RFA strongly modulates the immune system and anti-tumor immune responses in metastatic liver patients

CHIARA NAPOLETANO[1*], FEDERICA TAURINO[1*], MAURO BIFFONI[2], ADRIANO DE MAJO[3], GIORGIO COSCARELLA[3], FILIPPO BELLATI[4], HASSAN RAHIMI[1], SIMONA PAUSELLI[1], ILENIA PELLICCIOTTA[1,7], JOY M. BURCHELL[5], LUCIO ACHILLE GASPARI[3], LUCIA ERCOLI[6], PIERO ROSSI[3] and AURELIA RUGHETTI[1]

[1]Department of Experimental Medicine, University of Rome 'Sapienza', Rome; [2]Medical Oncology Section, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome; [3]Department of Surgery, University of Rome 'Tor Vergata', Rome; [4]Department of Gynecology, Obstetrics and Perinatology, University of Rome 'Sapienza', Rome, Italy; [5]Breast Cancer Biology Group, King's College London School of Medicine, Guy's Hospital, London, UK; [6]Department of Public Health, University of Rome 'Tor Vergata', Rome, Italy

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Abstract. Radiofrequency tumor ablation (RFA) is a therapeutic modality for liver cancer patients inducing localized tumor necrosis with maximal preservation of normal liver parenchyma. We investigated the immunomodulatory effects exerted by RFA treatment in liver cancer patients with metastatic liver lesions (13 patients) or hepatocellular carcinoma (HCC) (4 patients). Analysis of lymphocyte subsets by flow cytometry revealed that after RFA, $CD3^+$ T cells, in particular $CD4^+$, were decreased in metastatic cancer patients, while no change was observed in HCC patients. Moreover, RFA induced trafficking of naïve and memory $CD62L^+$ T cells from circulation to tissues. When characterizing the function of T cells, proliferative response to PHA was strongly increased after 48 h from RFA in metastatic cancer patients. Furthermore, T cells produced IFN-γ in response to the tumor associated MUC1 antigen. In contrast, humoral immune responses against tumor antigens such as MUC1 and HCV proteins were unaffected by RFA treatment, although increase of circulating B cells was observed only in metastatic cancer patients. These results indicate that RFA application can exert an activating effect on the immune system in metastatic cancer patients, favouring trafficking of lymphocyte subsets and enhancing tumor antigen specific cellular immune responses.

Correspondence to: Dr Aurelia Rughetti, Department of Experimental Medicine, Viale Regina Elena 324, 00161 Rome, Italy
E-mail: aurelia.rughetti@uniroma1.it

Present address: [7]Department of Tumor Immunology, Catholic University of Rome, Rome, Italy

[*]Contributed equally

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Introduction

Hepatocellular carcinoma (HCC) and carcinoma metastases (in particular from colon carcinoma) are the most frequent liver malignancies worldwide. Surgical resection is regarded as the gold standard treatment, although only 10 to 20% of patients are candidates for resection because of extensive disease or medical co-morbidities. Recently, progress in imaging has allowed the development of interstitial therapies with *in situ* destruction of liver tumors and saving of normal tissue such as transcatheter arterial chemoembolization (TACE), interstitial ablative therapies, including percutaneous alcohol injection (PEI), cryotherapy, laser, microwaves and radiofrequency thermal ablation (RFA) (1,2).

In this scenario, RFA has received increasing interest, both for the possibility of treating patients unsuitable for surgical resection and for the low morbidity and mortality related to the ablation technique itself. Moreover, RFA is an interesting therapeutical option for liver cancer patients with limited liver reserve as a result of chronic disease (3). This technique is designed to induce tumor destruction by delivering a high frequency alternating current, through an active needle-electrode introduced into the neoplastic tissue itself. The needle-electrode can be positioned percutaneously (under US or TC guidance), by laparoscopy or laparotomy. Frictional heating is caused when the ions in the tissue attempt to follow the changing directions of the alternating current thus causing cell death by means of coagulative necrosis. Already at 43˚C in 30-60 sec apoptosis is seen, cellular death occurs in a few minutes at the temperature of 50˚C; in a few seconds at 55˚C and almost instantaneously at temperatures above 60˚C (4). Low morbidity and mortality rates, effective tumor ablation and maximal preservation of

Table I. Patient clinical characteristics.

Patient[a]	Gender	Age	Primary tumor	Tumor nodules	Nodule diameter[b]	Time of RFA treatment[c]
P1	F	67	Breast	1	1.8	24
P2	F	61	Breast	1	1.2	12
P3	F	74	Breast	3	2.8/1.2/2.1	26
P4	F	53	Breast	4	3/3/1.5/1.5	36
P5	M	70	Pancreas	3	4/2.5/1	30
P6	M	53	Pancreas	1	5	46
P7	M	70	Stomach	3	1.2/2/2.3	25
P8	M	77	Colon	1	6	46
P9	F	63	Colon	3	1.8/2/2	26
P10	M	78	Colon	1	5	24
P11	M	76	Colon	1	5	36
P12	F	63	Colon	2	2/3.6	46
P13	F	54	Breast	4	1.5/1/3/3	36
PH1	M	60	HCC (HCV^+/HBV^+)	2	2/3	16
PH2	M	74	HCC (HCV^+)	2	3/3	25
PH3	M	73	HCC (Alcohol)	2	2.5/2	21
PH4	M	73	HCC (HCV^+)	1	2	7

