Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.03.051.

Appendix A

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CLINICAL LIVER

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Participating investigators from the Hokuriku Liver Study Group are listed in Appendix A.
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Conflicts of Interest
The authors disclose no conflicts.

Supplementary Materials and Methods

Plasma Amino Acid Analysis

Plasma sample amino acid concentrations were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry followed by derivatization.[1] An MSQ Plus LC/MS system (Thermo Fischer Scientific, Waltham, MA) equipped with an electrospray ionization source was used in positive ionization mode for selected ion monitoring. Xcalibur version 1.4 SR1 software (Thermo Fischer Scientific, Yokohama, Japan) was used for data collection and processing. The high-performance liquid chromatography separation system consisted of an L-2100 pump, L-2200 autosampler, and L-2300 column oven (Hitachi High-Technologies Corporation, Tokyo, Japan). A Wakosil-II 3C8-100HG column (100, 2.1, 3 mm; Wako Pure Chemical Industries, Osaka, Japan) was used for the separation, and the mobile phase consisted of eluent A (25-mmol/L ammonium formate in water, pH 6.0) and eluent B (water:acetonitrile = 40:60).

Western Blotting

The expression of HCV core protein, Socs3, Foxo3a, phospho-Foxo3a (Ser253) (pFoxo3a), STAT1, pSTAT1 (Tyr701), S6K, pS6K, p-mTOR (Ser2448), Raptor, and β-actin were evaluated with mouse anti-core (Affinity BioReagents, Golden, CO), mouse anti-Socs3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxo3a, rabbit anti-β-actin (Sigma-Aldrich, St Louis, MO), rabbit anti-phospho-Foxo3a (Ser253), rabbit anti-STAT1, rabbit anti-p-STAT1 (Tyr701), rabbit anti-p70 S6K, rabbit anti-pS6K, rabbit anti-p-mTOR (Ser2448), and rabbit anti-Raptor (Cell Signaling Technology, Beverly, MA), respectively. Densitometric analysis was conducted directly on the blotted membrane using a charge coupled device camera system (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software (Frederick, MD).

Primer Sequences for PCR and siRNA

Primer sequences for PCR and siRNA were as follows: 2′5′OAS: forward 5′- CTC AGA AAT ACC CCA GCC AAA TC-3′, reverse 5′-GTG GTG AGA GGA CTG AGG AA-3′; Socs3: forward 5′-TAC CAC CTG AGT CTC CAG CTT CTC-3′, reverse 5′-CCT GGC AGT TCT CAT TAG TTC AGC ATT C-3′; Foxo3a: forward 5′-TGC TGT ATG CAA GAA CTT TCC AGT AGC AG-3′, reverse 5′-ACT CTA GCC CCC ATG CTA CTA GTG-3′; glyceraldehyde-3-phosphate dehydrogenase: forward 5′-GAA GGT GAA GGT CGG AGT-3′, reverse 5′-GAA GAT GGT GAT GGG ATT TC-3′, siFoxo3a (SASI_Hs01_00119127; Sigma) sense: 5′-GAA UGA UGG GCU GAC UGA AdTdT-3′, antisense: 5′-UUC AGU CAG CCC AUC AUU CdTdT-3′. Small interfering Raptor was purchased as part of KIAA1303 siGENOME SMART pool siRNA reagents from Dharmacon, Inc (Lafayette, CO).

Construction of ISRE-Luc Reporter and FBEmut-luc Reporter Plasmids

Oligonucleotides containing the ISRE tandem repeat sequence (sense 5′-TCG AGA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA A-3′, antisense 5′-AGC TTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT C-3′, consensus 5′-GAA Ann GAA ACT-3′) were annealed, and integrated into Xho I and Hind III sites of the pGL4.23 luciferase vector (Promega). The human Socs3 promoter region (-109/+217) was amplified by genomic PCR using specific primers (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG-3′ and reverse, 5′-CCG TGA AGT CCA CAA AGG AGC CTT C-3′) and cloned into the EcoR V site of the pGL4.10-luc2 reporter vector (Promega). The Socs3 FBE mutant reporter vector was created by substituting 2 adenines in the putative FBE with guanines (wild-type sequence 5′-CTAAACA-3′, mutated sequence 5′-CTGAGCA-3′).

ChIP Assay

For the ChIP assay using the anti-ISGF3γ antibody, 1×10^6 Huh-7 cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid medium for 6 hours. For ChIP using the anti-Foxo3a antibody, 1×10^6 Huh-7 cells were cultured in low-amino-acid medium for 24 hours.

