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Therapeutics, Targets, and Chemical Biology

Cancer Research

Antitumor Effect after Radiofrequency Ablation of Murine Hepatoma Is Augmented by an Active Variant of CC Chemokine Ligand 3/Macrophage Inflammatory Protein-1α

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Abstract

Several chemokines are used for immunotherapy against cancers because they can attract immune cells such as dendritic and cytotoxic T cells to augment immune responses. Radiofrequency ablation (RFA) is used to locally eliminate cancers such as hepatocellular carcinoma (HCC), renal cell carcinoma, and lung cancer. Because HCC often recurs even after an eradicative treatment with RFA, additional immunotherapy is necessary. We treated tumor-bearing mice by administering ECI301, an active variant of CC chemokine ligand 3, after RFA. Mice were injected s.c. with BNL 1ME A.7R.1, a murine hepatoma cell line, in the bilateral flank. After the tumor became palpable, RFA was done on the tumor of one flank with or without ECI301. RFA alone eliminated the treated ipsilateral tumors and retarded the growth of contralateral non–RFA-treated tumors accompanied by massive T-cell infiltration. Injection of ECI301 augmented RFA-induced antitumor effect against non–RFA-treated tumors when administered to wild-type or *CCR5*-deficient but not *CCR1*-deficient mice. ECI301 also increased CCR1-expressing $CD11c^+$ cells in peripheral blood and RFA-treated tumors after RFA. Deficiency of *CCR1* impairs accumulation of $CD11c^+$, $CD4^+$, and $CD8^+$ cells in RFA-treated tumors. Furthermore, in IFN-γ-enzyme-linked immunospot assay, ECI301 augmented tumor-specific responses after RFA whereas deficiency of *CCR1* abolished this augmentation. Thus, we proved that ECI301 further augments RFA-induced antitumor immune responses in a CCR1-dependent manner. *Cancer Res; 70(16); 6556–65. ©2010 AACR.*

Introduction

Chemokines are a class of candidate molecules for immunotherapy. Chemokines are presumed to play an essential role in the regulation of leukocyte trafficking and dendritic cell-T-cell interactions (1–4). In animal experiments, intratumoral use of chemokines, such as monocyte chemoattractant protein-1/CC chemokine ligand 2 (CCL2), macrophage inflammatory protein (MIP)-1α/CCL3, or MIP-3α/CCL20, succeeds in decreasing tumorigenesis accompanied by increase in the numbers of tumor-infiltrating dendritic, natural killer, or T cells (5–7). Thus, application of chemokines in immunotherapy is promising but needs further refinement before they can be used in clinical situations.

Radiofrequency ablation (RFA) is an eradicative treatment against cancers, such as hepatocellular carcinoma (HCC), renal cell carcinoma, and lung cancer. RFA of HCC can generate HCC-specific T cells in peripheral blood (8). Activation of dendritic cells in human peripheral blood is also observed after this treatment (9). Thus, RFA can induce immunogenic tumor cell death and subsequently tumor-specific immune responses (8–11). However, multicentric development of HCC in the cirrhotic liver frequently results in tumor recurrence even after the apparent curative treatment of HCC by RFA (12). These observations suggest that RFA-induced tumor-specific immune responses are often not sufficient to prevent tumor recurrence. Thus, additional treatment modalities are required to augment HCC-specific immune responses.

CCL3/MIP-1α can augment immune responses but problems arise because of its tendency to form large aggregates at high concentrations when administered systemically. Unlike human naïve CCL3, BB-10010 is generated by a single amino acid substitution of Asp26 to Ala and exhibits similar biological potencies, but rarely forms large aggregates (13). Based on its activity to mobilize bone marrow cells to peripheral blood, randomized clinical trials were performed to examine whether the combined administration of BB-10010 and chemotherapeutic agents can protect against chemotherapy-induced neutropenia. However, the myeloprotective effects of BB-10010 were not sufficient to warrant its use with chemotherapy (14). Concomitantly, several lines of evidence reveal that the administration of human recombinant CCL3

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can mobilize activated T-cell and dendritic cell precursors into circulation (15, 16).

ECI301, which has the same amino acid sequence as BB-10010, was generated using the fission yeast (*Schizosaccharomyces pombe*) expression system. ECI301 can augment irradiation-induced tumor regression when administered systemically to mice bearing multiple subcutaneous tumors (17). Of interest is the fact that the effects were observed in both unirradiated and irradiated tumors. Thus, systemic ECI301 treatment can augment irradiation-induced tumor-specific systemic immunity. These observations prompted us to investigate the effects of ECI301 on RFA-treated mice. Here, we show that ECI301 further augments RFA-induced antitumor immune responses in a CCR1-dependent manner.

Materials and Methods

Mice

Seven- to 9-week-old specific pathogen–free female BALB/c mice were purchased from Charles River Japan and designated as wild-type (WT) mice. BALB/c-*nu/nu* mice were purchased from CLEA Japan. *CCR1*-deficient ($CCR1^{-/-}$) mice were a gift from Dr. Philip M. Murphy (National Institute of Allergy and Infectious Disease, NIH, Bethesda, MD); *CCR5*-deficient ($CCR5^{-/-}$) mice were a gift from Dr. Kouji Matsushima (Department of Molecular Preventive Medicine, Tokyo University, Tokyo, Japan). All mice were backcrossed to BALB/c mice for 8 to 10 generations. All animal experiments were performed under specific pathogen–free conditions in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University (Japan).

Tumor cell line

A murine HCC cell line, BNL 1ME A.7R.1 (BNL), was purchased from the American Type Culture Collection in 1998 and kept at low passage throughout the study. The cells were screened for bacteria, fungus, and *Mycoplasma* contamination by direct culture method in 2006 before start of the study. The cells were cultured in DMEM (Sigma Chemical Co.) containing 10% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids, 1 μmol/L sodium pyruvate, 2 mmol/L L-glutamine, 50 μg/mL streptomycin, and 100 units/mL penicillin (Life Technologies, Inc.).

Animal models

ECI301 was generated as previously described and provided by Effector Cell Institute, Inc. (17, 18). The left and right flanks of 7- to 9-week-old female WT, $CCR1^{-/-}$, $CCR5^{-/-}$, and *nu/nu* mice were injected s.c. with 5×10^5 BNL cells in 100 μL of PBS. Fourteen days later, when tumor size reached a diameter of 6 to 8 mm, tumors of one flank were treated using a radiofrequency generator (RITA 500PA, RITA Medical Systems) and needle as described below. On days 0, 2, and 4 after RFA, 20 μg of ECI301 in 100 μL of PBS were injected i.v. via the tail vein, whereas mice treated with RFA alone were injected with 100 μL of PBS. Untreated tumor-bearing mice were used as controls. In another schedule, 2 μg of ECI301 in 100 μL of PBS were injected i.v. from day 0 to day 4 (5 consecutive days). The sizes of non–RFA-treated tumors on the contralateral flank were evaluated twice a week using calipers, and tumor volumes were calculated using the following formula: tumor volume (mm^3) = (longest diameter) × (shortest diameter)^2 / 2.

RFA-treated or non–RFA-treated tumors were excised at the indicated time intervals for immunohistochemical analysis and quantitative real-time reverse transcription-PCR (RT-PCR). Spleens and peripheral blood were removed from the mice at the indicated time intervals for flow cytometric analysis and enzyme-linked immunospot assay (ELISPOT).