[a]P, Metastatic cancer patient; PH, HCC patient; [b]Diameters are expressed as cm; [c]Time in minutes of the all RFA applications.

normal liver parenchyma are the main advantages of RFA therapy for the management of liver lesions (5). In fact, it is considered an efficacious therapy as a bridge to orthotopic liver transplantation in HCC patients (6). Moreover, recent studies have suggested a survival benefit for patients with colorectal liver metastases undergoing RFA compared with historical controls undergoing chemotherapy alone and have shown that number and size of tumor nodules and serum marker levels can be considered predictors of survival after RFA treatment (7).

In animal models RFA has been shown to mimic a pathological stimulus inducing strong inflammatory signals: T cells are present at the site of the ablated lesions and release of the necrotic tumor tissue activates the immune system against the tumor (8,9). Recent reports have also shown that the *in vitro* proliferative response to tumor extracts is amplified in HCC patients following RFA (10,11), although RFA impacts on important parameters of biological tumor behaviour such as metastatic spreading and viral load are still unclear (12). So far, little is known about the effect of RFA treatment on the immune response in metastatic cancer patients.

We report that in metastatic cancer patients thermal ablation by radiofrequency induces a consistent modulation of circulating immune effector cells, conversely to that observed in HCC patients. However, in both group of patients, RFA treatment induced a redistribution of both naïve and memory $CD62L^+$ T cells. We studied the specific anti-tumor T cell response using as target antigen the MUC1 glycoprotein. MUC1 is a tumor-associated antigen, commonly overexpressed in breast, colon and pancreatic cancers. Cancer transformation undergoes aberrant *O*-glycosylation thus generating tumor-associated carbohydrate epitopes such as GalNAc-*O*-S/T (Tn) and Gal-GalNAc-*O*-S/T (T) (13) on the peptide core (tandem repeat, TR) of its extracellular domain. We report the first evidence that in metastatic cancer patients T cell response against the MUC1 antigen was increased. These results may contribute to better define the immunological aftermath induced by RFA and its possible relevance in designing combinatorial immuno-radio-surgical therapies for the treatment of liver cancer lesions.

Materials and methods

Patient characteristics. Seventeen patients with mean age of 67 (range 53-77 years) were enrolled in this study: 13 metastatic liver cancer patients and 4 hepatocellular carcinoma (HCC) patients (Table I). Among metastatic liver cancer patients, breast cancer was the primary tumor for 5 patients with a total of 13 liver metastasis, colorectal carcinoma for other 5 with 8 liver metastasis, pancreatic carcinoma for 2 patients with 4 metastasis, gastric cancer for 1 patient with 3 metastases. Mean diameter of the 28 metastases was 3.2 cm (range: 1.2-6 cm). HCC patients were all cirrhotic, as a consequence of HCV(n=2) and HCV/HBV (n=1) infection and of alcohol abuse (n=1) respectively and had a total of 7 HCC nodules. Mean diameter of the 7 HCC nodules was 2.5 cm (range: 2-3 cm).

RFA treatment. All patients signed informed consent for the treatment. RFA procedures were performed percutaneously except in one (*P11*), 6 under general anaesthesia and 11

Table II. Summary of the circulating immune cell populations assesed in the study by flow cytometry.

Target population	Predominant reactivity
$CD3^-/CD19^+$	B cells
$CD3^+$	T cells
$CD3^+/CD4^+$	T cell subset
$CD3^+/CD4^+/CD45RA^+$	Naïve $CD4^+$ cells
$CD3^+/CD4^+/CD45RA^-$	Memory $CD4^+$ cells
$CD3^+/CD8^+$	T cell subset
$CD3^+/CD4^-/CD45RA^+$	Naïve $CD8^+$ cells
$CD3^+/CD4^-/CD45RA^-$	Memory $CD8^+$ cells
$CD3^-/CD16^+/CD56^+$	NK cells

under conscious sedation with Midazolam and Fentanyl. Local anaesthesia was achieved by using 2% lidocaine. All procedures were performed under ultrasound (US) guidance using Hitachi Spazio EUB-404 with B-314 probe for single needle or Ca-11 probe for cluster needle and AL-33S probe for RFA during laparotomy. The procedures were performed using the RF Generator Cool-Tip System (Series 3; Radionics, MA). The single RF electrode was 17 gauge, with internal dual channels for chilled water. The clustered electrode comprised three needles spaced 0.5 cm apart. A peristaltic pump (Watson-Marlow, Wilmington, MA) was used to deliver 0˚C saline through the electrodes at 10-25 ml/min to maintain a tip temperature of 20-25˚C. In 3 patients (1 breast cancer and 2 colorectal cancer with 2 and 1 metastatic nodules, respectively) treatment was repeated in order to complete necrosis.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (1.077 g/ml, Pharmacia, LKB, Sweden) density gradient centrifugation from heparinized blood obtained at different time points before (day 0) and after RFA (day 2 and 7) and frozen immediately. The phenotype of circulating PBMCs was defined using the Multistaining Kits (Becton-Dickinson, San, Diego, CA). Samples were analyzed using a FACScalibur (Becton-Dickinson) and analysis performed employing the CellQuest software (BD Bioscience). Specific lymphocyte populations were gated by SSC and FSC and expression of specific markers (Table II) and results plotted as a percentage of positive cells.

