NS*

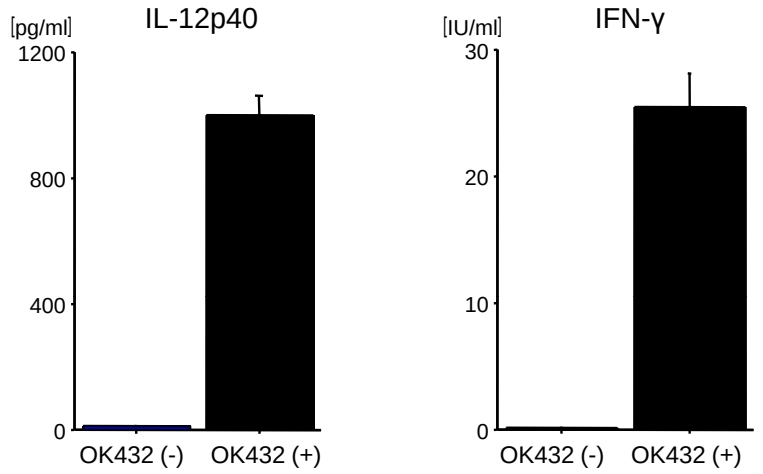
Results are expressed as means ± SD. *Mann-Whitney U test. †Fisher's exact test. TAE, transcatheter arterial embolization; OK-DC, OK432-stimulated dendritic cells; ALT, alanine transaminase; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; NS, not significant.

Fig.1A


A
OK432 (-)
OK432 (+)
CD80
CD83
CD86
CCR7
CD 11c
37.7 %
80.8 %
3.72 %
43.9 %
58.3 %
85.2 %
2.88 %
11.1 %
CD11c+CD80+
CD11c+CD83+
CD11c+CD86+
CD11c+CCR7+
[%]
OK432(-)
OK432(+)


B
FITC dextran uptake
OK432 (-)
OK432 (+)
Relative cell number
10 0
10 1
10 2
10 3
10 4
Fluorescence intensity
FITC dextran (-)
FITC dextran (+)
[MFI]
200
100
0
FITC dextran (-) (+) (-) (+)
OK432 (-)
OK432 (+)

C


IL-12p40
[pg/ml]
1200
800
400
0
OK432 (-)
OK432 (+)
IFN-γ
[IU/ml]
30
20
10
0
OK432 (-)
OK432 (+)

D

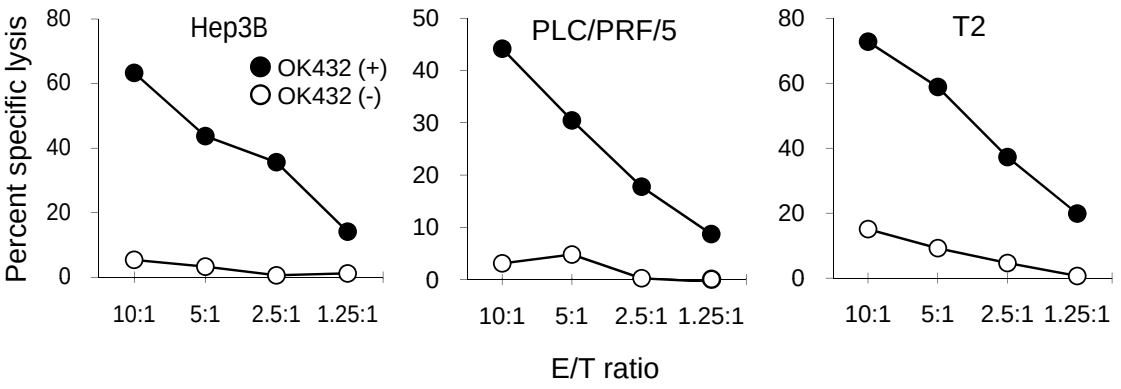

Hep3B
PLC/PRF/5
T2
OK432 (+)
OK432 (-)
Percent specific lysis
E/T ratio
10:1
5:1
2.5:1
1.25:1
0
20
40
60
80
10
30
50