Radiofrequency ablation

Mice were anesthetized by i.p. injection of Somnopentyl (Schering-Plough Animal Health) and carefully shaved in the tumor area. After placing the mice onto an aluminum plate attached with an electricity-conducting pad, an RFA needle of expandable electrode with maximum dimension of 20 mm (70SB 2 cm; RITA Medical Systems) was inserted into the middle of the tumors and expanded at 2 or 3 mm. RFA treatments were done using a radiofrequency generator at a power output of 25 W for 1.5 minutes and the temperature of the needle tips reached 70°C to 80°C.

Immunohistochemical analysis

The removed tumor tissues were embedded in Sakura Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek) as frozen tissues. Cryostat sections of the frozen tissues were fixed with 4% paraformaldehyde in PBS and stained with rat anti-mouse CD4 (BD Biosciences), rat anti-mouse CD8a (BD Biosciences), hamster anti-mouse CD11c (BD Biosciences), and rat anti-mouse F4/80 antibodies (Serotec) overnight at 4°C. The sections were then incubated with biotinylated rabbit anti-rat IgG (DakoCytomation) or biotinylated mouse anti-hamster IgG (BD Biosciences) for 1 hour at room temperature. The immune complexes were visualized using the Catalyzed Signal Amplification System (DakoCytomation) or the Vectastain Elite ABC and DAB substrate kits (Vector Laboratories) according to the manufacturer's instructions. As a negative control, rat IgG (Cosmo Bio) or hamster IgG (BD Biosciences) was used instead of specific primary antibodies. The numbers of positive cells in each animal were counted in 10 randomly selected fields at 400-fold magnification by an examiner without any prior knowledge of the experimental procedures.

Double-color immunofluorescence analysis

Tumor tissues were embedded in OCT compound as frozen tissues. After fixation with 4% paraformaldehyde/PBS, cryostat sections were stained with the combinations of anti-CD4 and goat anti-mouse CCR1 (Santa Cruz Biotechnology), anti-CD8a and anti-CCR1, anti-F4/80 and anti-CCR1, phycoerythrin (PE)-conjugated hamster anti-CD11c (BD Biosciences) and anti-CCR1, anti-F4/80 and goat anti-mouse CCL3 (R&D Systems), and anti-F4/80 and goat anti-mouse CCL4 antibodies (R&D). After extensive washing, AF488 donkey anti-rat IgG (Invitrogen) was used as a secondary antibody to detect $CD4^+$, $CD8a^+$, or $F4/80^+$ cells. Simultaneously,

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AF546- or AF488-donkey anti-goat IgG (Invitrogen) was used to detect $CCR1^+$, $CCL3^+$, or $CCL4^+$ cells. The sections were observed using a confocal microscope (LSM 510 META, Zeiss).

Quantitative real-time RT-PCR

Total RNA was extracted from the resected tumor using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After treating the RNA preparations with RNase-free DNase I (Qiagen) to remove residual DNA, cDNA was synthesized as described previously (19). Quantitative real-time PCR was done on a StepOne Real-Time PCR System (Applied Biosystems) using the comparative C_T quantification method. TaqMan Gene Expression Assays (Applied Biosystems) containing specific primers and probes [accession numbers: CCL3, Mm00441258_ml; CCL4, Mm00443111_m1; CCL5, Mm01302428_ml; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm99999915_g1] and TaqMan Fast Universal PCR Master Mix were used with 10 ng of cDNA to quantify the expression levels of CCL3, CCL4, and CCL5. Reactions were performed for 20 seconds at 95°C followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. GAPDH was amplified as an internal control and its C_T values were subtracted from the C_T values of the target genes (ΔC_T). The ΔC_T values of tumors after RFA with or without ECI301 were compared with the ΔC_T values of tumors of untreated mice.

Enzyme-linked immunospot assay

To prepare tumor lysates, BNL or CT26 cells were suspended in PBS and subjected to four cycles of rapid freezing in liquid nitrogen and thawing at 55°C. The lysate was spun at 15,000 rpm to remove particulate cellular debris. After harvesting murine spleens on day 21 after RFA, mononuclear cells were isolated by centrifugation through a Histopaque-1083 density gradient (Sigma Chemical). ELISPOT was performed using an IFN-γ-ELISPOT kit (Mabtech). Ninety-six-well plates coated with anti-mouse IFN-γ antibody were blocked for 2 hours with RPMI 1640 (Sigma Chemical) containing 10% FBS. Two hundred fifty thousand splenic mononuclear cells were added in triplicate cultures of RPMI 1640 containing 10% FBS together with BNL or CT26 lysates at a tumor cell-to-mononuclear cell ratio of 2:1. After 48 hours of culture, the plates were washed eight times with sterile PBS and further incubated for 2 hours with biotinylated anti-mouse IFN-γ antibody. After another eight washes, alkaline phosphatase–conjugated streptavidin was added to these