Evaluation of IFN-γ production to MUC1 tumor-associated antigen by ELISPOT assay. MUC1 glycopeptides carrying *O*-linked GalNAc or Gal-GalNAc (Tn and T carbohydrate epitopes, respectively) were chemically synthesized (14) and kindly provided by Professor H. Paulsen (Hamburg University, Germany) (Table III). PBMCs were grown in RPMI-1640 (Hyclone) with AB Human Serum (HS; Sigma) and IL-2 (20 UI/ml; R&D System, Germany) and MUC1 glycopeptide (25 μg/ml). As control antigens, Tetanus Toxoid (TT) antigen and *C. albicans* extracts (kindly

Table III. Tumor-associated MUC1 based glycopeptides.

MUC1 naked peptide	AHG	A	H	G	V	T	S	A	P	D	T	R	P	A	P	G	S	T	A	P	P
	A4	A	H	G	V	T	S | Gal-GalNAc	A	P	D	T	R	P	A	P	G	S	T	A	P	P
Tumor associated MUC1 glycopeptides	A4GN	A	H	G	V	T	S | GalNAc	A	P	D	T	R	P	A	P	G	S	T	A	P	P
	A4M1	A	H	G	V	T	S | Gal-GalNAc	A	P	**E**	**S**	R	P	A	P	G	S	T	A	P	P
	A4M3	A	H	G	V	T	S | Gal-GalNAc	A	P	**E**	**S**	R	P	A	P	G	S	T	A	P	**A**

MUC1 glycopeptides based on the tandem repeat sequence of MUC1 carrying the tumor associated *O*-linked carbohydrate epitopes GalNAc (Tn antigen) or Gal-GalNAc (T antigen). Amino acid substitutions corresponding to alternative sequence of MUC1 TR are indicated in bold.

