Radiofrequency Thermal Ablation of Hepatocellular Carcinoma Liver Nodules Can Activate and Enhance Tumor-Specific T-Cell Responses

Alessandro Zerbini,[1] Massimo Pilli,[1] Amalia Penna,[1] Guido Pelosi,[1] Claudia Schianchi,[1] Atim Molinari,[1] Simona Schivazappa,[1] Carlo Zibera,[2] Francesco F. Fagnoni,[2] Carlo Ferrari,[1] and Gabriele Missale[1]

[1]Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy and [2]Laboratory of Experimental Oncology, Scientific Institute of Pavia, Fondazione Salvatore Maugeri-Clinica del Lavoro e della Riabilitazione, IRCCS, Pavia, Italy

Abstract

Radiofrequency thermal ablation (RFA) destroys tumoral tissue generating a local necrosis followed by marked inflammatory response with a dense T-cell infiltrate. In this study, we tested whether hepatocellular carcinoma thermal ablation can induce or enhance T-cell responses specific for hepatocellular carcinoma–associated antigens. Peripheral blood mononuclear cells derived from 20 patients with hepatocellular carcinoma were stimulated before and a month after RFA treatment with autologous hepatocellular carcinoma–derived protein lysates obtained before and immediately after RFA treatment. The effect of thermal ablation on memory T-cell responses to recall antigens [tetanus toxoid, protein purified derivative (PPD), *Escherichia coli*] was also assessed. T-cell reactivity was analyzed in an IFN-γ enzyme-linked immunospot assay and by intracellular IFN-γ staining. Treatment was followed by a significant increase of patients responsive either to tumor antigens derived from both the untreated hepatocellular carcinoma tissue ($P < 0.05$) and the necrotic tumor ($P < 0.01$) and by a higher frequency of circulating tumor-specific T cells. T-cell responses to recall antigens were also significantly augmented. Phenotypic analysis of circulating T and natural killer cells showed an increased expression of activation and cytotoxic surface markers. However, tumor-specific T-cell responses were not associated with protection from hepatocellular carcinoma relapse. Evidence of tumor immune escape was provided in one patient by the evidence that a new nodule of hepatocellular carcinoma recurrence was not recognized by T cells obtained at the time of RFA. In conclusion, RFA treatment generates the local conditions for activating the tumor-specific T-cell response. Although this effect is not sufficient for controlling hepatocellular carcinoma, it may represent the basis for the development of an adjuvant immunotherapy in patients undergoing RFA for primary and secondary liver tumors. (Cancer Res 2006; 66(2): 1139-46)

Introduction

Hepatocellular carcinoma is the most common primary liver cancer with increasing incidence in the last two decades in Europe, the United States, and Japan (1, 2), which is expected to continue to increase for the next few decades because of the large pool of people infected by HBV and HCV. Orthotopic liver transplantation and surgical therapy offer the best opportunities to cure patients with hepatocellular carcinoma. However, both options are limited in their application because of the scarce organ availability for transplantation and because of the size and location of the tumor, associated vascular invasion and thromboses, and the characteristics and severity of the underlying liver disease. For these reasons, hepatocellular carcinoma management is mainly accomplished by local ablative therapies that can compete in efficiency with surgical resection of hepatocellular carcinoma nodules (3). Moreover, local ablation can be applied to a larger number of patients with more advanced liver disease, with multifocal presentation and other clinical conditions that can represent contraindications for liver surgery. These local techniques, however, are frequently followed by hepatocellular carcinoma recurrences; therefore, there is urgent need to develop novel therapies with systemic activity to avoid spontaneous progression of hepatocellular carcinoma.

Among different techniques, radiofrequency thermal ablation (RFA) is designed to destroy tumoral tissue by delivering a high-frequency alternating current with ionic agitation and frictional heating. At temperatures above 45°C to 50°C, cell membranes are destroyed, proteins are denatured, and a region of necrosis surrounding the electrode is generated (4, 5). Thermal ablation is followed by a marked local inflammatory response with a dense T-cell infiltrate in the liver of tumor-free domestic pig (6) and in the liver of rabbits implanted with a papilloma-induced epithelial tumor (7). Moreover, in the rabbit model and in mice injected with a melanoma cell line, thermal ablation can induce an antigen-specific T-cell response (7, 8). Taken together, these observations support the hypothesis that heat shock delivery and massive necrotic cell death by RFA may favor immune activation and presentation of otherwise cryptic antigens, thus inducing a tumor-specific T-cell response. The implications of such hypothesis are relevant with respect to two main perspectives. First, RFA is extensively and increasingly applied not only in the treatment of hepatocellular carcinoma but also for metastatic liver nodules and other extrahepatic solid malignancies, such as renal cell carcinoma, lung cancer, prostate cancer, metastatic bone lesions, and others. Second, combination of tumor destruction by RFA and active immunotherapy could represent a relatively simple and potentially successful strategy for combating hepatocellular carcinoma.

In this study, our aim was to verify whether thermal ablation could activate an antitumor specific T-cell response in patients who developed hepatocellular carcinoma in the context of liver cirrhosis. Accordingly, the study was designed to test the following: (*a*) if tumor-specific T-cell responses could be generated or enhanced by the treatment, (*b*) if thermal ablation could enhance non–tumor-specific memory T-cell responses, (*c*) if the inflammatory response triggered by RFA could affect the composition of

Requests for reprints: Gabriele Missale, Divisione Malattie Infettive ed Epatologia, Azienda Ospedaliero-Universitaria di Parma, Via Gramsci 14, 43100 Parma, Italy. Phone: 39-0521-702056; Fax: 39-0521-703857; E-mail: missale@tin.it.

doi:10.1158/0008-5472.CAN-05-2244

the circulating pool of T and natural killer (NK) lymphocytes, and (*d*) if the activation of the tumor-specific T-cell response could prevent hepatocellular carcinoma relapse.