Cells were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C, and the reaction was stopped with 250 mmol/L glycine for 10 minutes. Cells were suspended in sodium dodecyl sulfate-lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid [EDTA], 50 mmol/L Tris-HCl [pH 8.1]), complete protease inhibitor cocktail (Roche Applied Science), and incubated for 30 minutes at 10°C. Cell lysate was sonicated with Bioruptor (Cosmo Bio, Tokyo, Japan) to obtain chromatin fragments and diluted 10-fold in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl, complete protease inhibitor cocktail). Chromatin fragments were incubated with 2 μg ISGF3γ antibody (Santa Cruz Biotechnology), 2 μg Foxo3a antibody (H-144; Santa Cruz Biotechnology), or normal rabbit immunoglobulin G for 18 hours at 4°C. Dynabeads (30 μL) protein G (Invitrogen) was added and incubated for 1 hour at 4°C. The beads were washed with low-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl), high-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 500 mmol/L NaCl), LiCl wash buffer (250 mmol/L LiCl, 1% NP-40, 1% de-

oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO3, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2′5′OAS promoter region (forward, 5′-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3′, reverse, 5′-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3′) or putative FBE in the Socs3 promoter region (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3′, reverse, 5′-AGC GGA GCA GGG AGT CCA AGT C -3′). Values were normalized by the measurement of input DNA.

pH77S.3/GLuc2A

pH77S.2 is a modification of pH77S[2] containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5′- ATA ATA TTA CGC GTA *TGG GAG TCA AAG TTC TGT TTG CC*-3′ (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5′-ATA AAT AGAT ATC *GTC ACC ACC GGC CCC CTT GAT CTT*-3′ (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5′- ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3′ and 5′-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3′ (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln_{41} with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.[3,4] GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.[3,4] In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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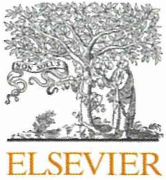

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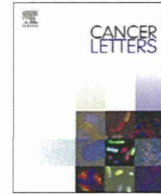

Frequency of $CD45RO^+$ subset in $CD4^+CD25^{high}$ regulatory T cells associated with progression of hepatocellular carcinoma

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The purpose of this study was to assess the properties of $CD4^+CD25^{high/low/negative}$ T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of $CD45RO^+$ cells in $CD4^+CD25^{high}$ T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the $CD45RO^+$ subset in $CD4^+CD25^{high}$ Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1–3].

$CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4–6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients with several different tumor types [3–12]. It has been reported that, in HCC patients, increased Tregs are correlated with $CD8^+$ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8,13–15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation [16,17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be

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implicated in the regulation of immune responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF-β1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of $CD4^+CD25^{high}$ $Foxp3^+$ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than $CD4^+CD25^{low/negative}$ cells. Furthermore, the proportion of $CD45RO^+$ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and healthy controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

Table 1
Clinical characteristics of hepatocellular carcinoma, liver cirrhosis, chronic hepatitis patients and healthy control.

Hepatocellular carcinoma (n = 62)	
Age (yrs)	68.9 ± 9.5
Gender (M/F)	37/25
Etiology of liver disease	
HBV/HCV/HBV + HCV/NBNC	19/34/2/7
TNM stages I/II/III/IV-A/IV-B	18/12/20/6/6
Largest tumor (mm)	
Child-Pugh A/B/C	37.6 ± 34.4
AFP (ng/mL)	41/8/3
DCP (mAU/mL)	10–35,093 (52)
Liver cirrhosis (n = 16)	
Age (yrs)	10–32,818 (34)
Gender (M/F)	58.3 ± 10.3
Etiology of liver disease	11/5
HBV/HCV/NBNC	4/7/5
Chronic hepatitis (n = 17)	
Age (yrs)	58.9 ± 10.4
Gender (M/F)	8/9
Etiology of liver disease	
HBV/HCV/NBNC	0/17/0
Healthy controls (n = 41)	
Age (yrs)	46.1 ± 19.1
Gender (M/F)	16/25

Note: Results except for AFP and DCP are expressed as means ± SD. AFP and DCP values are expressed as range (median). The reference range of normal values for the laboratory values: AFP < 10 ng/mL, DCP < 40 mAU/mL. M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

2.2. Isolation of PBMC and $CD4^+$ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma–Aldrich, St. Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. $CD4^+$ T cells were isolated from fleshly isolated PBMC by negative magnetic selection using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated $CD4^+$ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE, anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpha Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells " Plasmacytoid Subset " and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset " (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and intracellular staining

To determine the frequency of $CD4^+CD25^{high}$ T cells and the surface marker profile, $CD4^+$ T cells (at least 2×10^5 cells/tube) were stained with mAbs in the above described panel for 30 min on ice. Apropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2×10^5 $CD4^+$ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37 °C in a humidified CO_2 incubator for 4 h. The activated cells were first incubated with anti-CD4-PerCP for 15 min on ice, followed by fixation and permeabilization of the activated cells for 20 min at room temperature with BD Cytofix/Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD

Biosciences) for 15 min at room temperature. Apropriate isotype controls were included for each sample.

2.5. Flow cytometric analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6. Statistical analysis

Data are indicated as means ± SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric *U* test. $P < 0.05$ was considered significant.

3. Results

3.1. Frequencies of CD4⁺CD25^high T cells

To evaluate the frequencies of $CD4^+CD25^{high}$ T cell subsets that contain Tregs, MACS-sorted $CD4^+$ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and B). Although the frequencies of $CD4^+CD25^{high}$ T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that $CD4^+CD25^{high}$ T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and cytokine production of the $CD4^+CD25^{high}$ T cell subset in HCC patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22–24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was larger than that of $CD4^+CD25^{low/negative}$ subset, and it was also significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients (Fig. 2B). Thus, not only is the number of $CD4^+CD25^{high}$ T cells in HCC patients larger, but also the frequency of $Foxp3^+$ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of $CD4^+CD25^{high}$-$Foxp3^+$ T cell subset was quantitated following the stimulation with PMA/ionomycin using the specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the $CD4^+CD25^{high}$ subsets. In addition, the levels of IL-4 and IL-10 were high in the $CD4^+CD25^{high}Foxp3^+$ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the $CD4^+CD25^{high}Foxp3^+$ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.3. Phenotypes of the $CD4^+CD25^{high}$ T cell subset in HCC patients