Fig.2

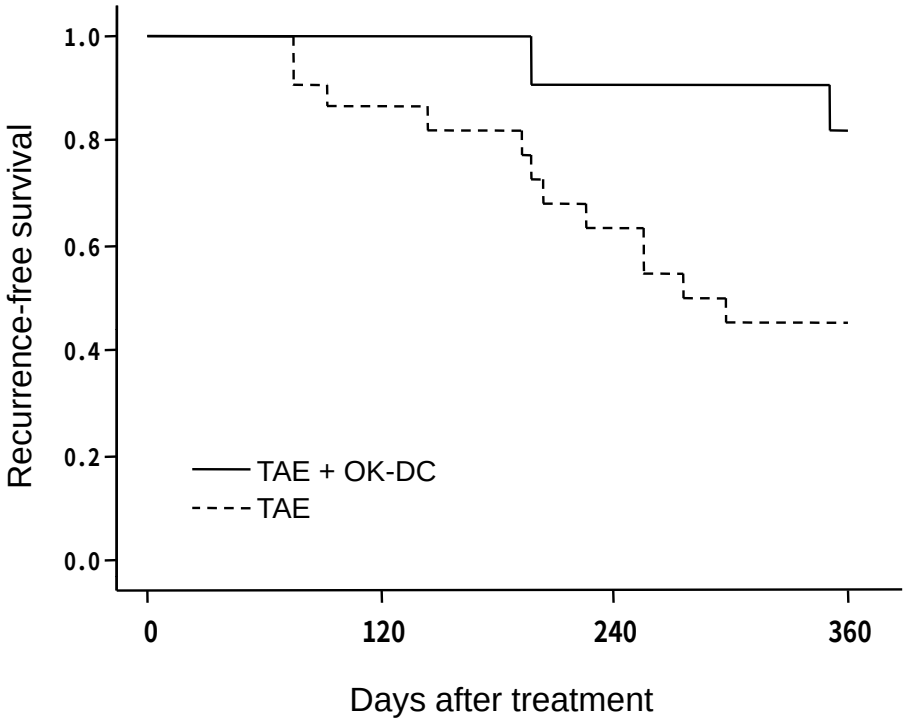

Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
0.0
TAE + OK-DC
TAE
0
120
240
360
Days after treatment