Patients and Methods

Patients. Twenty patients undergoing RFA for hepatocellular carcinoma in liver cirrhosis were studied (Table 1). Hepatocellular carcinoma diagnosis was defined by concordant ultrasounds and contrast-enhanced computed tomography (CT) scan. All patients were treated for a single hepatocellular carcinoma nodule with the exception of patient 16 who was treated for two nodules; hepatocellular carcinoma size ranged between 1.5 and 6.0 cm in diameter. Patients 4, 7, 15, 18, and 20 had been previously treated by RFA during the past 3 years, and patient 17 had been treated 3 months before by trans-arterial chemoembolization on a different hepatocellular carcinoma nodule. Liver cirrhosis was due to HCV infection in 14 patients, to HBV infection in one patient, and HBV/HCV coinfection in one patient; four of the HCV monoinfected patients were also HBcAb positive, suggesting a probable occult HBV carriage. Liver cirrhosis of patients 3 and 17 was due to alcohol intake, and liver cirrhosis of patient 19 was likely related to a previous HBV infection (Table 1).

Patients gave written informed consent before entering the study, and the study protocol, approved by the local ethical Committee, conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment. RFA was done under conscious sedation and ultrasound guidance. A 14-gauge needle with nine arrays and an active trocar tip (StarBurst XL) connected to the RF generator (Model 1500× RF generator, RITA Medical Systems, Inc., Mountain View, CA) was used for all treatments. Depending on the site of the hepatocellular carcinoma, 15- or 25-cm-long needles were employed. At the end of tumor ablation, thermocoagulation was done along the needle track to avoid hepatocellular carcinoma seeding. Patients were evaluated clinically and with contrast-enhanced CT at 1 month after ablation and by contrast-enhanced (Sonovue, Bracco, Italy) ultrasounds at 3 months after ablation.

Peripheral blood mononuclear cells and tissues collection. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density-gradient centrifugation and resuspended at 1×10^6/mL in RPMI 1640 supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 50 mg/mL gentamicin, and 10% FCS (complete medium). CD8 cells depletion (>96%) was done with the use of antibody-coated MACS microbeads (Miltenyi Biotec, Auburn, CA) followed by magnetic separation according to the manufacturer's instructions.

For tissue collection, a fine-needle (22 gauge) aspiration cytology of the liver tumor and of the nontumor surrounding liver were done just before RFA. Necrotic tumor tissue was collected on the tips of the nine arrays retracted at the end of treatment; this approach avoided a second fine-needle aspiration. These specimens were immediately resuspended in 200 μL PBS, and 2 mL of ammonium chloride (0.15 mol/L) were added to obtain erythrocytes lysis, removing contaminating blood. Samples were then washed with PBS and resuspended in 200 μL of PBS and stored at −80°C. Necrotic tissue from a control normal liver was obtained from a surgical liver resection done for rectum cancer liver metastasis. Immediately after resection, a section of the liver free of tumor tissue underwent thermal ablation with the same type of needle and RF generator employed for hepatocellular carcinoma treatment; necrotic tissue close to the needle tips was collected and treated as the other samples.

Antigen preparation. Tissue samples derived from aspiration cytology of the liver tumor and of the nontumor surrounding liver or 5×10^6 cells of tumoral lines were lysed by three repeated steps of freezing and thawing to obtain a protein lysate in the supernatant of the centrifuged specimens. Protein quantification was obtained with the Bio-Rad Protein Assay method (Bio-Rad Laboratories, Hercules CA) as recommended by the manufacturer.

Non–tumor-related antigens used to test the effect of thermal ablation on non–tumor-specific memory T-cell responses were tetanus toxoid

Table 1. Patient characteristics and treatment outcome

Patient	Age	Nodule size	α-FP	HBsAg	HBsAb	HBcAb	HCV-Ab	Complete ablation	Recurrence, 3 months	Recurrence, 6 months
1	57	3.8	5.0	+	−	+	−	Y	N	U
2*	70	2.0	20.0	−	−	−	+	Y	N	U
3	58	2.0	2.9	−	−	−	−	Y	N	N
4*	72	3.0	16.0	−	−	+	+	Y	M†	M
5	77	2.5	106	−	+	+	+	Y	N	U
6	59	2.5	6.6	−	−	−	−	Y	N	N
7*	74	3.5	6.9	−	−	−	+	Y	N	N
8	57	2.5	18.7	+	+	+	+	Y	N	U
9*	68	2.0	8.4	−	−	−	+	Y	N	N
10	47	3.0	32.2	−	−	−	+	Y	N	N
11	73	2.5	18.0	−	−	−	+	Y	N	N
12	58	3.0	34.0	−	−	−	+	Y	U	U
13	76	2.2	1.4	−	−	+	+	Y	N	N
14	72	4.0	908	−	+	+	+	Y	U	U
15*	72	3.0	20.5	−	−	−	+	Y	U	U
16	78	2.0-3.0	11.0	−	−	−	+	Y	M	M
17	65	2.5	12.0	−	−	−	−	Y	N	N
18*	71	1.5	255	−	−	−	+	Y	N	N
19	48	6.0	6.8	−	+	+	−	N	U‡	U‡
20*	69	2.0	12.8	−	−	−	+	Y	N	N

Abbreviations: Y, yes; N, no; U, uninodular recurrence; M, multinodular recurrence.
*Patients previously treated with PEI and/or RITA or TACE.
†Disease recurrence 1 month after RFA.
‡Persistence of live tumor tissue in the treated nodule.

(Connaught Laboratories Ltd., Willondale, Ontario, Canada), PPD (Statens Serum Institute, Copenhagen, Denmark), and *E. coli* extract (Chiron Corp., Emeryville, CA).

Tumor cell lines. The melanoma cell line MZ2-MEL was kindly provided by Flavio Arienti (Istituto Tumori Milano, Italy). Human hepatocellular carcinoma cell lines Hep3B, PLC/PRF/5, and HuH7 were kindly provided by Giovanni Raimondo (University of Messina, Italy). Human hepatocellular carcinoma cell line HepG2 and the erythroleukemic line K562 were purchased from the American Type Culture Collection (Rockville, MD); MZ2-MEL and K 562 were cultured in RPMI 1640 supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 50 mg/mL gentamicin, and 10% FCS. HepG2, Hep3B, HuH7, and PLC/PRF/5 were cultured in DMEM supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 50 mg/mL gentamicin, and 10% FCS. Cells were subcultured every 3 days after treatment with Trypsin-EDTA and harvested at day 2 to obtain protein cell lysates.