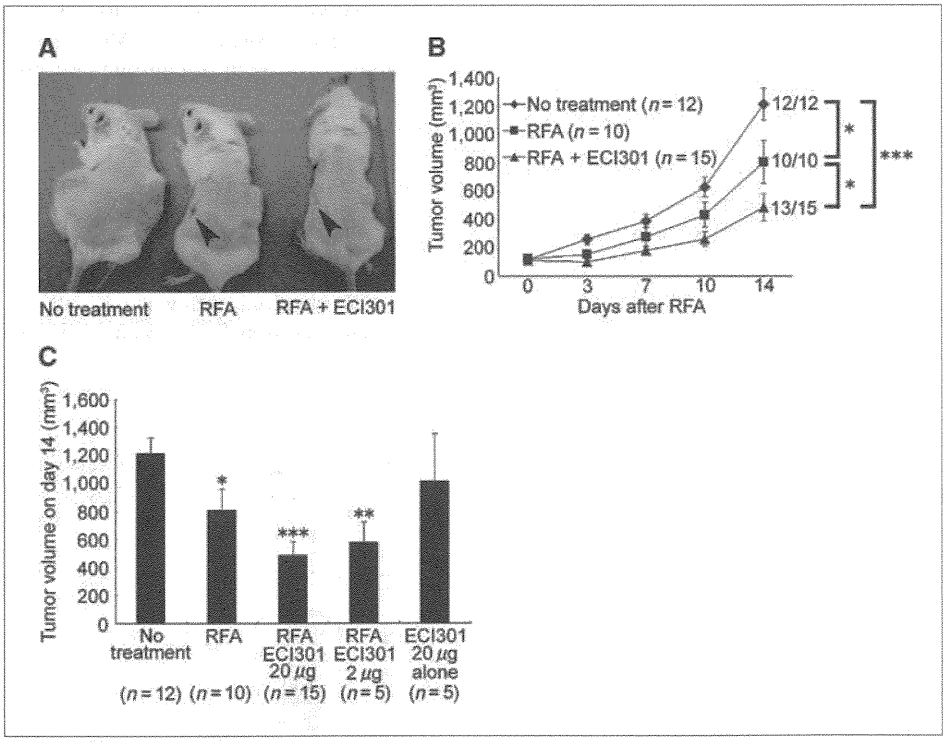

Figure 1. ECI301-induced augmentation of antitumor effects after RFA. WT mice were injected s.c. with 5×10^5 BNL cells into the left and right flanks. Fourteen days later, when tumors became palpable, tumors of one flank were treated using the RFA generator and needle. On day 0, 2, and 4 after RFA, 20 μg of ECI301 in 100 μL of PBS were injected i.v. into each mouse, whereas mice treated with RFA alone were injected with 100 μL of PBS. Tumor-bearing untreated mice were observed as controls. A, macroscopic appearances of the mice on day 14 after RFA are shown. Arrowheads indicate the scar after RFA. Representative results are from at least 10 mice in each group. B, non–RFA-treated tumor volumes after RFA with or without ECI301 were measured twice a week. Points, mean; bars, SE. *, $P < 0.05$; ***, $P < 0.001$. C, volumes of non–RFA-treated tumors on day 14 after RFA. In addition to the groups described in B, tumor volumes were determined in animals receiving 2 μg of ECI301 in 100 μL of PBS i.v. from day 0 to day 4 (5 consecutive days) after RFA and those receiving 20 μg of ECI301 alone without RFA. Columns, mean; bars, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with untreated mice.

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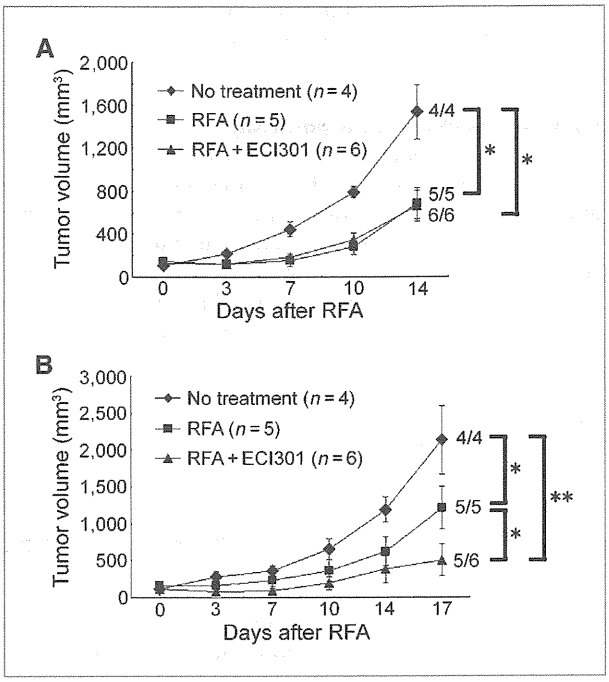

Figure 2. Deficiency of *CCR1* abrogates ECI301-augmented antitumor effects after RFA. $CCR1^{-/-}$ or $CCR5^{-/-}$ mice were inoculated with BNL cells and treated as described in the legend to Fig. 1. Non–RFA-treated tumor volumes were measured twice a week in $CCR1^{-/-}$ (A) and $CCR5^{-/-}$ (B) mice. Points, mean; bars, SE. *, $P < 0.05$; **, $P < 0.01$.

plates and incubated for 1 hour. Finally, the spots were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution. The number of specific spots was determined by subtracting the number of spots in wells without lysates from the number of spots in wells with tumor lysates. Wells were considered positive if they had more than 10 spots per well and were at least 2-fold greater than control.

Flow cytometric analysis

After harvesting blood samples from mice, mononuclear cells were isolated by centrifugation through a Histopaque-1083 density gradient (Sigma Chemical). The resultant single-cell preparations were stained with various combinations of allophycocyanin (APC)-labeled anti-CD8, APC-labeled anti-CD11c, FITC-labeled anti-CD4 (BD Biosciences), PE-labeled anti-CCR1 (Santa Cruz Biotechnology), and FITC-labeled anti-F4/80 monoclonal antibodies (Serotec). APC-rat IgG, APC-hamster IgG, and FITC-rat IgG were used as isotype controls (BD Biosciences). For each determination, at least 20,000 stained cells were analyzed on a FACSCalibur system (BD Biosciences). The data were expressed as the proportion of positive cells (compared with cells stained with an irrelevant control antibody).

Depletion of macrophages/monocytes

Clodronate liposome was prepared and systemic depletion of monocytes/macrophages was performed as previously described (20, 21). WT mice were i.p. injected with 200 μL of clodronate liposome five times: days −2, 0, 3, 6, and 10 after RFA treatment. Depletion of CD11c-negative monocytes in blood was confirmed by flow cytometry after injection of clodronate liposome.

Statistical analysis

Mean and SD or SE were calculated for the obtained data. Data were analyzed statistically using one-way ANOVA followed by Fisher's protected least significant difference test, except for the data of tumor growth, which were analyzed with two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

ECI301 augments RFA-induced antitumor effects

To investigate the effects of RFA against RFA-treated and non–RFA-treated tumors, each bilateral flank of BALB/c mice was injected with 5×10^5 BNL cells. Fourteen days later, when tumor size reached a diameter of 6 to 8 mm, tumors of one flank were treated with RFA. On the day after RFA, ulceration occurred in RFA-treated tumors, and these tumors started to shrink (data not shown). On day 14 after RFA, RFA-treated tumors were covered with scars without any macroscopic tumors (Fig. 1A). Moreover, RFA treatment also retarded the growth of contralateral non–RFA-treated tumors compared with the tumors in untreated mice (Fig. 1B and C). ECI301 (20 μg/mouse) administered on days 0, 2, and 4 after RFA augmented RFA-induced growth retardation of contralateral non–RFA-treated tumors (Fig. 1B and C). Furthermore, non–RFA-treated tumors completely disappeared in 2 of 15 mice treated with RFA and ECI301 but not in the other treatment groups (Fig. 1B and C). Therapeutic effects were observed, even when ECI301 (2 μg/mouse) was injected consecutively for 5 days from day 0 to day 4 after RFA.

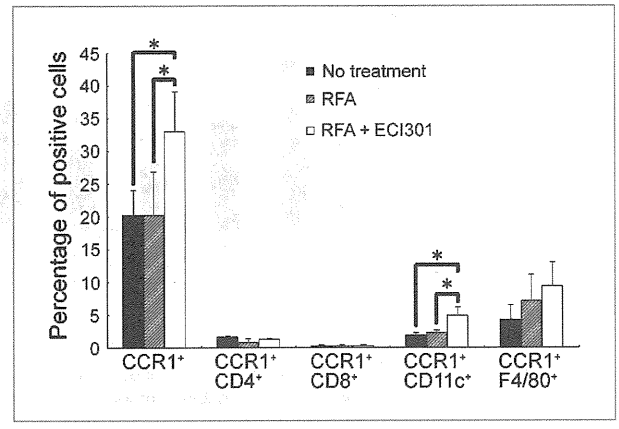

Figure 3. ECI301 increases CCR1-expressing cells in peripheral blood. Peripheral blood sample was harvested 8 h after RFA. Mononuclear cells were separated and stained with the indicated antibodies as described in Materials and Methods. Columns, mean percentages of $CCR1^+$, $CCR1^+CD4^+$, $CCR1^+CD8^+$, $CCR1^+CD11c^+$, or $CCR1^+F4/80^+$ cells ($n = 3$); bars, SD. *, $P < 0.05$.