Fig.3

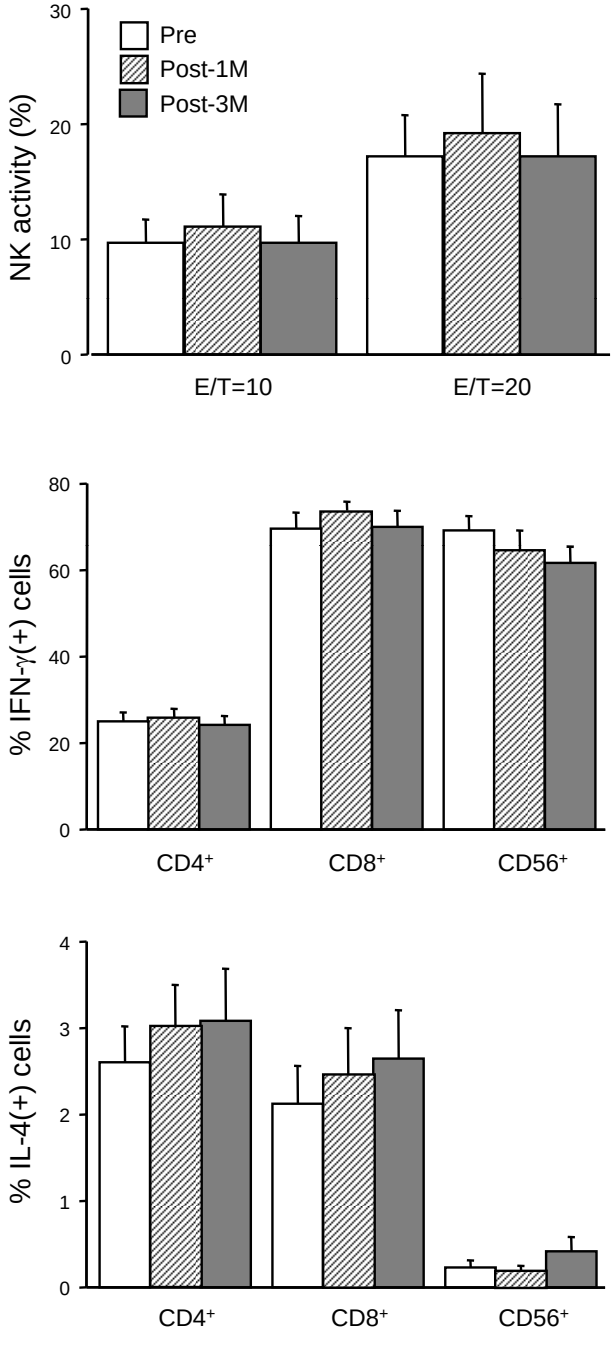

Pre
Post-1M
Post-3M
NK activity (%)
30
20
10
0
E/T=10
E/T=20
% IFN-γ(+) cells
80
60
40
20
0
CD4+
CD8+
CD56+
% IL-4(+) cells
4
3
2
1
0
CD4+
CD8+
CD56+