Enzyme-linked immunospot assay. Polyvinylidene plates (96 wells; Millipore, Bedford, MA) were precoated with 5 μg/mL anti-IFN-γ monoclonal antibody (mAb), 1-DIK (Mabtech, Nacka, Sweden) overnight at 4°C. Plates were then washed four times with PBS and blocked with RPMI/10% FCS for 2 hours at 37°C. Cryopreserved PBMCs were thawed and plated in the presence or absence of 25 μg/mL protein lysates, 0.5 μg/mL *E. coli* extract, 2 μg/mL tetanus toxoid, 10 μg/mL PPD, and 1 μg/mL phytohemagglutinin as positive control at 200,000 per well in a total volume of 100 μL. PBMCs were seeded in duplicates for antigenic stimulation and in triplicates for controls. The plates were incubated overnight at 37°C, 5% CO_2 and washed seven times with PBS/0.05% Tween before addition of the second, biotinylated anti-IFN-γ mAb, 7-B6-1 biotin (Mabtech) at 1 μg/mL, and incubated for 3 hours. After a further washing step, anti-biotin alkaline phosphatase–conjugated antibody (Mabtech) was added for 90 minutes. Following another washing step, individual cytokine-producing cells were detected as dark spots after a 5- to 10-minute reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase–conjugated substrate (Bio-Rad Laboratories). Spots were enumerated as IFN-γ spot forming units using an enzyme-linked immunospot (ELISPOT) plate reader (AID). The number of specific IFN-γ secreting T cells was calculated by subtracting the unstimulated control value from the stimulated sample. Wells were considered as positive if they were at least twice above background with at least 10 spots per well.

Intracellular IFN-γ staining. Intracellular cytokine assessment was done on antigen-specific $CD4^+$ or $CD8^+$ cells using flow cytometry. Autologous adherent cells, obtained by plating freshly derived PBMCs in a 96 flat-bottomed plate for 3 hours, were incubated with 25 μg/mL of the tissues protein lysates or 0.5 μg/mL *E. coli* extract or 2 μg/mL tetanus toxoid or 10 μg/mL PPD, for 2 hours followed by coculture with 4×10^5 nonadherent cells per well for 14 hours in complete medium in a humidified 5% CO_2 incubator at 37°C. Brefeldin-A (10 μg/mL; Sigma Chemical Co., St Louis, MO) was added 1 hour after beginning of coculture. Cell suspensions were stained with PE-anti-CD4, APC-anti-CD8, and PerCP-anti-CD3 mAb (Becton Dickinson, San Jose, CA), fixed and permeabilized (Fix&Perm, Caltag Laboratories, Burlingame, CA) at room temperature, and stained with FITC-labeled anti-human-IFN-γ (Sigma Chemical). Intracellular cytokine expression was analyzed on a FACSCalibur (Becton Dickinson) flow cytometer using the CELLQuest software (Becton Dickinson). Frequencies of CD4 and CD8 T cells expressing IFN-γ were always ≤0.01% when stimulated with nontumor liver tissue protein lysates.

Immunofluorescence cell surface analysis of lymphocytes. PBMCs were resuspended in PBS containing 1% FCS, 1% human serum, 10% mouse serum, and 0.01% sodium azide, then stained for 30 minutes on ice with combinations of saturating amounts of fluorochrome-conjugated murine mAbs. The following fluorochrome-conjugated mAbs were obtained from PharMingen BD Biosciences (Becton Dickinson Italia S.p.A., Milan, Italy): FITC-conjugated anti-CD4 (clone RPA-T4, IgG1), anti-CD45RA (HI100, IgG2b), anti-CD95 (DX2, IgG1); anti-CD16 (3G8, IgG1); PE-conjugated anti-CD8 (HIT8a, IgG1), anti-CD28 (CD28.2, IgG1); anti-CD3 (UCHT1, mouse IgG1); cychrome-conjugated: anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD8 (HIT8a). Phycoerythrin-cyanin 5.1 (PC-5)-conjugated mAbs directed to CD3 (UCHT1) and CD56 (N901, mouse IgG1) were purchased from Beckman Coulter, Inc. (Instrumentation Laboratory, Milan, Italy). For setting quadrants and gates limits of nonspecific immunoglobulin cell binding (negative controls), we used fluorochrome-conjugated IgGs of irrelevant specificity obtained from the same manufacturer. Naive and memory T cells within $CD4^+$ and $CD8^+$ T-cell subsets were defined as previously described (9) based on CD28 versus either CD95 or CD45RA expression. Lymphocytes with NK activity were identified as $CD3^-CD56^+$ and $CD56^+Fc\gamma RIII(CD16)^+$ cells.

Cell surface marker analysis was done in 11 of the 20 patients studied (patients 1, 2, 3, 5, 6, 7, 8, 12, 13, 15, and 16).

Statistical analysis. Statistical analysis of differences between mean values of the spots generated with different protein lysates was done using Wilcoxon signed rank test for paired data. Comparison of responsive patients to tumor antigens before and after RFA treatment and analysis of patients responsive to tumor antigens with or without disease recurrence were done by χ^2 analysis. $P < 0.05$ was considered significant.

Results

T-cell responses to hepatocellular carcinomas derived antigens. Frequency of tumor-specific T cells before and 4 weeks after RFA treatment was tested *ex vivo* in an IFN-γ ELISPOT assay in 20 patients. Before treatment, three patients showed a specific T-cell response stimulating T cells with tumor protein lysate and two patients with protein lysate derived from the post-RFA necrotic hepatocellular carcinoma tissue (Table 2). None of the patients showed a T-cell response directed to the nontumor liver tissue. Four weeks after treatment, a T-cell response to autologous tumor tissue was detected in two of the three patients reactive before treatment and in seven additional patients ($P < 0.05$). Stimulating with post-RFA necrotic tumor tissue a T-cell response was detected in 10 patients ($P < 0.01$).

After treatment, T-cell responses were also detected against autologous nontumor liver tissue in two patients due to a possible autoimmune effect or enhancement of anti-HCV T-cell response in patient 16.