To determine the phenotypical properties of $CD4^+CD25^{high}$ T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RA, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ cells were higher in the $CD4^+CD25^{high}$ T cell subset in all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$) (Fig. 3A and B). The percentage of $CD45RO^+$ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the $CD4^+CD25^{high}$ T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

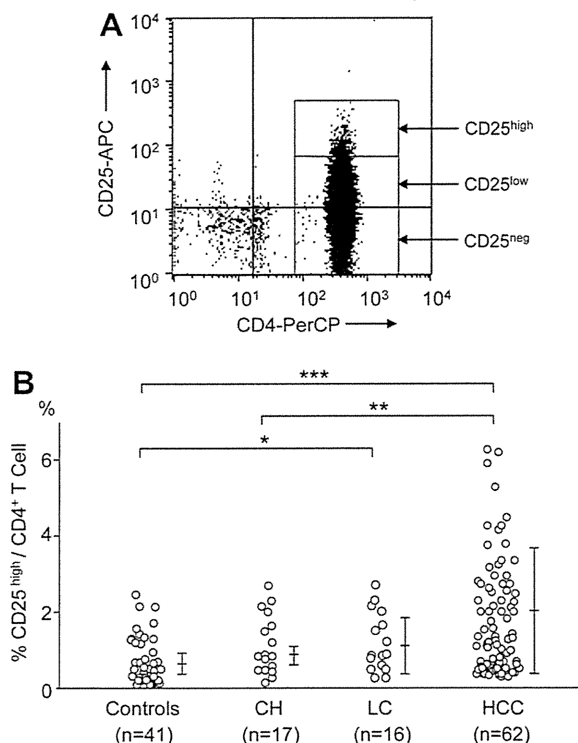

Fig. 1. Frequencies of $CD4^+CD25^{high}$ T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of $CD4^+CD25^{high}$ T cells in the peripheral blood of HCC ($n = 62$), LC ($n = 16$), CH ($n = 17$) patient, and healthy controls ($n = 41$). Percentages for individual patient analyzed are shown. The percentages represent the proportions of $CD4^+CD25^{high}$ T cells in total $CD4^+$ cells. The prevalence of $CD4^+CD25^{high}$ T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

3.4. $CD4^+CD25^{high}$ T Cell subset and dendritic cells of HCC patients

Several reports have suggested that the $CD4^+CD25^{high}$ T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells "Plasmacytoid Subset" and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset". HCC patients were divided into two groups according to the frequencies of $CD45RO^{positive}$ cells in $CD4^+CD25^{high}$ T cell subsets ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained >83.8% positive cells in $CD4^+CD25^{high}$ T cells. The frequencies of $CD123^+$ plasmacytoid DCs were significantly higher in $CD45RO^{++}$, group ($P < 0.05$) (Fig. 5A and B), although those of $CD33^+$ myeloid DCs were not correlated with the subsets in $CD4^+CD25^{high}$ cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of $CD4^+CD25^{high}$ T cells.

3.5. $CD4^+CD25^{high}$ T cell subset and tumor progression

To evaluate the association between $CD4^+CD25^{high}$ T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child-Pugh scores