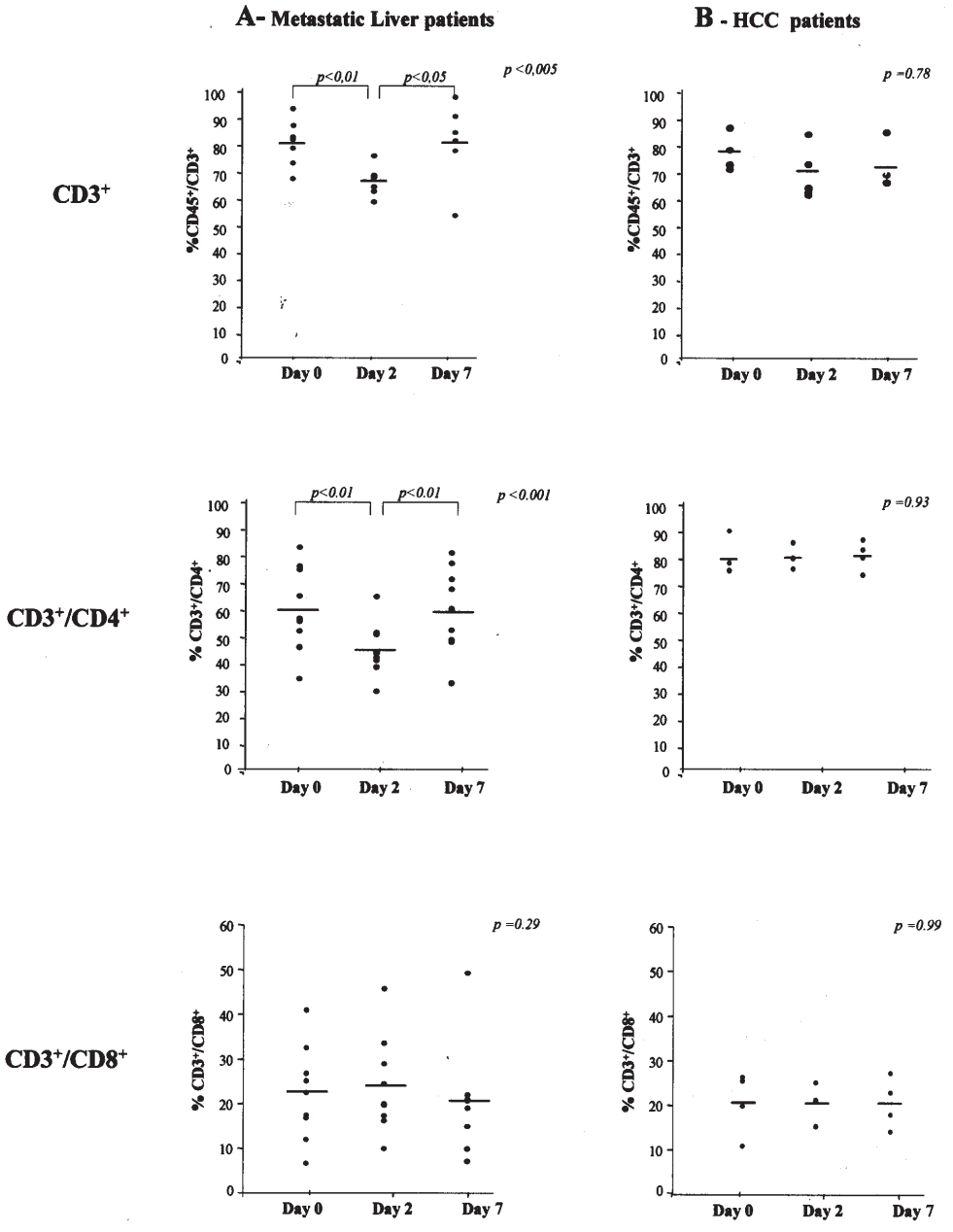

Figure 1. Scatterplots comparing the proportions of $CD3^+$ lymphocyte populations just before RFA treatment (day 0), and after 2 and 7 days from RFA in liver metastatic patients (A) and HCC patients (B). Values express percentage of blood circulating $CD3^+$ T cells, $CD3^+/CD4^+$ and $CD3^+/CD8^+$ lymphocytes in first, second and third row, respectively. Significant statistical differences were found in the distribution of $CD3^+$ T cells following RFA in metastatic liver patients ($p<0.005$) by two-tail ANOVA test and in particular statistic significance was found between day 0 vs. day 2 groups and therein day 2 vs. day 7 as assayed by Bonferroni test. Similar effects were observed in the $CD3^+/CD4^+$ subset in metastatic cancer patients (day 0 vs. day2, $p<0.01$; day 2 vs. day 7, $p<0.01$). No significant changes were observed in the HCC patients (B).

provided by Dr Toro Santucci, IIS, Rome, Italy) were used at 10 μg/ml. After a week of culture, T cells were boosted with peptide pulsed autologous irradiated PBMCs (1:1) and IFN-γ response to specific antigens was evaluated by ELISPOT. Plates (Millipore, Bedford, MA) were coated with 100 μl of MoAb anti-human IFN-γ (10 μg/ml; clone NIB42, Pharmingen, San Diego, CA), (overnight at 4˚C). After blocking with PBS-1% bovine serum albumin (BSA) (Sigma) (200 μl/well), PBMCs were plated in quadruplicate ($5x10^4$ cells/well) for 24 h. Cells were removed and plates washed and IFN-γ production was detected with biotinylated anti-IFN-γ mAb (clone 4S.B3, Pharmingen) (2 h RT, 5 μg/ml, 50 μl/well), revealed with streptavidin-alkaline phosphatase (Pharmingen) (2 h; 1:2,000; 50 μl/well) and chromogen substrate (50 μl/well). Spots were counted using the Immuno-Spot Image Analyzer.

Proliferative response to PHA. PBMCs, isolated at day 0, day 2 and day 7 from RFA were seeded at $5x10^5$ cells/ml (200 μl/well) in a 96-well round bottom plate (Corning Incorporated, NY) with phytohemoagglutinin (PHA; Sigma) 5 μg/ml in RPMI-5% HS. After 4 days, proliferative response was analysed by [^{3}H]-thymidine incorporation (ICN, Costa Mesa, CA) (1 μCi/well). The Proliferation Index (P.I.) was

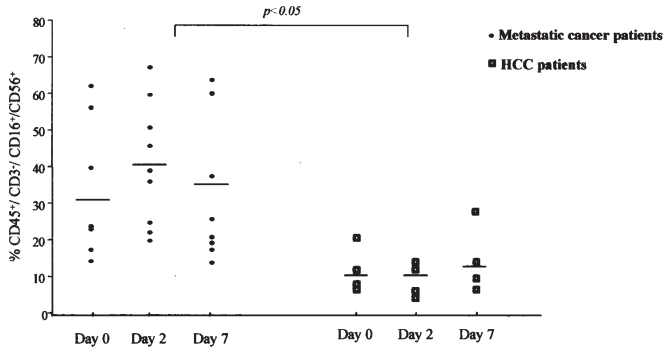

Figure 2. Scatterplots comparing circulating NK cells ($CD45^+/CD3^-/CD56^+/CD16^+$) during RFA treatment (day 0, 2 and 7) in liver metastatic patients (closed circle) and HCC patients (open square). The percentage of circulating NK cells was higher in metastatic patients than HCC during the study period ($p<0.05$). Following RFA application an increase of circulating NK cells was observed in metastatic liver patients, while no change was observed in HCC patients.

calculated by dividing the average cpm obtained for PHA stimulated PBMCs cell samples by the average cpm obtained for PBMCs alone.