Although no significant difference was observed between mean values of spots generated with nontumor liver tissues and tumor tissues before treatment, after treatment this difference was highly significant ($P < 0.001$), as well as the difference between nontumor liver tissue and necrotic tumor tissue ($P < 0.01$; Fig. 1). The comparison of the mean values of spots generated before and after treatment with untreated and necrotic tumor tissues were also statistically significant ($P < 0.05$).

To rule out the possibility that T-cell stimulation was a nonspecific effect caused by the necrotic tissue rather than induced by hepatocellular carcinoma–related antigens, post-RFA necrotic tissue from a control normal liver was used to stimulate PBMCs from 11 of the 20 hepatocellular carcinoma patients (patients 1, 2, 3, 6, 7, 12, 14, 15, 18, 19, and 20). Number of spots per well ranged from 1 to 12 with a maximum delta spot (subtracted of spots in presence of medium) of four spots per well, and no increase of spots was detected after RFA treatment (not shown).

T-cell response to tumor cell lines. To confirm the effect of RFA on the antitumor T-cell response, six tumor cell lines were used to stimulate PBMCs in an IFN-γ ELISPOT assay before and 4 weeks after RFA treatment. The hepatoma PLC/PRF/5 cell line was the most immunogenic. The protein lysate obtained from this cell line stimulated at least a two times increase of the background spots in 7 of 20 patients before and 16 of 20 patients after RFA treatment ($P < 0.01$). One of 13 patients tested showed a response to the Hep3B hepatoma cell line and 4 of 17 patients to the HU-H7

Table 2. T-cell response to hepatocellular carcinoma and liver nontumor surrounding tissue by ELISPOT assay before and after thermal ablation

	Before treatment			After treatment		
	Liver tissue	Tumor tissue	Post-RFA tumor tissue	Liver tissue	Tumor tissue	Post-RFA tumor tissue
Patient 1	15	0	45	0	0	0
Patient 2	15	0	0	15	**205**	**185**
Patient 3	0	0	0	**50**	**95**	**85**
Patient 4	0	15	0	0	0	25
Patient 5	0	**675**	**60**	20	**995**	**35**
Patient 6	25	**35**	25	30	5	5
Patient 7	0	0	0	25	**50**	25
Patient 8	30	30	**35**	0	15	5
Patient 9	0	5	10	0	0	40
Patient 10	10	0	0	10	5	25
Patient 11	5	5	10	0	40	15
Patient 12	5	5	5	5	60	50
Patient 13	20	10	15	0	0	20
Patient 14	5	**90**	20	10	**110**	**45**
Patient 15	20	5	25	10	15	**40**
Patient 16	0	10	15	**35**	**35**	**30**
Patient 17	0	0	0	0	5	10
Patient 18	0	10	10	0	10	**25**
Patient 19	10	0	0	20	20	40
Patient 20	5	0	0	0	**70**	**45**

NOTE: Average values of duplicates spots per 10^6 PBMCs after subtracting responses to medium alone. In bold are average spots values twice above background (medium).

hepatoma cell line before treatment. After RFA treatment, 3 of 13 patients showed a response to Hep3B and 6 of 17 patients to HU-H7 (Table 3). Interestingly, none of the 18 patients tested showed a significant reactivity before or after RFA to the melanoma and erythroleukemic lines (Mel MZ2 and K562; Table 3). Comparison of mean spots before and after RFA treatment showed a significant increase of the T-cell responses to the cell line PLC/PRF/5 ($P < 0.05$).

T-cell response to tumor nonrelated antigens. To test if thermal ablation could enhance memory T-cell responses to nontumor recall antigens, ELISPOT assay was done, stimulating PBMCs of 15 of the 20 patients undergoing treatment with tetanus

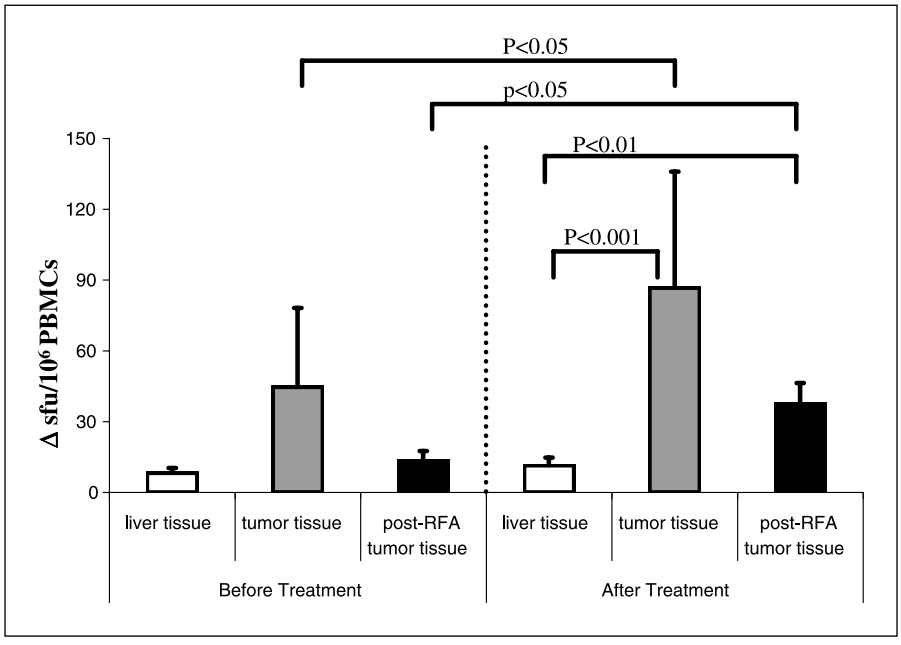

Figure 1. RFA can induce a significant increase of the hepatocellular carcinoma–specific T-cell response. *Columns,* mean values of spots generated in an IFN-γ ELISPOT assay in the presence of nontumor liver protein lysates and hepatocellular carcinoma protein lysates obtained before and after thermal ablation. PBMCs were derived on the day of RFA (*before treatment*) and 4 weeks later (*after treatment*).

toxoid, PPD, and *E. coli* extract. A positive T-cell response to tetanus toxoid, PPD, and *E. coli* could be detected in 2 of 15, 8 of 15, and 9 of 15 patients, respectively, before treatment and in 4 of 15, 11 of 15, and 11 of 15 patients after treatment. The strength of the response was also augmented after RFA treatment, with an increase of mean values of spots per 10^6 PBMCs generated with tetanus toxoid (23.8 ± 63.8 versus 51.9 ± 120.5; P = not significant) and a significant increase of mean values of spots generated with PPD (126.3 ± 156.6 versus 377.2 ± 567.7; $P < 0.02$) and *E. coli* (122.8 ± 142.2 versus 420.6 ± 502.8; $P < 0.01$).