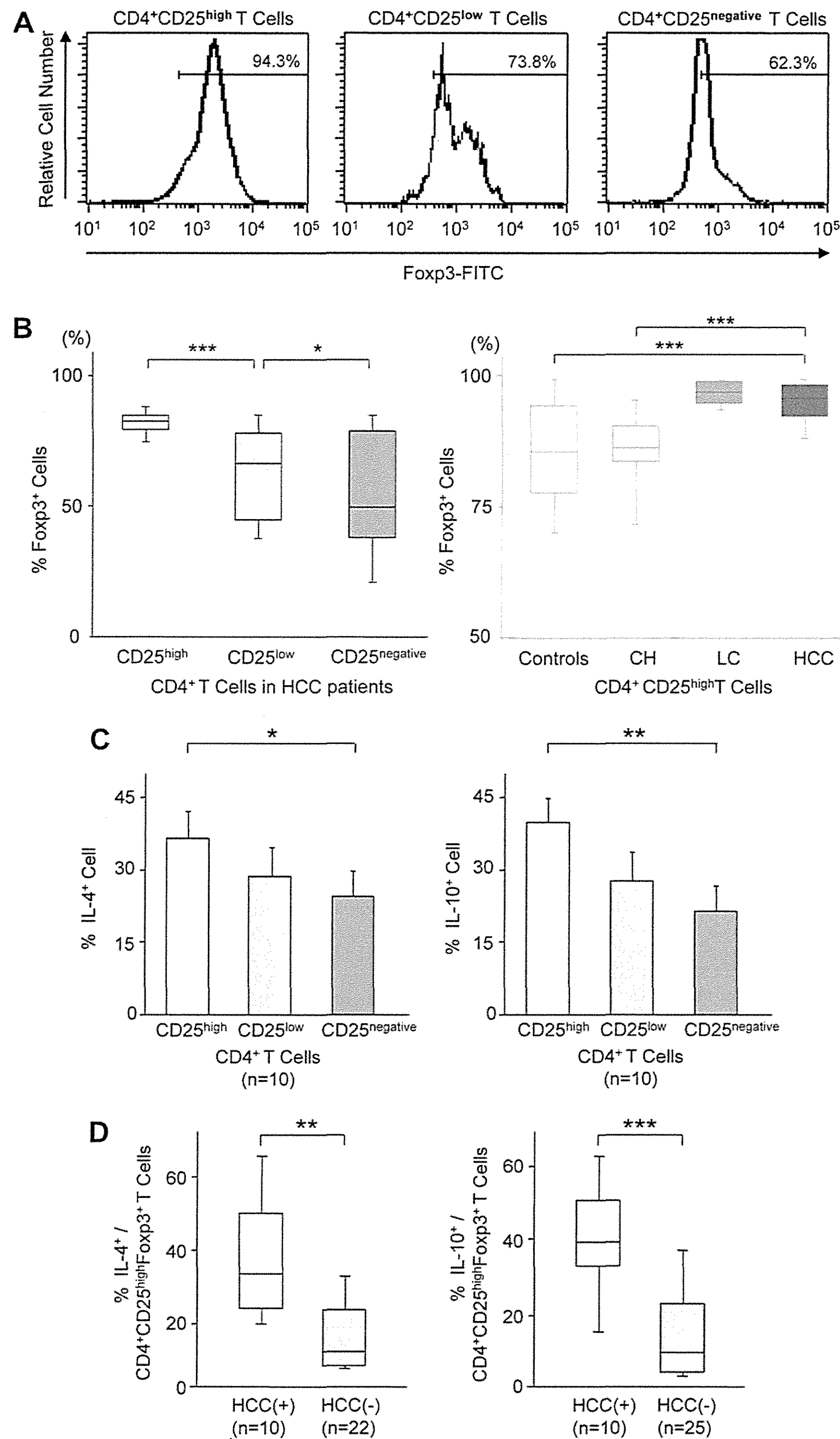

Fig. 2. Analysis of intracellular Foxp3 expression and cytokine production in $CD4^+$ $CD25^{high/low/negative}$ T cell subsets in HCC patients. (A) Representative expression of Foxp3 in $CD4^+$ T cells from an individual subject. Intracellular Foxp3 was stained following membrane permeabilization. Intracellular Foxp3 was detected by the specific mAb. (B) Statistical analysis in the left side panel shows that the percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{low/negative}$ T cell subsets, and in the right side panel shows that that of $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients. (C) Statistical analysis shows that the levels of Th2 cytokines IL-4 and IL-10 were remarkably high in the $CD4^+CD25^{high}$ T cell subset. (D) Comparison of intracellular cytokine production in $CD4^+CD25^{high}$ T cell subsets between patients with and without HCC. Healthy controls, patients with chronic hepatitis and liver cirrhosis were included in the HCC (−) column. IL-4 and IL-10 levels were higher in the $CD4^+CD25^{high}$ T cell subset in HCC patients. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