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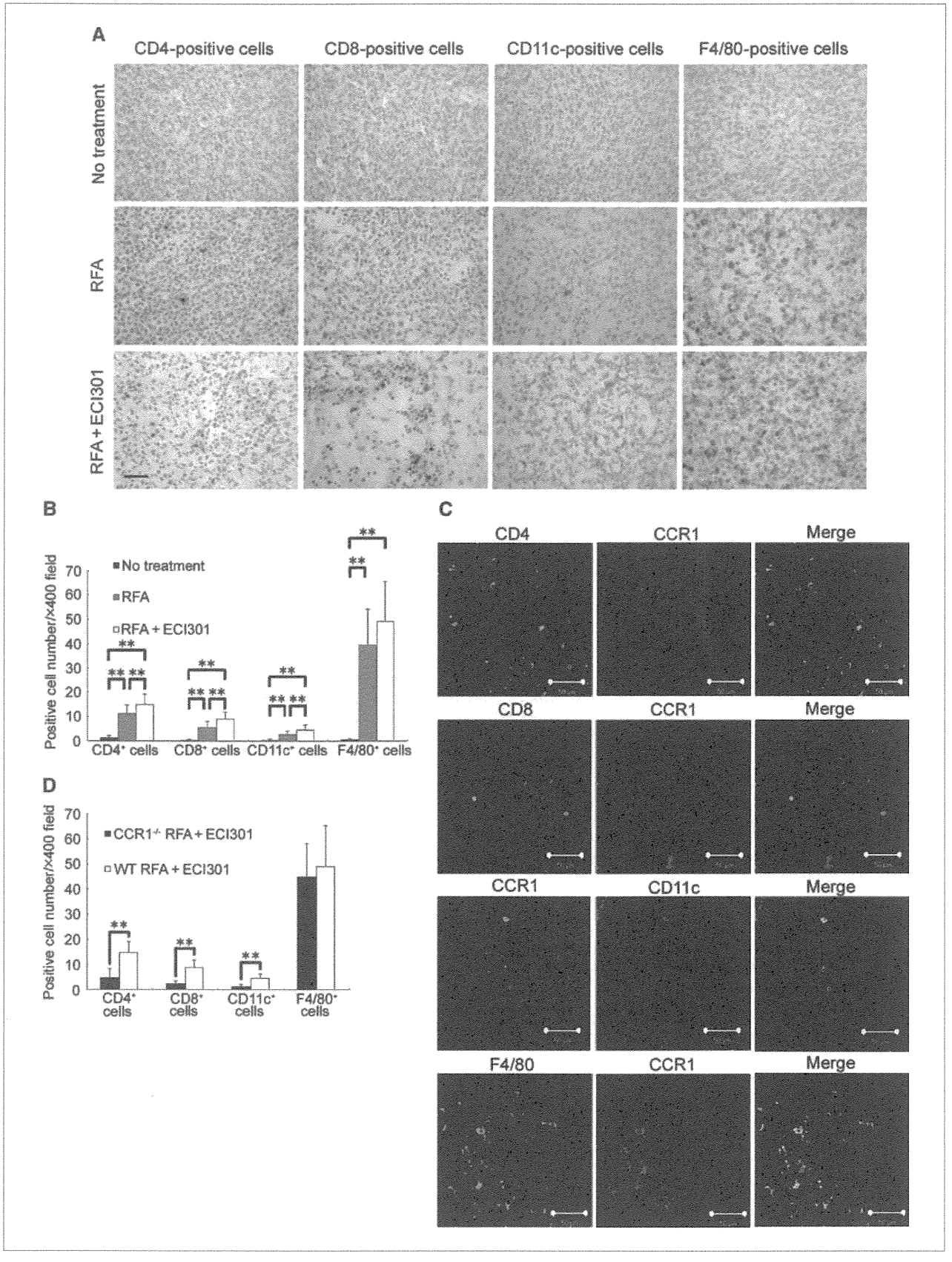

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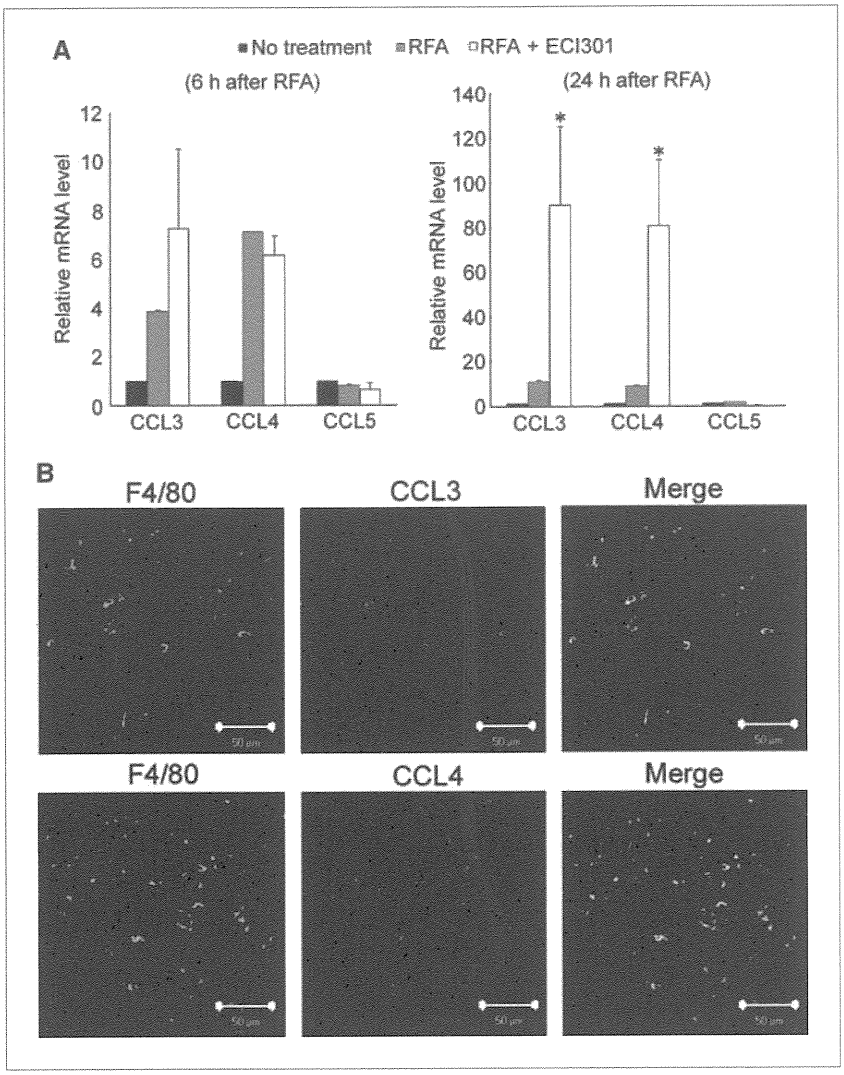

Figure 5. Increased CCL3 expression in RFA-treated tumors. A, real-time RT-PCR was performed on total RNA extracted from RFA-treated tumors of WT mice. The tumors were harvested 6 h (left) or 24 h (right) after RFA. Chemokine mRNA levels were normalized to GAPDH mRNA levels. Columns, mean (n = 3); bars, SD. *, $P < 0.05$, compared with untreated mice. B, RFA-treated tumors were removed from WT mice on day 1 after RFA plus ECI301 treatment and immunostained with the indicated combinations of antibodies as described in Materials and Methods. Right, digitally merged images. Representative results from three individual animals. Original magnification, ×400. Bar, 50 μm.