Identification of the T-cell subset responsible for tumor-specific T-cell responses. To define if CD4 or CD8 T cells were responsible for IFN-γ production in the ELISPOT assay, PBMCs from patients 9, 17, and 20 were stained with anti-CD4 and anti-CD8 mAbs and tested for IFN-γ production upon antigen stimulation by intracellular cytokine staining and fluorescence-activated cell sorting analysis. Autologous tumor, nontumor liver tissue, and PLC/PRF/5 protein lysates were used *ex vivo* for antigenic stimulation. Patient 20 showed an antigen-specific T-cell response when stimulated with the autologous hepatocellular carcinoma after treatment, and the T-cell response was mainly sustained by the CD4 subset although a weaker response was also detected with CD8-positive cells (Fig. 2). A prevalent CD4 response was observed against PLC/PRF/5 in patient 17 before (0.04% of CD4 cells) and after treatment (0.55% of CD4 cells) and in patient 20 only after treatment (0.035% of CD4 cells). In patient 20, PLC/PRF/5 elicited also a CD8-mediated response (0.06% of CD8 cells) only after treatment. IFN-γ production in presence of medium or of the nontumor liver tissue was always ≤0.01%.

In patients 9, 17, and 20, T-cell response to recall antigens (PPD and *E. coli*) was also confirmed by intracellular IFN-γ staining showing a response sustained by the CD4-positive T-cell subset (data not shown).

Phenotypic analysis of circulating lymphocytes and NK cells. Eleven patients were selected randomly to assess the effect of RFA treatment on the circulating pool of T and NK lymphocytes. The proportion of T cells among the overall lymphocyte pool (mean ± SD, 69.93 ± 6.68% before versus 68.94 ± 6.4% after RFA), as well as the proportion of $CD4^+$ (60.68 ± 6.5% versus 60.5 ± 5.9%) and $CD8^+$ (34.55 ± 5.76% versus 34.82 ± 6.13%) subsets among T cells were not significantly modified at 1 month after treatment (Table 4). Similarly, neither the proportion of naive (32.4 ± 17.38% versus 31.8 ± 15.6%) and memory (62.56 ± 17.1% versus 62.7 ± 14.5%) $CD4^+$ T cells, nor the proportion of naive (10.85 ± 4.5% versus 10.95 ± 5.1%) and memory (27.35 ± 10.1% versus 21.61 ± 10.4%) $CD8^+$ T cells seemed modified. However, when we compared by paired analysis the proportion of T cells expressing CD56 (9.47 ± 4.16% versus 10.99 ± 4.8%; $P = 0.05$), as well as $CD28^-$ T cells within the $CD8^+$ subset (60.58 ± 12.0% versus 64.69 ± 9.2%; $P < 0.05$), we found a significant increase after therapy. Similarly, $CD3^-CD56^+$ (11.12 ± 6.2% versus 13.54 ± 6.8%; $P < 0.05$) and $CD56^+CD16^+$ (9.47 ± 4.1% versus 11.0 ± 4.8%; $P < 0.05$) NK lymphocytes increased after RFA (Table 4). On the whole, these data indicate that RFA is accompanied by an activation and/or mobilization of lymphocytes with a phenotype of cytotoxic effector cells.

Treatment outcome. Contrast enhanced CT at 1 month after RF treatment showed a complete hepatocellular carcinoma ablation in 19 of 20 patients. In patient 19, ablation of the 6-cm hepatocellular

Table 3. T-cell response to tumor cell lines by ELISPOT assay before and after thermal ablation

	Before treatment						After treatment					
	Mel MZ2	K562	HepG2	PLC-PRF5	HU-H7	Hep3B	Mel MZ2	K562	HepG2	PLC-PRF5	HU-H7	Hep3B
Patient 1	0*	40	0	**185**	105	ND	0	45	30	**95**	**100**	ND
Patient 2	0	70	95	**275**	105	80	60	110	100	**290**	110	80
Patient 3	0	0	0	35	55	30	0	15	0	10	0	0
Patient 4	10	10	15	**225**	**105**	0	0	5	0	**130**	55	10
Patient 5	0	10	20	40	0	0	5	15	0	**185**	25	25
Patient 6	5	20	5	**80**	**60**	45	30	20	25	**320**	**100**	35
Patient 7	15	20	20	**65**	25	35	20	0	20	**55**	0	5
Patient 8	0	0	0	20	0	ND	0	10	10	**115**	25	ND
Patient 9	ND	ND	ND	0	ND	ND	ND	ND	ND	0	ND	ND
Patient 10	10	0	0	10	**30**	15	0	15	0	**30**	**85**	**40**
Patient 11	0	15	0	25	15	20	0	0	0	95	10	0
Patient 12	0	0	15	**40**	**45**	**75**	10	20	25	**45**	**35**	**65**
Patient 13	5	5	15	20	0	0	0	0	0	**90**	0	0
Patient 14	0	0	0	0	0	ND	20	10	0	**55**	15	ND
Patient 15	0	0	0	25	0	15	0	0	0	10	0	0
Patient 16	5	0	20	5	0	0	15	15	20	**35**	**30**	**30**
Patient 17	ND	ND	ND	**35**	ND	ND	30	ND	0	**495**	ND	ND
Patient 18	10	5	0	25	0	10	15	0	0	**45**	10	20
Patient 19	0	0	0	15	0	ND	30	20	45	**220**	**65**	ND
Patient 20	0	0	5	15	ND	ND	10	0	5	**60**	ND	ND

NOTE: In bold are average spots values twice above background (medium).
Abbreviation: ND, not done.
*Average values of duplicates spots per 10^6 PBMCs after subtracting responses to medium alone.