Fig.4

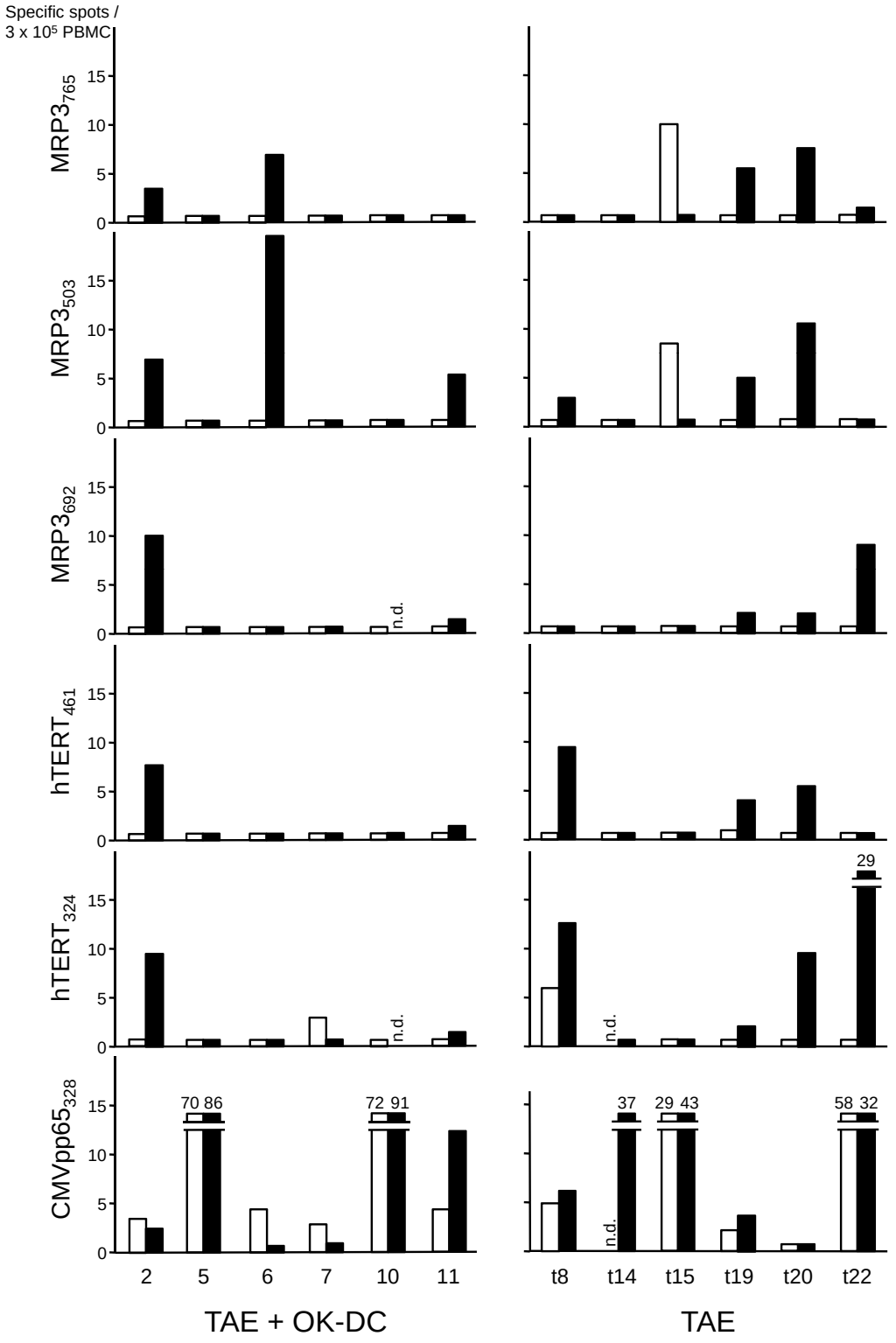

Specific spots / 3 x 10^5 PBMC
MRP3765
MRP3503
MRP3692
hTERT461
hTERT324
CMVpp65328
0
5
10
15
n.d.
70 86
72 91
37
29 43
58 32
29
2
5
6
7
10
11
t8
t14
t15
t19
t20
t22
TAE + OK-DC
TAE