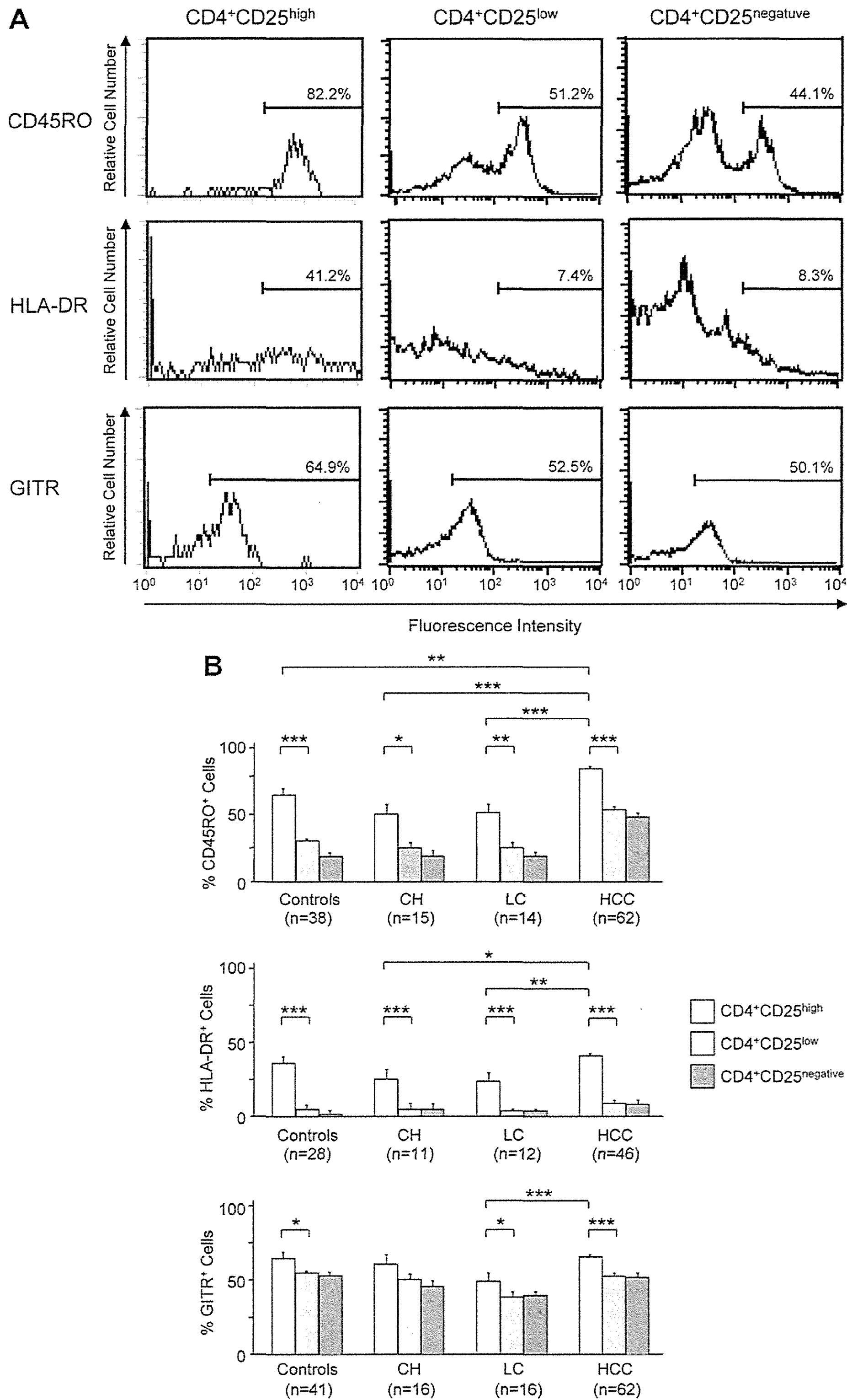

Fig. 3. Phenotypic analysis of CD4$^+$CD25$^{high/low/negative}$ T cell subsets in HCC patients. Fleshly isolated CD4$^+$ T cells (at least 2 × 10^5 cells/tube) from HCC patients were labeled with anti-CD4, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR7, anti-CTLA-4, anti-HLA-DR and anti-GITR mAbs. (A) Representative CD45RO, HLA-DR, and GITR expression profiles in CD4$^+$ T cell subsets that differ in CD25 expression. (B) Statistical analysis shows that the proportions of CD45RO$^+$, HLA-DR$^+$ and GITR$^+$ were elevated in the CD4$^+$CD25^{high} T cell subsets of all patient groups compared to the CD4$^+$CD25$^{low/negative}$ T cell subsets, except for GITR$^+$ cells in CH patients ($P < 0.05$). The percentage of CD45RO$^+$ cells in HCC patients was elevated compared to the patients with advanced liver diseases and healthy controls. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

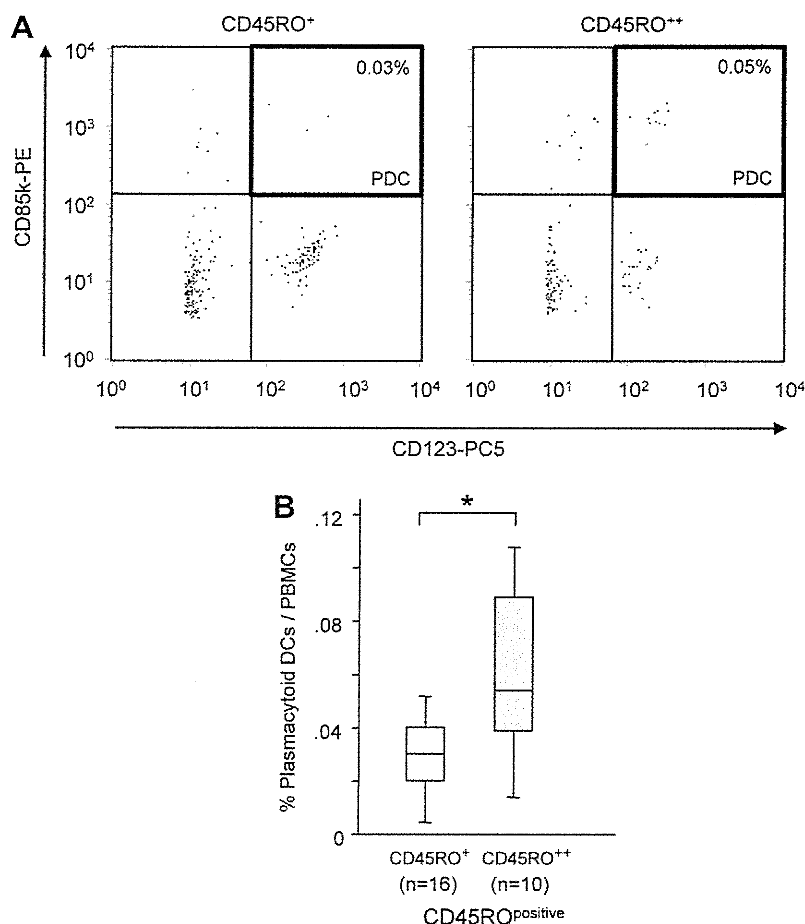

Fig. 4. Frequencies of plasmacytoid DCs in peripheral blood of HCC patients. Whole blood cells were analyzed by flow cytometry following staining with a combination of the mAbs. HCC patients were divided into two groups according to the frequencies of $CD45RO^{positivee}$ cells in $CD4^{+}CD25^{high}$ T cell subset ($CD45RO^{+}$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained > 83.8% positive cells in $CD4^{+}CD25^{high}$ T cells. (A) Representative dot plots of plasmacytoid DCs. Plasmacytoid DCs of $CD45RO^{+}$ group are shown in the left panel and $CD45RO^{++}$ group in the right panel. (B) Statistical analysis shows that the frequencies of plasmacytoid DCs were significantly higher in $CD45RO^{++}$ group. *Indicates $P < 0.05$.