Detection of anti-MUC1 and anti-HCV circulating human antibodies. Serum samples were collected at day 0, 2 and 7 from tumor ablation and stored frozen at -80˚C until use. For detection of MUC1 antibodies EIA/RIA plates (Corning Incorporated) were precoated overnight with streptavidin (Sigma) (1 μg/ml), then blocked with PBS-5% BSA. Biotinylated MUC1 60 mer (1 μg/ml) was added for 2 h; 100 μl patient sera (1:100) were added to the wells in triplicate. Peroxidase-conjugated α-human IgG (Dako, Denmark) (1:1000) and OPD-substrate (Sigma) were used to reveal reaction; absorbance was measured at 492 nm. Anti-HCV Igs levels were detected in HCC patients sera at the same time-point as above by chemiluminescent microparticle immunoassay (Architect kit, Abbot Diagnostic, Germany) following the manifacturer's instructions (15). Results are expressed as a ratio between the Relative Light Units (RLU) of the sample and the RLU of the standard control. Values above 1 were considered positive.

Statistical analysis. Descriptive statistics (average and standard deviation) were used to describe the groups of data. Repeated measures ANOVA with post-test for matched pairs was used to test for overall differences among the groups, followed by Bonferroni's significant difference adjustment for multiple comparisons.

Results

Modulation of circulating $CD3^+$ T cell subsets and NK cells in liver cancer patients following RFA. Effects of thermal ablation by radiofrequency on T cells were characterized, studying circulating CD3 T cell subsets ($CD4^+$ and $CD8^+$) in liver metastatic cancer patients before (day 0) and after RFA application (day 2 and day 7) (Fig. 1A). Also effects of such treatment on circulating lymphocyte subsets were monitored in the group of HCC patients (Fig. 1B).

At day 0, levels of circulating $CD3^+$ T cells were similar in both groups of patients. Following RFA, a significant modulation of $CD3^+$ T cells was observed in patients with liver metastasis ($p<0.005$). In particular, circulating $CD3^+$ T cells significantly decreased 2 days following RFA treatment ($p<0.01$) and original baseline values were recovered only at day 7 ($p<0.05$) (Fig. 1A), whereas in HCC patients $CD3^+$ cell count remained unmodified ($p=0.78$) (Fig. 1B). The reduction observed in $CD3^+$ T cells in metastatic patients was mainly due to a modification of the $CD4^+$ T cell subset ($p<0.001$). A statistically significant decrease was observed at day 2 ($p<0.01$) vs. day 0 and vs. day 7 in $CD3^+/CD4^+$ cells. Moreover, a consistent but not significant decrease was observed at day 7 in the $CD3^+/CD8^+$ cells ($p=0.29$). In HCC patients, both $CD4^+$ and $CD8^+$ counts remained unaffected by RFA treatment (Fig. 1B).

The effects of RFA on the natural immune effector cells were also studied (Fig. 2). In metastatic cancer patients, NK cells showed a weak increase, although not significant, while in HCC patients this was not observed. Moreover, the percentage of circulating NK cells was statistically higher in metastatic as compared to HCC patients at each time point ($p<0.05$) (Fig. 2). Thus, RFA treatment seemed to strongly modulate the T cell subsets in patients with metastatic liver lesions.

RFA modulates $CD62L^+$ T cells in liver cancer patients. Since RFA modulates circulating CD4 and CD8 T cells, the redistribution of these T cell subsets was analyzed on the basis of naïve ($CD45RA^+$) and memory ($CD45RA^-$) phenotype and on the basis of CD62L expression, a marker associated with the migratory phenotype of T cells (16) (Fig. 3). $CD62L^+$ T cells (CD4 and CD8) of both naïve and memory subsets showed a gradual decline following RFA treatment in the two groups of patients, with statistical significance for naïve T cells in the metastatic cancer patient group (CD4: $p<0.05$; CD8: $p<0.05$) (Fig.3A). In the memory T cell compartment, a general decline of T cell count was detected, however, this was significant only for the $CD62L^+CD4^+$ cell subset ($p<0.01$).

When analyzing the $CD62L^-$ T cells (Fig. 3B), naïve T cells were significantly increased in metastatic cancer patients after 7 days (CD4, $p<0.01$; CD8, $p<0.05$), while no change was observed in HCC patients. In both groups of patients, $CD62L^-$ memory T cells did not appear to be modulated by RFA.

RFA treatment modulates proliferative T cell response and enhances anti-MUC1 cellular response in metastatic cancer patients. The effects exerted by RFA on the general performace of the immune system of metastatic cancer patients were investigated by evaluating PBMC proliferation to strong aspecific stimuli such as PHA before (day 0) and after (day 2 and 7) tumor ablation (Fig. 4A). A strong increase in T cell response to PHA was observed at day 2 following RFA, while at day 7 proliferation returned to the baseline values. The proliferation pattern was consistent in all the patients tested, despite the heterogeneity of