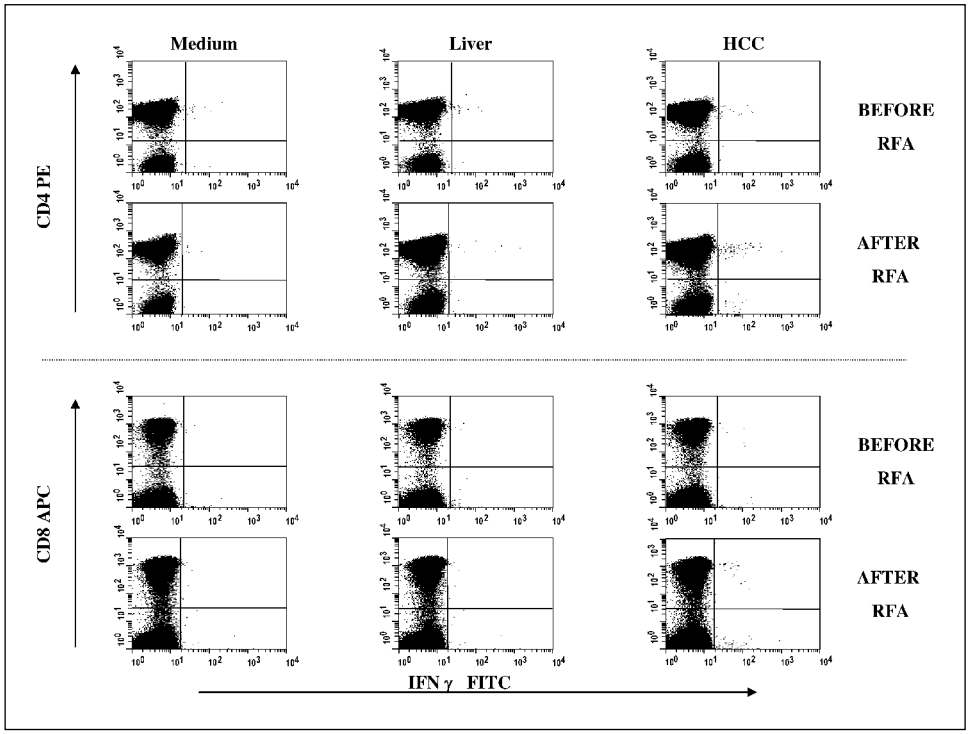

Figure 2. *Ex vivo* analysis of hepatocellular carcinoma–specific CD4 and CD8 cells from patient 20. The frequency of hepatocellular carcinoma–specific CD4 (*top*) and CD8-positive cells (*bottom*) was analyzed by intracellular cytokine staining for IFN-γ after PBMC stimulation in the presence of medium, autologous nontumor liver protein lysate, and autologous hepatocellular carcinoma protein lysate. Frequency of hepatocellular carcinoma–specific T cells after RFA treatment was 0.035% of total CD4-positive cells and 0.06% of total CD8 cells.

carcinoma nodule was not complete. Patient 4 showed early disease recurrence 1 month after treatment with the detection of a hepatocellular carcinoma nodule in a site different from that of the RF ablation. Patients were evaluated every 3 months thereafter for disease recurrence. At month 3, 4 of the 18 patients who where disease free at month 1 showed hepatocellular carcinoma recurrence, and four new patients presented hepatocellular carcinoma recurrence at month 6 at a different site from the treated hepatocellular carcinoma nodule (Table 1). At month 6, 50% (10 of 20) of the patients studied were disease free. Disease recurrence was at a different site from the treated nodules with the exception of patient 14, who, 3 months after ablation, still showed live tumor tissue at the site of RF treatment. Disease recurrence was uninodular in all cases with the exception of patients 4 and 16 presenting a multinodular hepatocellular carcinoma already 3 months after treatment (Table 1).

No protective effect of the antitumor T-cell response associated with RFA could be shown. In fact, T-cell responses directed to the autologous tumor antigens or the protein lysate of the most immunogenic hepatoma cell line PLC/PRF/5 measured before and after RF treatment were not significantly more expressed in the disease-free patients at 6 months of follow-up compared with the patients with hepatocellular carcinoma recurrence.

In patient 5, a new hepatocellular carcinoma lesion recurred 6 months after RFA. A fine-needle aspiration of the new nodule was available to stimulate in an ELISPOT assay PBMCs from four

Table 4. Analysis of circulating T and NK lymphocytes before and 1 month after RFA

	Before RFA	After RFA	*P*
T cells (% lymphocytes)	69.93 ± 6.68	68.94 ± 6.4	NS
$CD4^+$ cells (% T cells)	60.68 ± 6.5	60.5 ± 5.9	NS
Naive cells (% $CD4^+$ T cells)	32.4 ± 17.38	31.8 ± 15.6	NS
Memory cells (% $CD4^+$ T cells)	62.56 ± 17.1	62.7 ± 14.5	NS
$CD8^+$ cells (% T cells)	34.55 ± 5.76	34.82 ± 6.13	NS
Naive cells (% $CD8^+$ T cells)	10.85 ± 4.5	10.95 ± 5.1	NS
Memory cells (% $CD8^+$ T cells)	27.35 ± 10.1	21.61 ± 10.4	NS
$CD28^-$ (% $CD8^+$ T cells)	60.58 ± 12.0	64.69 ± 9.2	<0.05
$CD56^+$ cells (% T cells)	9.47 ± 4.16	10.99 ± 4.8	0.05
NK $CD3^-CD56^+$ (% lymphocytes)	11.12 ± 6.2	13.54 ± 6.8	<0.05
NK $CD3^-CD56^+CD16^+$ lymphocytes	9.47 ± 4.1	11.0 ± 4.8	<0.05

NOTE: Naive cells: $CD45RA^+$, $CD28^+$, and $CD95^-$. Memory cells: $CD45RA^-$, $CD28^+$, and $CD95^+$. Percentage of lymphocytes was evaluated in 11 randomly selected patients. Statistical analysis was done by two-tailed paired Student's *t* test.
Abbreviation: NS, not significant.

different time points: the day before RFA, month 1, month 3, and month 6 after treatment. In contrast to the vigorous response obtained, stimulating PBMCs with the first nodule-derived protein lysate, no T-cell response was generated stimulating PBMCs from the first two time points with the protein lysate of the new lesion, whereas a weak response was detected stimulating PBMCs derived at months 3 and 6 (Fig. 3).