Fig.5A

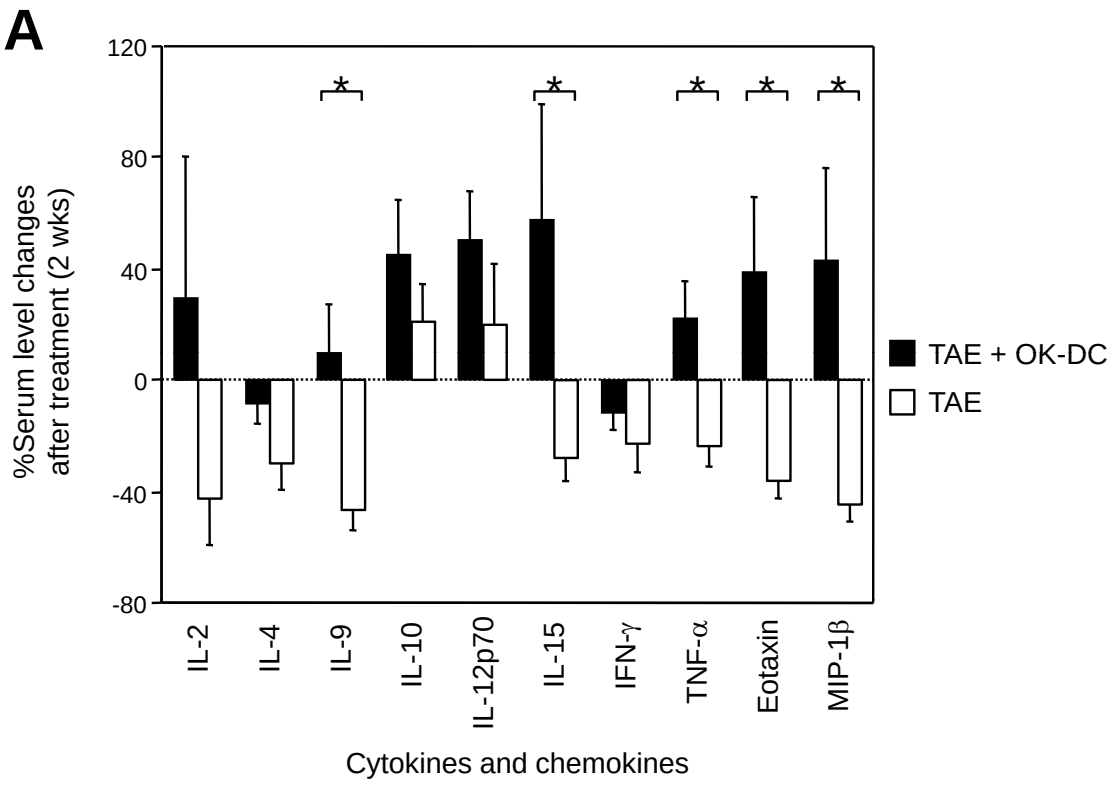
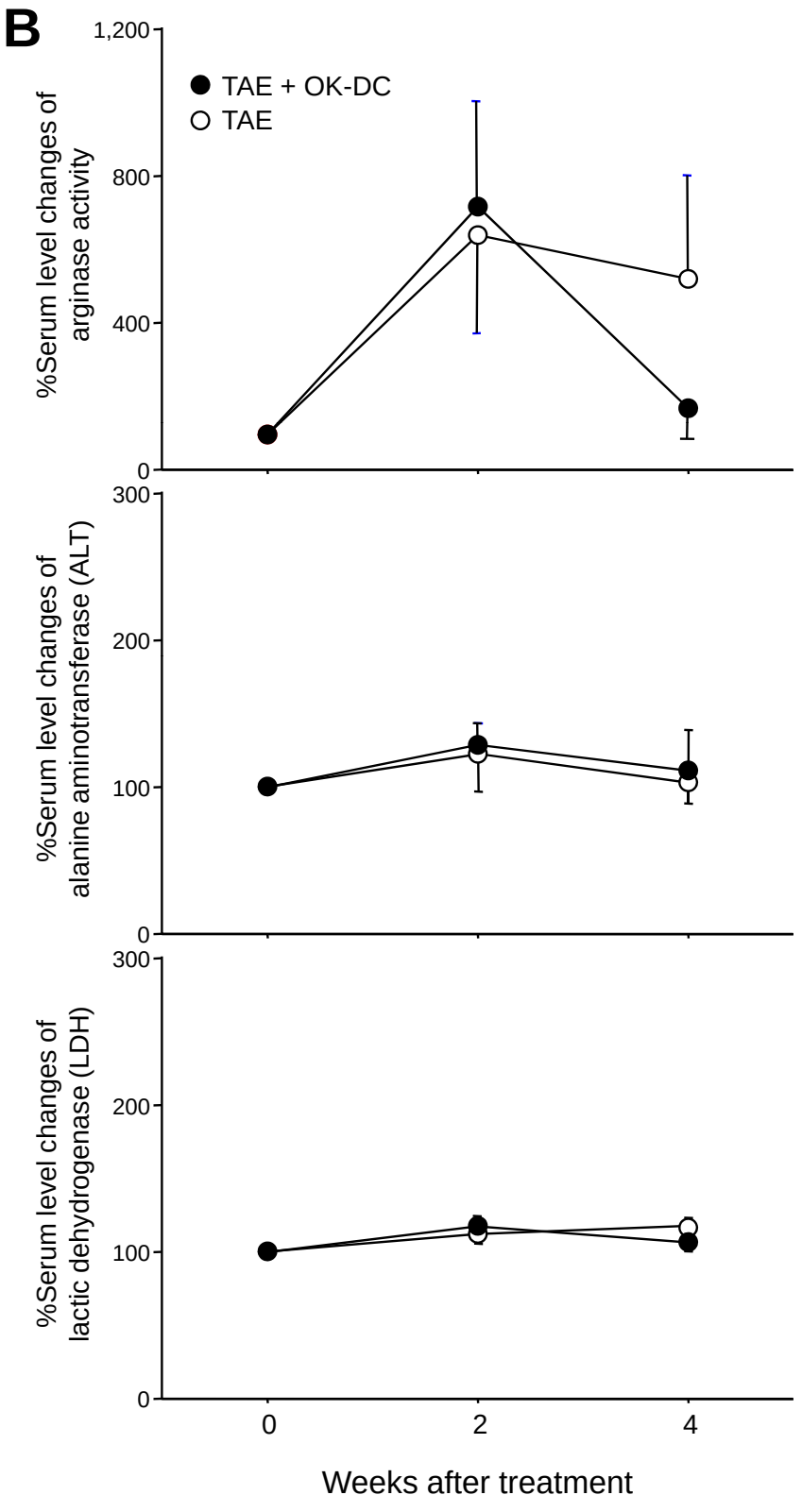

A
120
80
40
0
-40
-80
%Serum level changes after treatment (2 wks)
*
*
*
*
*
IL-2
IL-4
IL-9
IL-10
IL-12p70
IL-15
IFN-γ
TNF-α
Eotaxin
MIP-1β
Cytokines and chemokines
TAE + OK-DC
TAE