and fibrosis stages between two groups as described above. The levels of serum AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^{+}$ group (Fig. 4). Others were not significantly different between two groups. These results imply that a subset of Tregs may contribute to the progression of liver tumors.

4. Discussion

$CD4^{+}CD25^{high}$ $Foxp3^{+}$ regulatory T cells have been shown to increase in patients with malignancies to suppress the immune responses. In this study, we provide evidence that patients with HCC have increased frequencies of $CD4^{+}CD25^{high}$ T cells in their peripheral blood compared to healthy controls and chronic hepatitis patients. A large proportion of $CD4^{+}CD25^{high}$ T cells expressed Foxp3 and produced Th2 cytokines. We also showed that $CD4^{+}CD25^{high}$ T cells expressed high levels of CD45RO, HLA-DR and GITR, and, interestingly, the T cell frequencies expressing these surface molecules were associated with plasmacytoid DC numbers and maximum tumor diameters in HCC patients.

There are several reports of elevated numbers of Treg cells in the peripheral blood and tumor tissues of patients with different types of cancer [3–12]. The study of Unitt et al. provided the first report of increased $CD4^{+}CD25^{+}$ T cell frequency within tumor tissue compared to non-tumor tissue in HCC patients [13]. Ormandy et al. showed that the frequency of $CD4^{+}CD25^{high}$ T cells in peripheral blood of patients with HCC was significantly higher ($3.92 \pm 3.3\%$) than in healthy donors ($1.17 \pm 0.87\%$) and liver cirrhosis patients ($0.78 \pm 0.43\%$) [3]. Our data revealed that a minimal increase in $CD4^{+}CD25^{high}$ T cells was detected in LC patients and more pronounced changes were found in HCC patients.

We showed that higher percentages of $CD4^{+}CD25^{high}$ T cells produced Th2 cytokines IL-4 and IL-10 in HCC patients. Tregs were recently observed to produce IL-10 [25–27], which can be a major mediator of immune suppression [28–30]. Voo et al. reported that Tregs in the peripheral blood of healthy donors secreted IL-10 but not IL-2, IFN-γ, or IL-4 [31]. Schmitz-Winnenthal et al. demon-

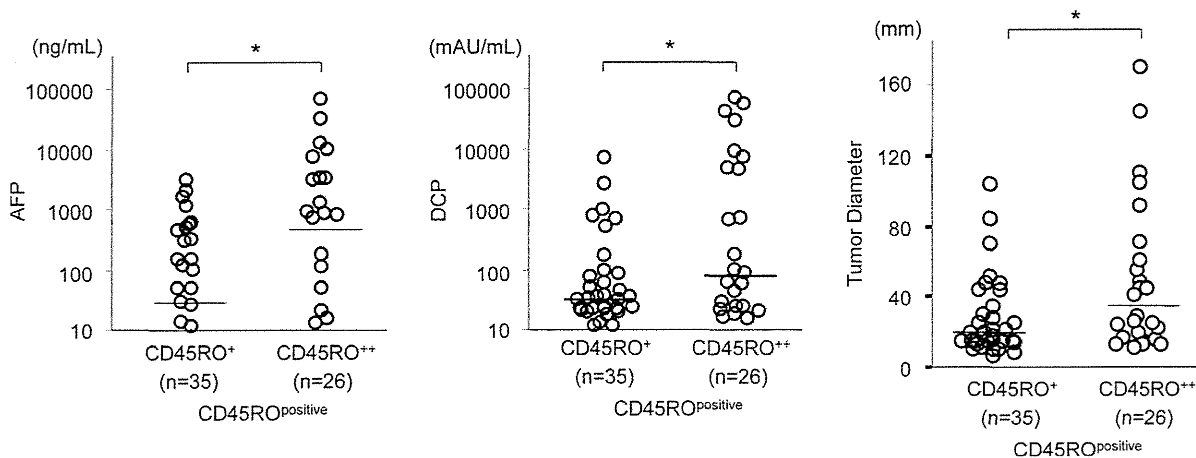

Fig. 5. Prevalence of CD4$^+$CD25$^{high/low/negative}$ T cell subsets and tumor progression. The levels of AFP and DCP and the maximum tumor diameters in CD45RO^{++} group were larger than those in CD45RO$^+$ groups. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyl prothrombin. *Indicates $P < 0.05$.

strated the presence of Treg secreting IL-10 but not IL-4 or IFN-γ upon antigen recognition in chronic pancreatitis patients [32]. The present data demonstrated that larger numbers of Tregs produced not only IL-10 but also IL-4 in HCC patients, which may contribute to the strong immunosuppressive properties of the T cells in liver malignancies.