Discussion

RFA destroys tumoral tissue generating a local necrosis followed by marked inflammatory response with a dense T-cell infiltrate (7), suggesting the activation of the adaptive T-cell response. Infectious agents can readily activate the innate immune response through interaction of conserved microbial products with receptors on dendritic cells (10). These pathogen-associated molecular patterns are key elements for a coordinated and efficient activation of the immune response. Although tumors can express neoantigens or can overexpress self-antigens, they lack non-self components of microbial origin, so that tumor cells, like their normal tissues counterpart, are prone to induce tolerance by default. Alternatively, immune responses can be also activated by danger signals of other origins. It is a matter of debate whether the danger signals generated by necrotic death of cancer cells can provide sufficient signals to trigger the induction of a tumor-specific T-cell response (11, 12). Among the activation signals released by necrotic cell death, the complement system, which is activated by necrosis and not by apoptosis, can activate dendritic cells at the site of the damaged tissue (13). Cellular stress up-regulates the expression of MHC class I–like molecules that activate NK, γδ, and CD8 αβ T cells committed to clear "stressed" cell elements (14). Moreover, heat shock proteins can be released *in vivo* by RFA for hepatocellular carcinoma and have been shown to enhance cell-mediated immune responses by activating the natural immunity and by augmenting hepatocellular carcinoma-specific cytotoxic T-cell response (15–17).

In the present study, a significant enhancement of the antitumor CD4 and CD8 T-cell response was detected after hepatocellular carcinoma RFA in patients with liver cirrhosis. Autologous hepatocellular carcinoma and a mixture of heterologous hepatocellular carcinoma protein lysates were used as the antigenic preparations to test the effect of thermal ablation on the tumor-specific T-cell response. This approach allowed the analysis of the T-cell response directed to the whole tumor-derived soluble antigen extract. To test if the tumor tissue could maintain its antigenic potential even after necrosis induced by thermal ablation, hepatocellular carcinoma protein lysates were obtained also at the end of treatment and were shown to successfully induce a tumor-specific T-cell response. T-cell responses were lower in terms of number of spots if compared with the ones generated with tumor tissue derived before ablation and responses were concordant in 7 of 10 responsive patients (Table 2). These inconsistencies could be explained by the use of tissue recovered at the end of treatment from the tip of the arrays of the needle that could have undergone a carbonizing effect, thus reducing its antigenic potential.

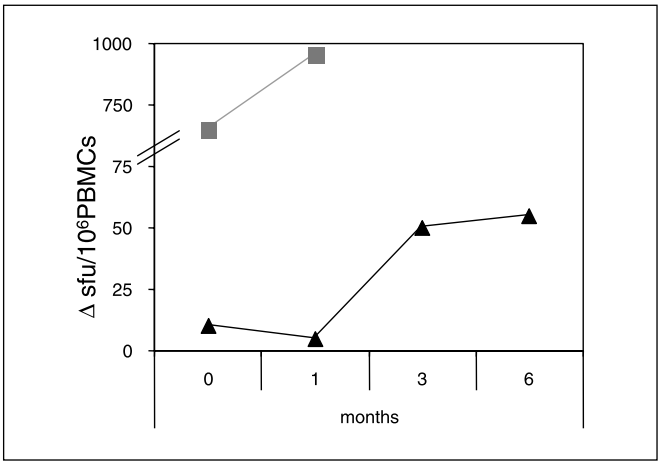

Figure 3. Hepatocellular carcinoma recurring after radiofrequency ablation is not recognized by T cells derived at the time of treatment. In patient 5, a new hepatocellular carcinoma lesion recurred 6 months after RFA. In contrast to the high immunogenicity of the protein lysate derived from the first hepatocellular carcinoma lesion, the protein lysate of the recurring hepatocellular carcinoma was not recognized by the PBMCs derived the day before RFA (time 0) and 1 month after RFA. This antigenic preparation could elicit a weak T-cell response 3 and 6 months after treatment. —■—, I protein lysate; —▲—, II protein lysate.

On one side, we found that RFA could induce/increase the recognition not only of autologous nonthermally ablated lysate but also of autologous thermally ablated tumor lysate. On the other side, in spite of cross-recognition between ablated and nonablated tissue, we found lack of response or a weak enhancement of response to autologous nontumor liver tissue. These data indicate that antigens released after RFA can be found and recognized also within untreated lesions of hepatocellular carcinoma and suggest that recognition may be limited to antigens present in the tumor but absent in the liver tissue.

The effect of thermal ablation on the enhancement of the tumor-specific T-cell response was confirmed by the results obtained with protein lysates of tumor cell lines. T-cell responses were only directed to hepatoma cell lines and not to the eritroleukemia (K562) and melanoma (Mel MZ2) cell lines (Table 3), suggesting a cross-reactive response with the autologous hepatocellular carcinoma tumor lysate, although an alloreactive T-cell response cannot completely be ruled out. Interestingly, PLC/PRF/5 was the most immunogenic cell line being recognized by 16 of the 20 patients after RFA. This new observation deserves future studies aimed at the identification of the immunogenic antigens expressed by this cell line. This cell line is known to secrete HBsAg, but no significant correlation between infection or previous exposure to HBV and PLC/PRF/5 T-cell reactivity was observed in our patient cohort.

As expected, the CD4 T-cell subset was shown to be the principal responsible for the antitumor response. However, phenotypic analysis of the whole lymphomononuclear cell population showed a concomitant loss of CD28 and acquisition of CD56 differentiation antigens by T cells along with an increase in frequency of NK lymphocytes, suggesting also a consistent activation of the cytotoxic arm (Table 4). On the whole, these data obtained a month after RFA, when transitory effects of the acute phase response are no longer detectable, indicate that various elements of the immune system with effector function are boosted by RFA.

The significant expansion of memory T-cell responses directed to recall antigens indicates that thermal ablation of liver tumors can not only induce a tumor-specific T-cell response but can also be responsible of a profound "adjuvant" effect. This could be explained by release of inflammatory mediators able to activate dendritic cells and T cells with different specificity. Anyhow, this effect of thermal ablation that was not reported in previous studies in animal models (7, 8) deserves to be studied in depth for its possible exploitation for an immunotherapeutic protocol.