On the contrary, administration of ECI301 without RFA did not result in a significant decrease in tumor size (Fig. 1C). These observations suggest that ECI301 can augment RFA-induced antitumor effects but fails to induce antitumor effects by itself.

Deficiency of *CCR1* abrogates increased antitumor effect of ECI301 after RFA

ECI301 uses two distinct chemokine receptors, CCR1 and CCR5. To elucidate the roles of these chemokine receptors, either tumor-bearing *CCR1*$^{-/-}$ or *CCR5*$^{-/-}$ mice were similarly treated with RFA plus ECI301. RFA retarded the growth of non–RFA-treated tumors in *CCR1*$^{-/-}$ mice similar to that in WT mice, but ECI301 failed to further accentuate RFA-induced growth retardation of non–RFA-treated tumors (Fig. 2A). In contrast, ECI301 augmented RFA-mediated inhibition of non–RFA-treated tumors in *CCR5*$^{-/-}$ mice, resulting in complete tumor eradication in one of six mice (Fig. 2B). These observations indicate that CCR1-expressing, but not CCR5-expressing, cells play an important role in ECI301-induced augmentation of tumor regression after RFA.

ECI301 increases CCR1-expressing cells in peripheral blood and RFA-treated tumors after RFA

The reported capacity of CCL3 to mobilize leukocytes into peripheral blood (15, 16) prompted the investigation of the

Figure 4. ECI301 increases infiltration of CCR1-expressing leukocytes into RFA-treated tumors after RFA. RFA-treated tumors were removed from WT or *CCR1*$^{-/-}$ mice 8 h after RFA. A, B, and D, immunohistochemical analysis was performed using anti-CD4, anti-CD8a, anti-CD11c, or anti-F4/80 antibodies. A, representative results from three individual WT mice in each group. Original magnification, ×400. Bar, 50 μm. B and D, the numbers of CD4$^+$, or CD8$^+$, CD11c$^+$, or F4/80$^+$ cells were counted. Cell density was determined in 10 randomly chosen tumor areas at 400-fold magnification. Columns, mean (n = 5); bars, SD. **, $P < 0.01$. C, RFA-treated tumor tissues were processed using double-color immunofluorescence analysis as described in Materials and Methods. Right, digitally merged images. Representative results from three individual animals. Original magnification, ×400. Bar, 50 μm.

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effects of ECI301 on peripheral blood. RFA alone had few effects on the numbers of CCR1-expressing cells, but subsequent ECI301 administration increased the numbers of CCR1-expressing cells in peripheral blood, particularly $CD11c^+$ cells, but not $CD4^+$ or $CD8^+$ cells (Fig. 3). Because immune cells need to accumulate in RFA-treated tumors at an early stage to initiate adaptive immune responses, CCR1 expression by tumor-infiltrating cells in RFA-treated tumors was examined 8 hours after treatment. RFA-induced $CD4^+$, $CD8^+$, $CD11c^+$, and $F4/80^+$ cell infiltrations into RFA-treated tumors were greater than those into tumors of untreated mice. Moreover, ECI301 further increased the numbers of $CD4^+$, $CD8^+$, and $CD11c^+$ cells infiltrating into RFA-treated tumors compared with the numbers of these cells infiltrating into tumors treated with RFA alone (Fig. 4A and B). In RFA-treated tumors, most $CD11c^+$ and $F4/80^+$ cells expressed CCR1, whereas few $CD4^+$ and $CD8^+$ cells expressed CCR1 (Fig. 4C). Furthermore, ECI301-induced $CD4^+$, $CD8^+$, and