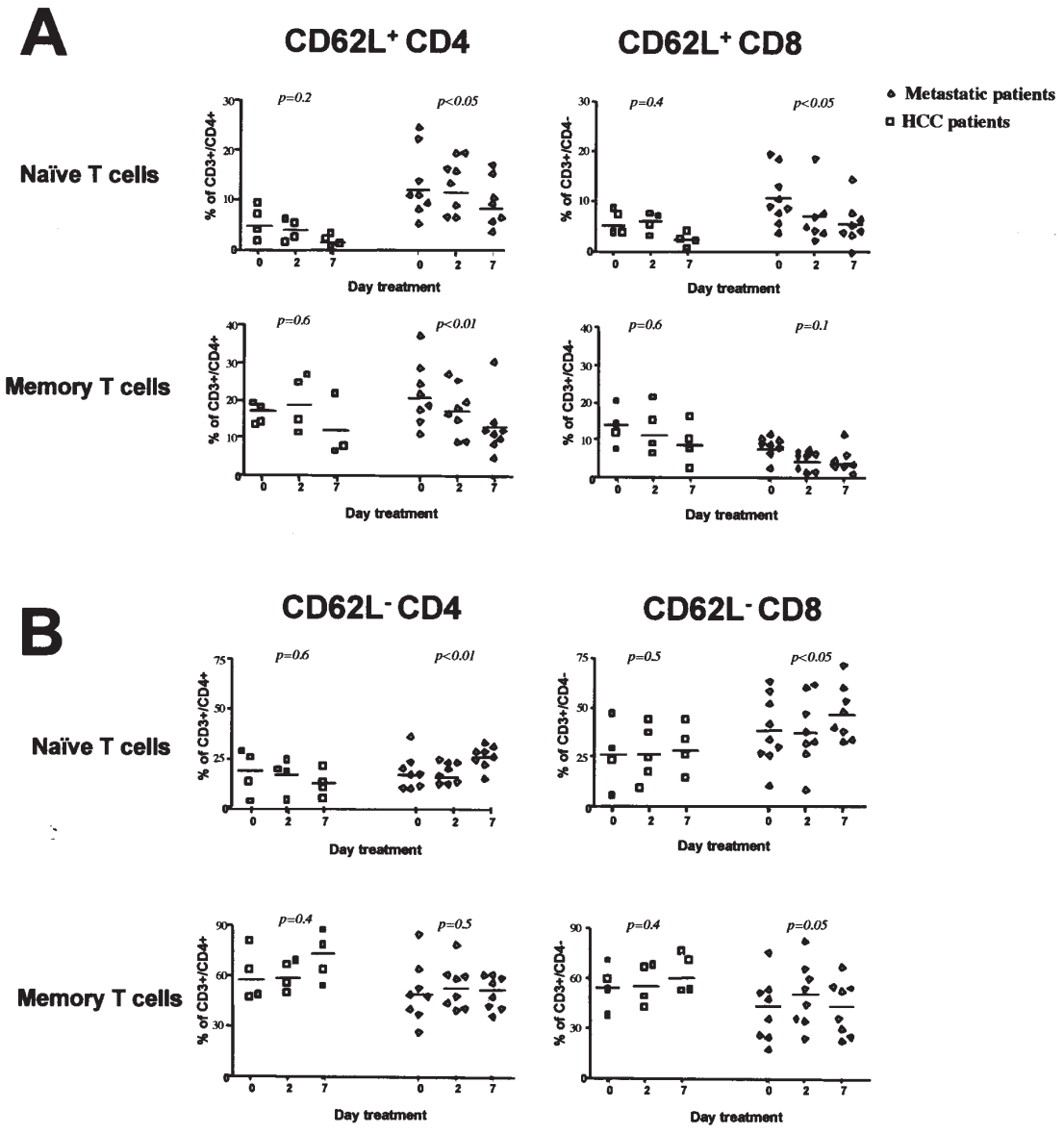

Figure 3. Effects of RFA on CD62L T cells of both naïve and memory T cell subsets. Cytofluorimetric analysis was performed on circulating lymphocytes following RFA treatment (day 0, day 2 and day 7) in HCC patients (open squares) and in metastatic liver patients (open triangles). T cells were simultaneously characterised for CD62L, CD4 and CD45RA expression. (A) Shows the circulating levels of $CD62L^+$ $CD4^+$ and $CD8^+$ T cells of both naïve ($CD45RA^+$) and memory ($CD45RA^-$) subsets, while in (B) results regarding $CD62L^-$ T cells are reported. Statistical significance in redistribution of T cell subsets following RFA was evaluated by ANOVA test.

proliferative responses observed at day 0 (ranging from 15 up to 97 P.I.).

To study whether the stimulatory effect observed on the aspecific immune response could imply any influence on the anti-tumor one, the cellular response to the MUC1 tumor epithelial antigen and in particular to tumor-associated glycoepitopes of MUC1, was evaluated as IFN-γ production in ELISPOT assay. PBMCs before and 7 day after RFA were stimulated using a panel of MUC1 tumor-associated glycopeptides (20 μg/ml, 20 mer) (Table III). The employed glycopeptides mimicked the glycopeptide structures found on the tumor-associated MUC1 molecules: A4, A4M1 and A4M2 glycopeptides carried the T carbohydrate residue (Gal-GalNAc), while the A4GN glycopeptide carried the Tn glycoepitope (GalNAc). Moreover, the A4M1 and A4M3 showed an alternative MUC1 peptide backbone as found *in vivo* (14). The AHG peptide corresponded to the unglycosylated MUC1 sequence. One week from priming, T cells were boosted with the specific antigen and IFN-γ secretion was evaluated by ELISPOT assay (Fig. 4B). Following RFA treatment, IFN-γ production to MUC1 glycopeptides markedly increased. A response to the unglycosylated MUC1 sequence (AHG peptide) was also observed. The IFN-γ response appeared to be independent upon the specific amino acid substitution occurring in the TR sequences *in vivo* (A4M1 and A4M3 vs. A4 glycopeptides). IFN-γ response to Tetanus Toxoid and *C. albicans* antigens did not significantly increase.