Fig.5B


B
1,200
800
400
0
TAE + OK-DC
TAE
%Serum level changes of arginase activity
300
200
100
0
%Serum level changes of alanine aminotransferase (ALT)
300
200
100
0
%Serum level changes of lactic dehydrogenase (LDH)
0
2
4
Weeks after treatment

[Changes are UNDERLINED]

Prolonged Recurrence-Free Survival following OK432-Stimulated Dendritic Cell Transfer into Hepatocellular Carcinoma during Transarterial Embolization

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Short Title: OK432-Stimulated DC Transfer into HCC during TAE

Keywords: hepatocellular carcinoma, dendritic cells, immunotherapy, transcatheter hepatic arterial embolization, recurrence-free survival.

SUMMARY

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumor recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a Streptococcus-derived anticancer immunotherapeutic agent, into tumor tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0.1 KE/ml OK432 for two days. Thirteen patients were administered with 5 x 10^6 of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs 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. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan-Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls (P = 0.046, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumor necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial antitumor effects against liver cancer.

INTRODUCTION

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumor recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumor liver tissues causes de novo development of HCC [3,4]. One strategy to reduce tumor recurrence is to enhance antitumor immune responses that may induce sufficient inhibitory effects to prevent tumor cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumor cells.

To enhance tumor antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and costimulatory molecules, e.g., CD40, CD80 and CD86 [9,10], and loaded with tumor-associated antigens including tumor lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NKT) cell activation, DCs have been stimulated and modified to produce larger amounts of cytokines, e.g., interleukin (IL)-12, IL-18 and type I interferons [10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g., C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of Streptococcus pyrogenes, were recently suggested to produce large amounts of T helper-1 (Th1)-type cytokines including IL-12 and interferon-γ (IFN-γ)

and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and prostaglandin E2 (PGE2)] [16]. Furthermore, because OK432 modulates DC maturation through TLR4 and the β_2 integrin system [16,17] and TLR4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of antitumor immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have been shown themselves to mediate strong, specific cytotoxicity toward tumor cells via CD40 / CD40 ligand interactions [19].

Recently, we have reported that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumor tissues and contributed to the recruitment and activation of immune cells in situ. However, this approach by itself yielded limited antitumor effects probably due to insufficient stimulation of immature DCs [the preparation of which seems closely related to therapeutic outcome [21,22]]. The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumor tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles, and, most importantly, experienced prolonged recurrence-free survival.

PATIENTS AND METHODS

Patients.

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of ≥70 %, an age of ≥20 years, informed consent, and the following normal baseline hematological parameters (within 1 week before DC administration): hemoglobin ≥8.5 g/dl; white cell count ≥2,000 /μl; platelet count ≥50,000 /μl; creatinine <1.5mg/dl, and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, hematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within four weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or antihistamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (4 women and 9 men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled in the study, with an age range from 56 to 83 years (**Table 1**). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of twenty-two historical controls (9 women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects one week later [24]. They underwent ultrasound, CT scan or magnetic resonance imaging (MRI) of the abdomen about one month after treatment and at a minimum of once every three months thereafter, and tumor 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 one 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 previously reported [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep^{TM} Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in 6-well tissue culture dishes (Costar, Cambridge, MA) at 1.4 x 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) 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 x 10^6 cells were suspended in 5 ml normal saline containing 1 % autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ) 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 leukocyte antigen (HLA)-DR^+.

Flow cytometry analysis.

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lin 1 (lineage cocktail 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), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA). Cells were analyzed on a FACSCalibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA).

DC phagocytosis.

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACSCalibur™ 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 ^{51}Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 (ATCC, Manassas, VA) and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labeled with ^{51}Cr. In a 96-well plate, 2.5 x 10^3 target cells per well were incubated with DCs for 8 h at different effector / target (E / T) ratios in triplicates. Percent specific lysis was calculated as follows: (experimental release - spontaneous

release) / (maximum release - spontaneous release) x 100. Spontaneous release was always < 20 % of the total.

NK cell activity.

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

Intracellular cytokine expression.

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) at 37 °C in humidified 7 % CO_2 for 4 h. To block cytokine secretion, brefeldin A (Sigma, St. Louis, MO) [27] was added to a final concentration of 10 µg/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^{TM} system (BD Pharmingen), resuspended in PBS containing 1 % paraformaldehyde (PFA), and analyzed on a flow cytometer (≈10,000 gated events acquired per sample).