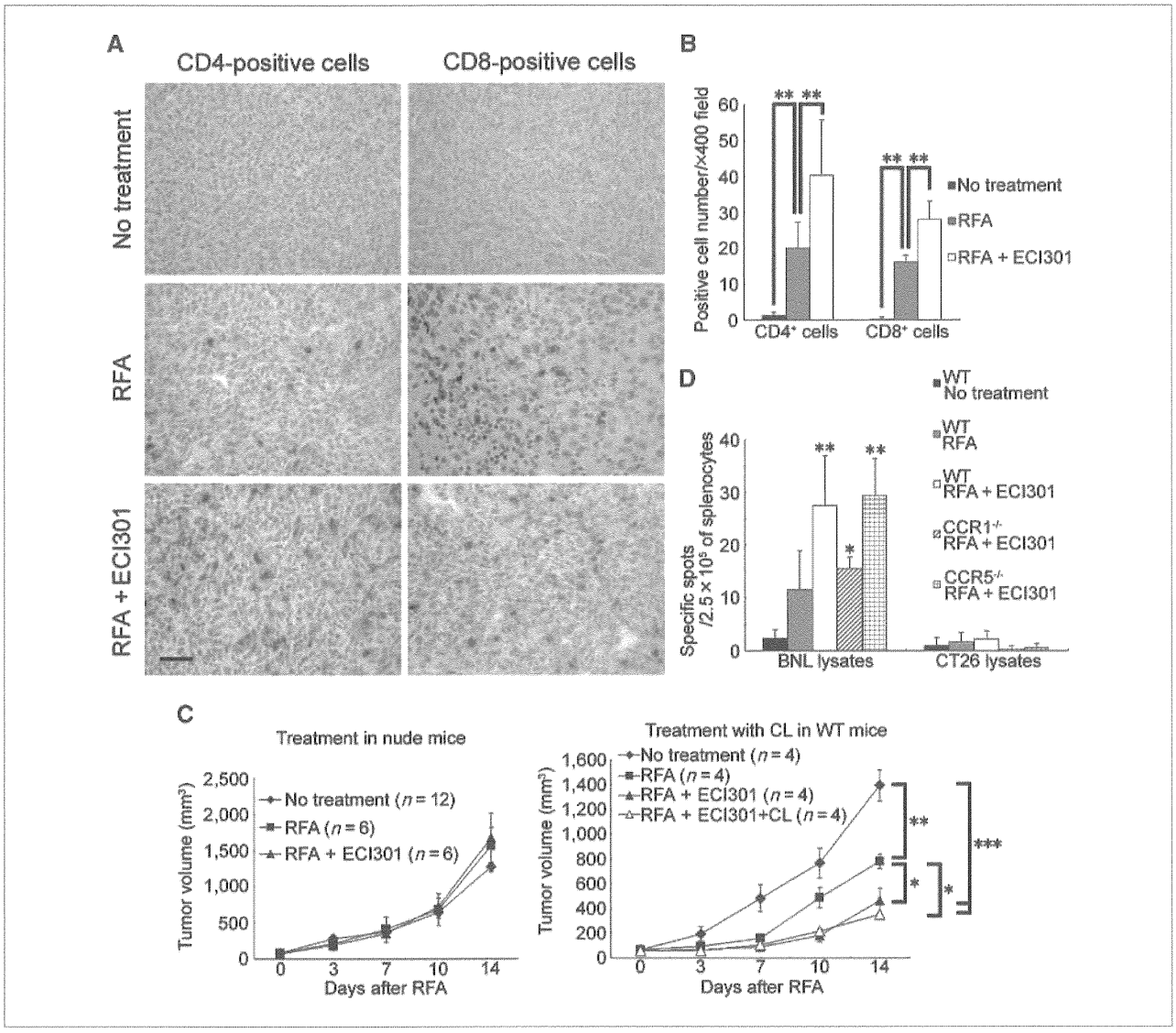

Figure 6. ECI301 augments tumor-specific immune responses after RFA. A, non–RFA-treated tumors were removed from WT mice on day 3 after RFA, and immunohistochemical analysis was done using anti-CD4 or anti-CD8a antibodies. Representative results from three individual animals in each group. Original magnification, ×400. Bar, 50 μm. B, the numbers of $CD4^+$ or $CD8^+$ cells were counted. Cell density was determined in 10 randomly chosen tumor areas at 400-fold magnification. Columns, mean (n = 5); bars, SE. **, $P < 0.01$. C, BALB/c-*nu/nu* mice (left) and BALB/c-WT mice (right) were inoculated with BNL cells and treated as described in the legend to Fig. 1. In the RFA + ECI301 + clodronate liposome (CL) group, the mice were injected with 200 μL of CL to deplete monocytes/macrophages as described in Materials and Methods. Non–RFA-treated tumor volumes were measured twice a week. Points, mean; bars, SE. D, spleens from WT, $CCR1^{-/-}$, or $CCR5^{-/-}$ mice were harvested on day 21 after RFA, and mononuclear cells were separated from the spleens for ELISPOT assay as described in Materials and Methods. The number of specific spots was determined by subtracting the number of spots in wells without lysates from the number of spots in wells with tumor lysates. Columns, mean (n = 3); bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with untreated WT mice.

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$CD11c^+$ cell infiltrations into ablated tumors were lesser in *CCR1*$^{-/-}$ mice than in WT mice, whereas $F4/80^+$ cells infiltrated RFA-treated tumors in *CCR1*$^{-/-}$ mice and WT mice to a similar extent (Fig. 4D). These observations suggest that ECI301 augments RFA-induced $CD4^+$, $CD8^+$, and $CD11c^+$ cell infiltrations into RFA-treated tumors in a CCR1-dependent manner.

ECI301 increases intratumoral expression of CCL3 after RFA

We showed that $CCR1^+$ cells were mobilized into blood by i.v. administered ECI301. However, the concentration of ECI301 in blood can go down rapidly as time passes (the peak is 5 minutes, and the half-life is <2 hours),[4] allowing ECI301-mobilized $CCR1^+$ cells to migrate into tissues where chemokines are highly produced. To prove this point, chemokine expression in RFA-treated tumors was examined. RFA plus ECI301 treatment increased *CCL3* mRNA expression level 6 hours after RFA. Moreover, 24 hours after treatment, *CCL3* and *CCL4* mRNA expression levels became almost 10-fold higher in tumors treated with RFA alone than in tumors of untreated mice, and ECI301 further increased the mRNA expression level of these chemokines in RFA-treated tumors (Fig. 5A). CCL3 and CCL4 were detected in tumor-infiltrating $F4/80^+$ cells (Fig. 5B). These observations indicate that RFA treatment causes local production of CCL3 and CCL4 in RFA-treated tumors and ECI301 further increases the expression of these chemokines. As the concentration of ECI301 in blood decreases, chemokines produced locally in RFA-treated tumor can attract CCR1-expressing $CD11c^+$ cells, thereby indirectly inducing CCR1-negative $CD4^+$ and $CD8^+$ cell infiltrations.

ECI301 augments RFA-induced tumor-specific immune responses accompanied by T-cell infiltrations into non–RFA-treated tumors

Non–RFA-treated tumors were analyzed histologically to clarify the mechanisms underlying the CCR1-dependent inhibitory effect of RFA plus ECI301 treatment against these tumors. Although few $CD4^+$ or $CD8^+$ cells were observed in the tumors of untreated mice, RFA treatment increased the numbers of $CD4^+$ and $CD8^+$ cells in the non–RFA-treated tumors 3 days after RFA. ECI301 further augmented RFA-induced $CD4^+$ and $CD8^+$ cell infiltrations into non–RFA-treated tumors (Fig. 6A and B). However, only a marginal number of $CD11c^+$ or $F4/80^+$ cells infiltrated into non–RFA-treated tumors of mice treated with RFA alone or RFA plus ECI301-treated mice (data not shown). Based on these findings, we hypothesized that ECI301-augmented tumor regression after RFA may be associated with T-cell–mediated antitumor immune responses. To clarify this point, *nu/nu* mice on a BALB/c background were treated by RFA with or without ECI301. Deficiency of T cells abrogated the tumor-inhibitory effect of ECI301 as well as the RFA-induced antitumor effect (Fig. 6C). Thus, both ECI301- and RFA-induced tumor regressions require T-cell–mediated antitumor immune response.

[4] Unpublished data from Effector Cell Institute.

However, $CD4^+$ or $CD8^+$ T cells rarely expressed CCR1 in blood and RFA-treated tumors. $CCR1^+$ cells in RFA-treated tumors were $CD11c^+$ cells and $F4/80^+$ cells, and only the former accumulate in RFA-treated tumors in a CCR1-dependent manner. These findings suggest that CCR1-positive $CD11c^+$ cells may activate antitumor T-cell responses and indirectly induce tumor retardation. Accordingly, we next examined the effect of depletion of monocytes/macrophages on ECI301-augmented tumor regression. I.p. injection of clodronate liposome depleted CD11c-negative monocytes in blood, although it did not change the number of $CD11c^+$ cells (data not shown). Depletion of these CD11c-negative monocytes did not cause any effects on ECI301-enhanced tumor regression, indicating that ECI301-augmented antitumor T-cell immunity was independent of CD11c-negative monocytes (Fig. 6C).

Finally, to prove the presence of systemic adaptive immune responses, IFN-γ ELISPOT assay was performed using mononuclear cells from the spleen. A greater number of spots against BNL cell lysates, but not against CT26 cell lysates, were generated by RFA plus ECI301–treated mice than that by mice treated with RFA alone or untreated mice. Moreover, ablation of *CCR1* gene, but not *CCR5* gene, reduced the number of spots against BNL cell lysates even when the mice were treated with RFA plus ECI301 (Fig. 6D). These observations suggest that ECI301 can further augment RFA-induced tumor-specific adaptive immune responses and subsequent tumor retardation in a CCR1-dependent manner.

Discussion

HCC occurs predominantly in individuals with chronic liver disease related to hepatitis B or hepatitis C virus infections (22–24). In addition to surgical resection, RFA treatment has been developed to eradicate solitary HCC lesions (25). RFA of HCC induces specific T-cell responses against liver tumors in human and rabbit (8, 11). Moreover, activated dendritic cells were detected in peripheral blood of HCC patients after RFA (9). These previous reports indicate that RFA treatment can induce antitumor immune responses against HCC (8–11). Likewise, we observed that RFA treatment generated tumor-specific IFN-γ–producing cells and inhibited the growth of non–RFA-treated tumors accompanied by massive T-cell infiltration into these tumors. However, even after successful ablation of HCC lesion by RFA, tumor recurrence often occurs probably because HCC develops in a multicentric manner in the cirrhotic liver (12). These observations indicate that RFA-induced augmentation in immune response may not be sufficient to prevent tumor recurrence. Thus, a novel therapeutic modality is required to further augment RFA-induced tumor-specific immune responses. Here, we showed that combined administration of ECI301 and RFA can augment tumor-specific immune responses against HCC.