Previous results obtained in rabbit (7) and mouse (8) animal models have shown that RFA can induce a tumor-specific proliferative T-cell response and even transplantable protective immunity. Our current data confirm that similar effects can be induced in patients with hepatocellular carcinoma and support the hypothesis that RFA can act as a strong "adjuvant" for active antitumor immune therapies.

Unfortunately, no correlation between enhancement of the antitumor T-cell response and lack of disease progression could be shown. Differently from the oligoclonal tumors studied in the animal models, however, human hepatocellular carcinoma is known to be more heterogeneous with different genetic profiles even among nodules of the same patient (18–20), making control of disease progression a more difficult task for the immune system in the natural human situation. Patient 5 presented a frequency of tumor specific T cells in the range of 1 of 1,000 circulating T cells before treatment, showing an increase of hepatocellular carcinoma-specific T cells after RFA (Fig. 3). In spite of this, hepatocellular carcinoma recurred within 6 months after ablation. Interestingly, the protein lysate of a fine-needle aspiration biopsy done on the relapsing hepatocellular carcinoma nodule did not elicit any IFN-γ secretion by the T cells derived before and 1 month after RFA. This protein lysate, however, could elicit a weak but significant T-cell response of PBMCs obtained 3 months before and the same day of the biopsy of the relapsing hepatocellular carcinoma nodule (Fig. 3). These data suggest that a mechanism of hepatocellular carcinoma immune escape or selection could have occurred in this patient, as it has been described for other cancers (21–23).

In conclusion, our results show for the first time in human cancer the immunostimulating effect induced by RFA. Although this immune response stimulated by this procedure alone may not be sufficient to prevent hepatocellular carcinoma relapse, the induced immune events could be exploited to design new immunotherapeutic strategies. Because RFA can be applied not only to hepatocellular carcinoma treatment but also to intrahepatic colangiocellular carcinoma, metastatic liver nodules and other extrahepatic solid malignancy, such as renal cell carcinoma, lung cancer, prostate cancer, and metastatic bone lesions, these novel immunotherapeutic approaches have the potential perspective of a wide range of applications to different types of malignancies.

Acknowledgments

Received 6/27/2005; revised 10/1/2005; accepted 11/2/2005.

Grant support: FIRB/Ministry of Education, University and Research grant RBNE013PMJ_006; EU grant LSHM-CT-2004-503359; and Fondazione Cassa di Risparmio di Parma, Italy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Eng J Med 1999;340:745–50.
2. Hassan MM, Frome A, Patt YZ, El-Serag HB. Rising prevalence of hepatitis C virus infection among patients recently diagnosed with hepatocellular carcinoma in the United States. J Clin Gastroenterol 2002;35:266–9.
3. Lam CM, Ng KK, Poon RT, Ai V, Yuen J, Fan ST. Impact of radiofrequency ablation on the management of patients with hepatocellular carcinoma in a specialized centre. Br J Surg 2004;91:334–8.
4. Le Veen RF. Laser hypertermia and radiofrequency ablation of hepatic lesions. Semin Intervent Radiol 1997; 14:313–24.
5. Lencioni RA, Allgaier HP, Cioni D, et al. Small hepatocellular carcinoma in cirrhosis: randomized comparison of radiofrequency thermal ablation versus percutaneous ethanol injection. Radiology 2003;228:235–40.
6. Hansler J, Neureiter D, Strobel D, et al. Cellular and vascular reactions in the liver to radio-frequency thermo-ablation with wet needle applicators. Study on juvenile domestic pigs. Eur Surg Res 2002;34:357–63.
7. Wissniowski TT, Hansler J, Neureiter D, et al. Activation of tumor-specific T lymphocytes by radiofrequency ablation of the VX2 hepatoma in rabbits. Cancer Res 2003;63:6496–500.
8. den Brok MH, Sutmuller RP, van der Voort R, et al. *In situ* tumor ablation creates an antigen source for the generation of antitumor immunity. Cancer Res 2004;64: 4024–9.
9. Fagnoni FF, Lozza L, Zibera C, et al. Cytotoxic chemotherapy preceding apheresis of peripheral blood progenitor cells can affect the early reconstitution phase of naive T cells after autologous transplantation. Bone Marrow Transplant 2003;31:31–8.
10. Geijtenbeek TB, van Vliet SJ, Engering A, 't Hart BA, van Kooyk Y. Self- and nonself-recognition by C-type lectins on dendritic cells. Annu Rev Immunol 2004;22: 33–54.
11. Bartholomae WC, Rininsland FH, Eisenberg JC, Boehm BO, Lehmann PV, Tary-Lehmann M. T cell immunity induced by live, necrotic, and apoptotic tumor cells. J Immunol 2004;173:1012–22.
12. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191: 423–34.
13. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 1996; 271:348.
14. Gleimer M, Parham P. Stress management: MHC class I and class I-like molecules as reporters of cellular stress. Immunity 2003;19:469–77.
15. Schueller G, Stift A, Friedl J, et al. Hyperthermia improves cellular immune response to human hepatocellular carcinoma subsequent to co-culture with tumor lysate pulsed dendritic cells. Int J Oncol 2003; 22:1397–402.
16. Schueller G, Kettenbach J, Sedivy R, et al. Heat shock protein expression induced by percutaneous radiofrequency ablation of hepatocellular carcinoma *in vivo*. Int J Oncol 2004;24:609–13.
17. Schueller G, Kettenbach J, Sedivy R, et al. Expression of heat shock proteins in human hepatocellular carcinoma after radiofrequency ablation in an animal model. Oncol Rep 2004;12:495–9.
18. Lee JS, Thorgeirsson SS. Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. Gastroenterology 2004;127:51–5.
19. Chen X, Cheung ST, So S, et al. Gene expression patterns in human liver cancers. Mol Biol Cell 2002;13: 1929–39.
20. Feitelson MA, Pan J, Lian Z. Early molecular and genetic determinants of primary liver malignancy. Surg Clin North Am 2004;84:339–54.
21. Bai XF, Liu J, Li O, Zheng P, Liu Y. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. J Clin Invest 2003;111:1487–96.
22. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;11:999–1005.
23. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991–8.