RFA and B-cell mediated response. B cell-mediated response is a key point in the memory immune response following inflammation and can be an important parameter in tumor

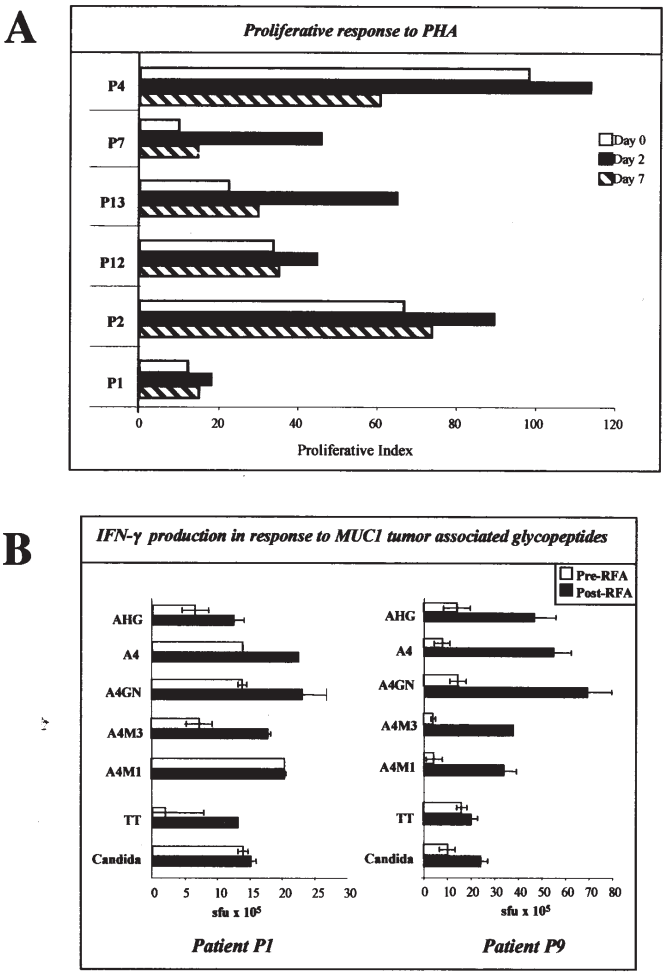

Figure 4. Immunostimulatory effect of RFA on anti-tumor immune response. (A) Proliferative assay of circulating PBLs before and after RFA treatment (day 0, open histogram; day 2, black histogram; day 7 striped histogram) following PHA stimulation for 72 h in metastatic liver patients (breast, P1, P2, P4, P13; stomach, P7; colon, P12). Proliferative response was evaluated as [^{3}H]-thymidine incorporation and results plotted as Proliferative Index (P.I). (B) Elispot assay for evaluating the production of IFN-γ after stimulation with specific tumor-associated MUC1 glycopeptides before and after RFA in metastatic liver patients P1 (breast) and P9 (colon). MUC1 based antigens were AHG, 20 mer corresponding to the unglycosylated TR, Gal-GalNAc (A4) and GalNAc (A4GN) carrying glycopeptides. A4M3 and A4M1 are MUC1 Gal-GalNAc carrying glycopeptides corresponding to peptide TR variants. As control antigens, TT and *C. albicans* extracts were used. PBLs were stimulated with the specific antigen and after seven days, 10^5 cells/well were plated and stimulated overnight in millipore plates coated with anti-IFN-γ antibody in triplicate for each condition. Results were evaluated as IFN-γ spot forming unit (sfu) by ELISPOT analyser and plotted as histograms (white and black histograms for before and after RFA treatment, respectively).

immunity. In metastatic cancer patients, RFA induced an increase of the circulating B cell subset ($p<0.05$), particularly in the 48 h following RFA ($p<0.05$) (Fig. 5A). Conversely, no B cell increment was observed in HCC patients, although baseline B cells were similar in both groups of patients (Fig. 5B). The B cell response was also analyzed as means of circulating IgGs specific for MUC1 tumor antigen in metastatic patients as well as anti-HCV IgGs in HCC patients (Table IV). In both groups of patients, anti-MUC1 or anti-HCV IgG titers were not significantly modified by RFA treatment.

Discussion

Different loco-regional therapies have been developed for unresectable liver tumors aimed at achieving local tumor control and prolonging patient survival. RFA and other ablative treatments have an increasing application for liver cancers showing high benefit for clinical management. In particular, RFA has a definitive role for HCC treatment both as curative intent and as a bridge to liver transplantation; moreover, it is the most widely used tumor ablative technique for treatment of colorectal liver metastasis (17-19). RFA destroys tumoral tissues by necrotic and apoptotic mechanisms (4) and these pathological events may influence the overall and anti-tumor host immune responses (20). Results reported herein show that indeed the RFA application may enhance the immune system reactivity in liver cancer patients, in particular in those with metastatic lesions.