It appears that Tregs consists of heterogenous populations within CD4$^+$ T cells, and that a subset of CD4$^+$CD25^{high} T cells could be subdivided into different functional subsets based on the expression of various surface molecules [6]. The proportions of Tregs expressing these molecules are reported to be different in the various forms of cancer. The prevalence of CD45RO$^+$ and GITR$^+$ Treg cells is higher in CD4$^+$CD25^{high} T cells than in CD4$^+$CD25$^{low/negative}$ T cells in renal cell carcinoma [4]. In head and neck squamous cell carcinoma, however, CD4$^+$CD25^{high} T cells express CTLA-4, Foxp3, and CD62L but little GITR, and CD25 $^{low/negative}$ T cells express intermediate to high levels of GITR and HLA-DR [8]. Our study showed that Tregs in HCC patients expressed significantly higher levels of CD45RO, HLA-DR and GITR compared to CD4$^+$CD25$^{low/negative}$ cells, suggesting that the activated populations of Tregs may contribute to the establishment of immunosuppressive microenvironments.

Little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated numbers of Treg cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells [20,33]. In peripheral blood, there are two distinct populations of DCs which can be distinguished based on phenotypical and morphological characteristics; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18,34]. Our data demonstrated that higher frequencies of CD45RO$^+$CD4$^+$CD25^{high} T cells were associated with higher frequencies of pDCs in the peripheral blood of HCC patients. When the tumor antigens are assumed by pDCs through Toll-like receptor 9 (TLR9) via receptor-mediated endocytosis, secretions of pro-inflammatory cytokines, such as type I interferons (IFNs), would be caused. On the contrary, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape. They exhibit reduced IFN-α production upon TLR9 stimulation and can induce IL-10 producing CD4$^+$ and CD8$^+$ Treg [35,36]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the tumor and by limiting anti-tumor cytolytic activity through induction of CD8$^+$ Treg.

Concerning the association of Tregs and prognosis, it has been reported that an increased number of circulating Tregs predicts poor survival of patients with renal cell carcinoma [4], gastric and esophageal cancers [7], myelodysplastic syndrome [37] and HCC [11]. In addition, tumor-infiltrating Tregs were associated with reduced survival in ovarian cancer [12] and HCC patients [1]. In addition, we found that CD45RO$^+$CD4$^+$CD25^{high} T cell subset was associated with larger tumor burdens, implying that a subset of Tregs may contribute to the promotion of tumor cell growth in the liver. However, it is also well possible that this just reflects stronger activation caused by a larger amount of antigen.

We performed the functional evaluation of Tregs derived from HCC patients by incubating with responder CD4$^+$CD25$^-$ T cells (Tresp). We observed that CD45RO$^+$ CD4$^+$CD25^{high} T cells of HCC patients did not suppress the proliferation of responder T cells when co-cultured at Treg/Tresp ratios of 1:2 and 1:8 (data not shown). In contrast, Hoffmann et al. confirmed that the CD45RA$^+$ CD4$^+$CD25^{high} T cells of healthy volunteers give rise to a homogeneous and highly suppressive Treg cell population, whereas CD45RA-CD4$^+$CD25^{high} T cells generate cell lines with mixed phenotype and function [38]. Although the reasons of these conflicting data were not clarified in the current study, cell viability, apoptosis susceptibility, involvement of Th1 cytokines, and interaction to helper T cell subsets of Tregs obtained from HCC patients need to be evaluated in the future experiments.

This study may be helpful for a better characterization of Treg subsets in the peripheral circulation of patients with HCC, which may establish the immunosuppressive environment to promote tumor progression. Furthermore, to gain insights into changes in the Treg subsets

during the therapeutic option may lead to more effective immunotherapies against cancer and may improve prognosis.

Conflict of interest

None declared.

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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 μg/ml), which were AFP_{357} (EYSRRHPQL), AFP_{403} (KYIQESQAL) and AFP_{434} (AYTKKAPQL),[10] and 20 μg/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 $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), $MRP3_{765}$ (VYSDADIFL), $hTERT_{167}$ (AYQVCGPPL), $hTERT_{324}$

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(VYAETKHFL) and $hTERT_{461}$ (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 ($HIVenv_{584}$) 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 ($CMVpp65_{328}$).[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 peptides 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. 1*a*). 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. 1*b*).

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. 2*a*). On the other hand, the frequency of patients who showed both positive and increasing T-cell