IFN-γ enzyme-linked immunospot (ELISPOT) assay.

ELISPOT assays were performed as previously described with the following modifications [28-30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 $(SART2)_{899}$ (SYTRLFLIL), $SART3_{109}$ (VYDYNCHVDL), multidrug resistance protein 3 $(MRP3)_{765}$ (VYSDADIFL), $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), alpha-fetoprotein $(AFP)_{403}$ (KYIQESQAL), AFP_{434} (AYTKKAPQL), AFP_{357} (EYSRRHPQL), human telomerase reverse

transcriptase (hTERT)$_{167}$ (AYQVCGPPL) (unpublished), hTERT$_{461}$ (VYGFVRACL), hTERT$_{324}$ (VYAETKHFL), were used in this study. Negative controls consisted of an HIV envelope-derived peptide (HIVenv$_{584}$). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide (CMVpp65$_{328}$). The colored 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 two fold 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). Briefly, frozen serum samples were thawed at room temperature, diluted 1 : 4 in sample diluents, and 50 µl aliquots of diluted sample were added in duplicate to the wells of 96-well microtiter 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-1ß, 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 FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and 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 done 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 the manufacturer (BioAssay Systems, Hayward, CA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA) to remove urea, combined with the sample buffer in wells of 96-well plate, and incubated

at 37^{o}C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The color produced was read at 520 nm using a microtiter plate reader.

Statistical analysis.

Results are expressed as means ± SD. Differences between groups were analyzed for statistical significance by the Mann-Whitney U test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumor 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 three days. 54.6 ± 9.5 % (mean ± SD; 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 ± 14.2 % were CD11c-positive (myeloid DC subset) and 14.8 ± 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 costimulatory 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 the cytokines IL-12 and IFN-γ (**Fig. 1C**). In addition, they displayed high cytotoxic 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 monitored clinically and biochemically after DC infusion (**Table 2**). A larger proportion (12 / 13) of the patients were complicated with high fever compared to those previously treated with immature DCs (5 / 10) [20], probably due to the proinflammatory responses induced by OK432-stimulated DCs. However, there were no grade 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 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 tumor 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 compared 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; 2 / 13 and 12 / 22, respectively; P = 0.046, log-rank test) (**Fig. 2**). The results demonstrated that OK432-stimulated DC transfer during TAE therapy reduces tumor 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 one and three months after treatment and NK cell cytotoxicity against K562 erythroleukemia target cells measured using the ^{51}Cr-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 tumor antigens.

To assess the effects on T cell responses to tumor 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 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 tumor antigens MRP3 and hTERT were induced in 3 of 6 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 4 (numbers t8, t19, t20 and t22) of 6 HLA-A24-positive patients. These data indicate that T lymphocyte responses to HLA-A24 restricted peptide epitopes of tumor 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 concentrations 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-1β (CCL4) were markedly induced 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-1β.

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 6- or 7-fold in patients treated with TAE. However, this increase was independent of the presence or absence of OK432-stimulated DC transfer (**Fig. 5B**). Nonetheless, 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 level 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 alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) activities. We did not observed 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 DCs during TAE treatment may reduce the immunosuppressive activities of MDSCs, and assist in developing a favorable environment for the induction of antitumor immunity.

DISCUSSION

Although many novel strategies including immunotherapies have been developed in an attempt to suppress tumor 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-1β were markedly enhanced after DC transfer. In addition, serum levels of arginase activity were decreased following DC transfer. Collectively, this study demonstrated the feasibility, safety, and beneficial antitumor effects of OK432-stimulated DC infusion into tumor tissues for patients with cirrhosis and HCC, suggesting the ability of an active immunotherapeutic strategy to reduce tumor recurrence after locoregional treatment of HCC.