Several chemokines are used for immunotherapy against cancers because they can attract immune cells such as dendritic and cytotoxic T cells to augment tumor-specific immune responses (26). However, some chemokines can simultaneously attract myeloid-derived suppressor and regulatory T cells to promote neovascularization and induce immunosuppressive

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microenvironments (26–28). The double-edged activities of chemokines frequently preclude their use for tumor immunotherapy. Moreover, most chemokines exhibit a bell-shaped dose-response curve with a narrow effective dose window. Thus, determination of an optimal dose of chemokines is important to elicit efficient antitumor responses (29). Several lines of evidence show that intratumoral use of CCL3 reduces tumorigenicity (6, 30). Furthermore, there are no reports showing that use of CCL3 can promote tumor progression. We observed that systemic administration of ECI301 without RFA treatment induced neither reduction nor progression of tumors. On the contrary, systemic injection of ECI301 after RFA can inhibit the growth of non–RFA-treated tumors in the contralateral side. ECI301-enhanced tumor regression after RFA was both CCR1 and T-cell dependent, but T cells rarely expressed CCR1 in blood and RFA-treated tumors. Because depletion of monocytes/macrophages did not affect the retardation of ECI301-treated tumors, CCR1-expressing $CD11c^+$ dendritic cells might activate antitumor T-cell responses and indirectly induce tumor retardation via some mechanisms such as antigen presentation and cytokine production. ECI301 mobilized a large number of $CCR1^+$ cells into blood, and these mobilized cells may be attracted into highly CCL3-producing RFA-treated tumors and cause increased number of tumor-infiltrating $CCR1^+CD11c^+$ dendritic cells. Thus, $CCR1^+$ precursors in blood and $CCR1^+CD11c^+$ tumor-infiltrating dendritic cells might play important roles in ECI301-augmented antitumor effects (31–33).

ECI301 could not increase the number of $F4/80^+$ cells in the RFA-treated tumor sites. Accumulation of $F4/80^+$ cells in the tumor treated with ECI301 plus RFA was also independent of CCR1. $F4/80^+$ cells, which might include a large number of macrophages/monocytes, are usually attracted into the tumor by CCL2, CCL4, and CCL5 that are produced in the tumor sites. CCR2, the receptor for CCL2, and CCR5, the receptor for CCL4 and CCL5, might be responsible for migration of monocytes/macrophages (27, 34–36). However, it is still unclear whether monocytes/macrophages use CCR2 or CCR5 after massive tumor cell death caused by treatments such as RFA because tumor cell death induces different profiles of chemokine production in the tumors (4). Although ECI301 did not directly induce migration of $F4/80^+$ cells, the mechanism underlying the infiltration of $F4/80^+$ macrophages remains to be elucidated.

Breaking tolerance for tumor cells is necessary for induction of antitumor immunity. Several independent groups have suggested multiple mechanisms underlying immunogenic tumor cell death induced by anticancer chemotherapy or radiation therapy (37–40). Anthracyclin causes apoptosis along with translocation of calreticulin to the apoptotic tumor cell surface. Calreticulin exposure augments phagocytosis of apoptotic cancer cells by dendritic cells with an eventual increase in immune response (37, 38). Chemotherapy or irradiation kills tumor cells to release high mobility group box 1 (HMGB1). Released HMGB1 activates dendritic cells after binding to toll-like receptor 4 expressed by these cells (39). Apoptosis induced by local radiation therapy augments MHC class I expression by tumor cells, thereby facilitating their recognition by cytotoxic T cells (40). RFA induces the expression of heat shock proteins 70 and 90 on ablated tumor cells, and these proteins can activate toll-like receptor–expressing antigen-presenting cells (41, 42). In addition, we showed that RFA treatment alone caused local production of CCL3 in RFA-treated tumors accompanied by accumulation of T cells and $CD11c^+$ dendritic cells. These mechanisms may also account for the observed RFA-induced generation of tumor-specific immune responses.

We revealed that combined treatment of ECI301 and RFA augmented antitumor-specific immune responses, thereby inhibiting the growth of non–RFA-treated tumors in a CCR1-dependent manner. Thus, combined treatment of ECI301 and RFA can prevent human HCC from recurring after RFA treatment. The absence of any severe adverse effects in mice (data not shown) further warrants the clinical trial of ECI301 combined with RFA as a treatment regimen for HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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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" ($\leq$2 cm) or "large" (>2 cm), and tumor multiplicity was categorized as "multiple" ($\geq$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), $hTERT_{324}$

(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^{TM} 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

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

				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
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.

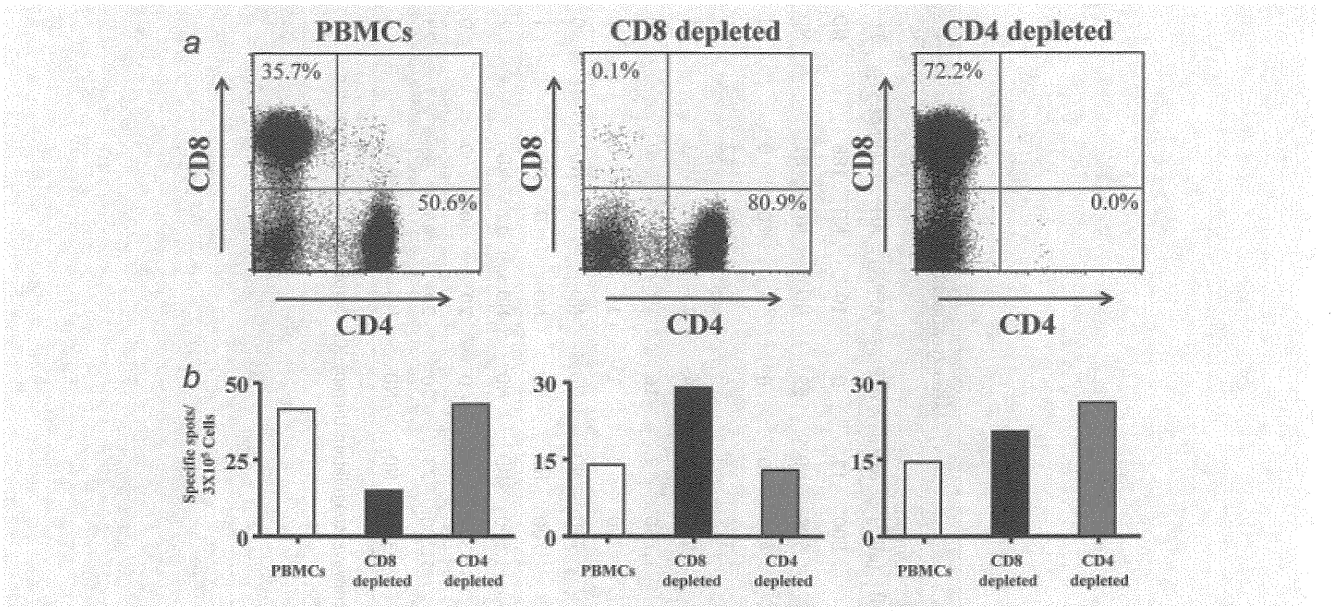

Figure 1. IFN-γ production of CD4- or CD8-depleted T cells against whole AFP. AFP-specific IFN-γ-producing T cells were analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed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

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.