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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	$CMVpp65_{328}$	TT
1	A2	RF	C	<10	1,600	ND	ND	ND	1	ND	0	<10	1,400	ND	ND	ND	0	ND	1
2	A26,A31	RF	C	61	1,700	ND	ND	ND	0	ND	**13**	23	900	ND	ND	ND	0	ND	0
3	A11,A26	No	–	100	1,700	ND	ND	ND	5	ND	1	50	1,500	ND	ND	ND	0	ND	0
4	A24	RF	C	18	700	0	7	0	6	0	**25**	16	500	1	**10**	1	1	2	**16**
5	A24,A33	RF	C	2,357	1,200	**13**	2	6	0	**13**	0	700	1,100	2	1	1	0	9	0
6	A24	RF	C	14	1,800	0	0	0	0	0	**42**	<10	1,400	**53**	**27**	**38**	**14**	**36**	**108**
7	A23,A33	No	–	96	500	0	0	0	5	**291**	0	138	800	**46**	0	0	3	**484**	0
8	A24,A26	No	–	142	600	1	0	0	0	0	0	126	500	2	0	0	0	**166**	1
9	A2,A24	RF	C	<10	700	6	1	0	0	9	0	<10	700	0	0	0	0	**32**	**15**
10	A24	PEIT	C	<10	1,300	8	4	8	8	**146**	5	<10	1,300	0	1	1	0	1	1
11	A24,A26	PEIT	N	18	1,100	0	0	0	1	ND	0	13	400	0	0	0	**15**	**10**	**55**
12	A24,A33	RF	N	11	800	3	2	0	4	**94**	**10**	11	700	0	0	0	0	**24**	0
13	A11,A24	PEIT	C	52	1,300	0	2	5	1	2	0	24	1,200	0	0	0	0	0	3
14	A24	RF	C	54	2,400	**25**	5	4	8	**12**	0	67	1,700	0	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	0
16	A3,A24	RF	C	2,876	900	0	1	0	**13**	0	5	3,285	700	0	0	0	0	0	0
17	A24,A33	No	–	205	400	4	2	3	2	**26**	9	220	100	2	1	0	1	**39**	1
18	A24,A30	RF	C	18	1,100	4	0	3	8	**14**	7	13	900	1	**16**	1	5	**12**	0
19	A2,A24	RF	C	330	1,500	2	0	0	0	**18**	1	36	1,100	0	4	0	3	8	1
20	A2,A33	RF	C	10	1,400	ND	ND	ND	**10**	ND	**68**	<10	800	ND	ND	ND	**31**	ND	**101**

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

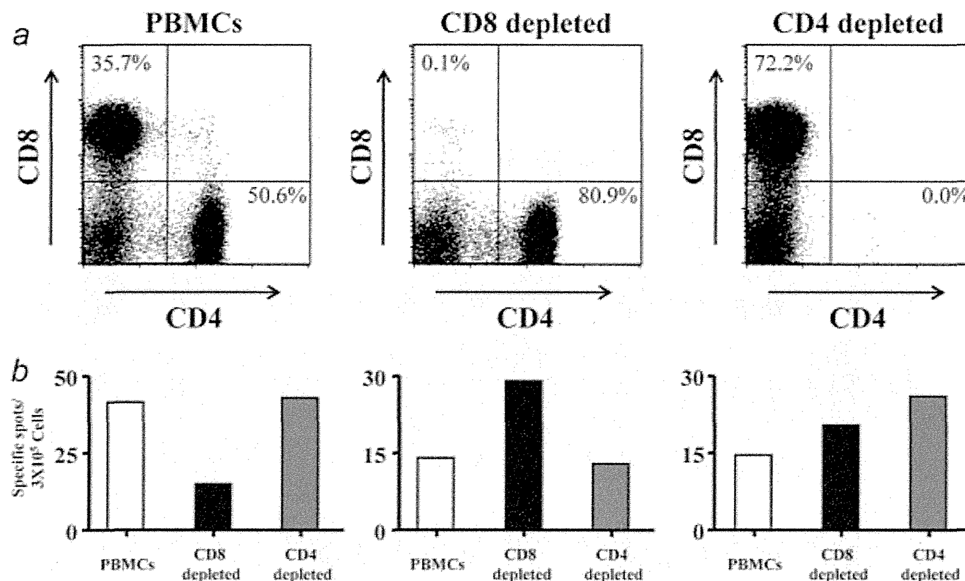

Figure 1. IFN-γ production of CD4- or CD8-depleted T cells against whole AFP. AFP-specific IFN-γ-producing T cells were analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed 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

	Before treatment						After treatment					
Patient	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$
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. 2*b* and 2*c*).

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. 3*a*–3*d*). 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. 3*e*). 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. 3*f*–3*h*). 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

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

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	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[1])	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

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

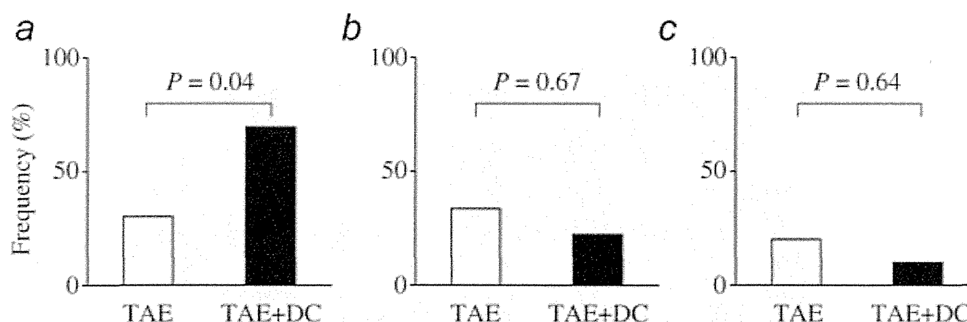

Figure 2. Frequency of the patients who showed enhancement of T-cell responses after treatment. The prevalence of antigen-specific T cells was determined by IFN-γ ELISPOT analysis using alpha-fetoprotein (AFP) and AFP-derived peptides (*a*), CMV pp65-derived peptide (*b*) or tetanus toxoid protein (*c*)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