DCs were stimulated with OK432 prior to infusion into tumor 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 directly infused into tumor tissues. Since 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]. The further studies are needed to determine the killing mechanisms of DCs derived from HCC patients in a direct (TNF, TRAIL, Fas ligand, 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, costimulation 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 antitumor effects were not clearly defined. The current study shows for the first time that OK432-stimulated DCs induce beneficial antitumor responses when transferred into tumor tissues during TAE therapy. The antitumor 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 of these alterations could contribute to the further enhancement of antitumor effects compared to those in our previous study with immature DCs [20]. Furthermore, the tumor 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 tumor environment.

NK cell activity and intracellular cytokine responses in $CD4^+$ and $CD8^+$ T lymphocytes and $CD56^+$ NK cell subsets in PBMCs were not significantly changed in patients treated with OK432-stimulated DCs. Furthermore, we did not observe tumor antigen-specific T lymphocyte responses clearly associated with DC administration. The data therefore suggest that the immune responses induced by the therapy applied here were not systemically detectable. Because cytotoxic T lymphocyte responses were

enhanced in patients receiving > 3 X 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 antitumor 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], tumor-specific effector T lymphocytes coexist with the tumors. Consistent with these observations, the current results suggest that cytotoxic T lymphocyte responses in PBMCs are not reliable predictors of beneficial antitumor 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-1β 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 hematopoietic 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 antitumor immune responses [57]. Eotaxin is known to selectively recruit eosinophils also contributing to antitumor effects [58,59], and MIP-1β 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 antitumor effects, although the precise molecular and cellular mechanisms associated with the actions of these cytokines and chemokines were not clearly defined in the current

analysis.

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DISCLOSURE

The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

Figure 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**) lin 1$^-$HLA-DR$^-$ subsets with [OK432(+)] and without [OK432(-)] stimulation were analyzed for surface expression of CD80, CD83, CD86 and CCR7. Dot plots of a representative case are shown in the left-hand panel. Mean percentages (± SD) 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 lin 1$^-$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) (± SD) 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 IL-12 and IFN-γ measured by ELISA. OK432-stimulated cells produced large amounts of the cytokines. The data indicate means ± SD of the groups with and without the stimulation. All comparisons in A - C [OK432(+) vs. OK432(-)] were statistically significant by the Mann-Whitney U test ($P < 0.005$). (**D**) Tumoricidal activity of DCs assessed by incubation with ^{51}Cr-labeled Hep3B, PLC/PRF/5 and T2 targets for 8 h at the indicated E / T (effector / target) cell ratios. OK432-stimulated cells displayed high cytotoxic activity against the target cells. The results are representative of the cases studied.

Figure 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with (TAE + OK-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, CT scan or 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; 2 / 13 and 12 / 22, respectively; $P = 0.046$, log-rank test).

Figure 3. Natural killer (NK) cell activity and intracellular cytokine production in PBMCs of patients treated with OK432-stimulated DCs during TAE therapy (n = 13). PBMCs were isolated before and one and three months after treatment and used for the analyses. Upper panel, NK cell cytotoxicity against K562 erythroleukemia target cells was evaluated at the 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 IFN-γ and IL-4. Percentages of cytokine-positive cells were quantitaed 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 ± SD of the groups.

Figure 4. Immune responses to HLA-A24-restricted peptide epitopes derived from tumor antigens in HLA-A24-positive patients treated with OK432-stimulated DCs during TAE therapy (numbers 2, 5, 6, 7, 10 and 11) and HLA-A24-positive historical controls treated with TAE without DC transfer (numbers t8, t14, t15, t19, t20 and t22). PBMCs were obtained before (open bars) and one month after the infusion (solid bars), pulsed with the peptides derived from SART2, SART3, MRP3, AFP and hTERT, and IFN-γ production was quantitated by ELISPOT. Negative controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml PMA or a CMV pp65-derived peptide ($CMVpp65_{328}$). 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.

Figure 5. Cytokine and chemokine profiling and arginase activity in **s**era of patients treated with OK432-stimulated DCs during 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 two weeks after the treatments were calculated as follows: [(posttreatment level – pretreatment level) / pretreatment level] X 100. The data are means ± SEM 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 four weeks after treatment, the difference did not reach statistical significance (*P* > 0.05). Percentage changes in serum levels two weeks after the treatments were calculated as follows: [(posttreatment level – pretreatment level) / pretreatment level] X 100. The data indicate means ± SEM of the groups.