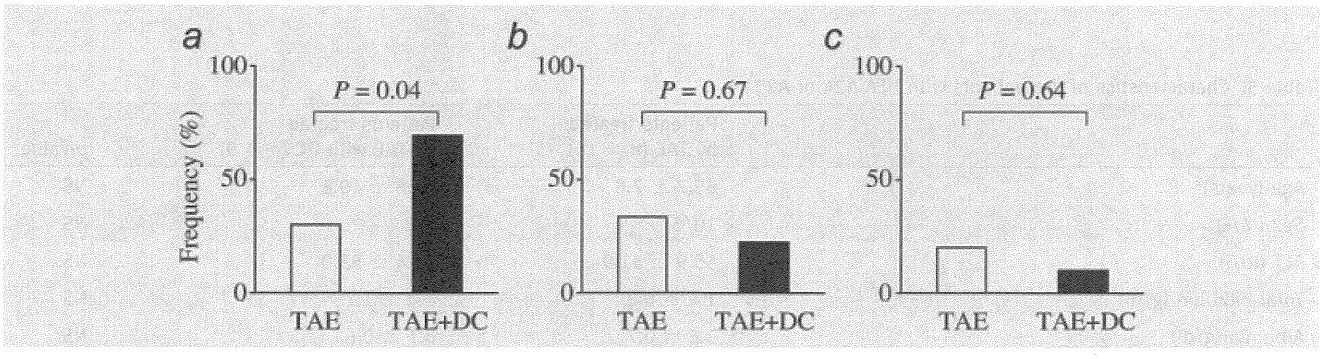

Figure 2. Frequency of the patients who showed enhancement of T-cell responses after treatment. The prevalence of antigen-specific T cells was determined by IFN-γ ELISPOT analysis using alpha-fetoprotein (AFP) and AFP-derived peptides (*a*), CMV pp65-derived peptide (*b*) or tetanus toxoid protein (*c*)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

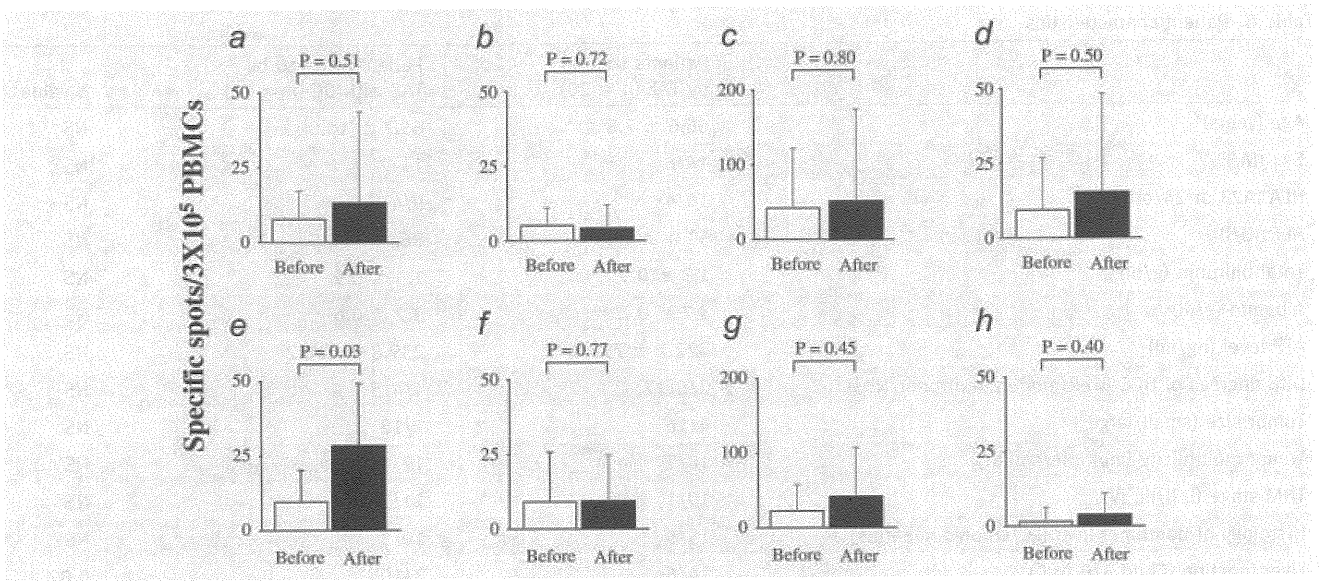

Figure 3. Comparison of direct *ex vivo* analysis (IFN-γ ELISPOT assay) before and after treatment of HCC. The assay was performed using PBMCs of patients who received TAE for AFP-derived peptides (*a*), AFP (*b*), CMV pp65-derived peptide (*c*) or tetanus toxoid protein (*d*). The same assay was performed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

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

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

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

The precise mechanism of this phenomenon is still unknown; however, in recent studies, several treatments to destroy tumor cells by necrosis and/or apoptosis have induced antitumor immune responses in animal models[14,44] and even in humans.[6–10] In the study of *in situ* tumor ablation, it is reported that tumor ablation creates a tumor antigen source for the induction of antitumor immunity.[9,44] In another study regarding photodynamic therapy (PDT),[45] it is reported that acute inflammation, expression of heat-shock proteins and providing tumor antigens to DCs caused by PDT induce tumor-specific immune responses.

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

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

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

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

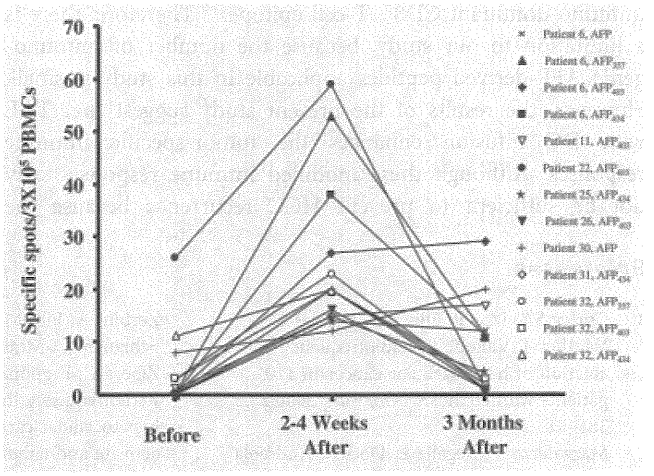

Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN-γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2–4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments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

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

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

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

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

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

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

immuno-dominant $CD8^+$ T-cell epitope.[52] Therefore, there is a limitation to our study, because the number of immunogenic AFP-derived peptides applicable in this study is small. However, the results of the present study suggest that TAE with DC infusion enhances the tumor-specific immune responses. Although these modified immune responses may not be sufficient to prevent HCC recurrence because the enhanced immune responses are transient and attenuate within 3 months, these results may contribute to the development of novel immunotherapeutic approach for HCC.

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