Several reports suggest that in animal models, RFA treatment could provide activatory signals and become a source of tumor antigens for the immune system (8,9,21). In humans, RFA treatment can exert modulatory effects on the immune system (22) and could boost lymphocyte proliferation and production of inflammatory cytokines in response to tumor extracts in HCC patients (10,11). Herein, we show for the first time that in metastatic cancer patients, RFA treatment can amplify the specific T cell response against an epithelial tumor antigen such as the MUC1 *O*-glycoprotein. MUC1 tumor antigen is target of humoral and cellular immune response in cancer patients and is considered an optimal target for cancer immunotherapy (23). In the change to malignancy, MUC1 is overexpressed and aberrantly glycosylated: novel cancer-associated carbohydrate moieties (such as T and Tn) exist on the extracellular domain thus generating MUC1 glycoepitopes that are targets of a tumor specific immune response both humoral and cellular (24-27). RFA treatment amplifies T cell mediated IFN-γ response towards these tumor-associated glycoepitopes, while the specific immune response to recall antigens is not significantly increased. The enhancement of the anti-tumor immune response could be a bystander effect induced by the overall inflammatory response generated by the tumor ablation procedure. However, the MUC1 specific immune reaction is still sustained after a week from RFA, while the proliferative response to PHA, regarded as a general indicator of immune activation, was augmented after 48 h and declined to baseline values after a week from RFA. These results suggest that in metastatic cancer patients, thermal ablation of the tumor mass may induce an antigen specific immune response that could be fostered independently by the overall inflammatory response.

No previous report exists on the influence of RFA on the humoral anti-tumor immune response. Other ablative tumor treatments have been shown to induce an anti-tumor IgM response (28). We characterized the humoral immune response monitoring the B cell pattern during RFA and the specific IgGs titers against the MUC1 tumor antigen and HCV capsidic proteins in metastatic and HCC patients,

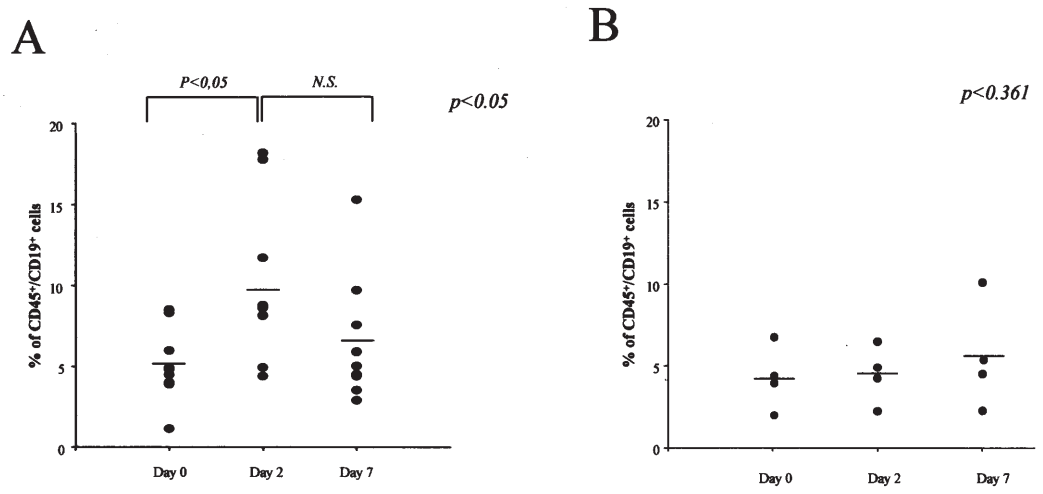

Figure 5. Effects of RFA treatment on circulating B cells. Circulating B cells ($CD45^+/CD3^-/CD19^+$) were analysed by flow cytometry at different time points of the RFA protocol in metastatic liver patients (A) and in HCC patients (B). Only in metastatic cancer patients, RFA treatment significantly modulates B cell counts as evaluated by ANOVA test ($p<0.05$), as showed by increase of B cell level at day 2 ($p<0.05$).

Table IV. Anti-tumor humoral response in liver cancer patients undergoing tumor ablation.

Metastatic patients	Primary tumor	Anti-MUC1 circulating IgGs[a]	
		Pre-RFA	Post-RFA
P1	Breast	0.69	0.80
P2	Breast	0.96	1.31
P3	Breast	0.53	0.50
P5	Pancreas	0.42	0.40
P6	Pancreas	0.54	0.61
P13	Breast	1.06	0.93
P8	Colon	0.67	0.75
P9	Colon	0.43	0.67
P10	Colon	0.48	0.76
P12	Colon	0.48	0.56
HCC patients	**Primary tumor**	**Anti-HCV circulating Igs[b]**	
		Pre-RFA	Post-RFA
PH1	HCC (HBV^+/HCV^+)	12.53	13.04
PH2	HCC (HCV^+)	15.68	15.14
PH3	HCC (HCV^-)	0.98	0.94
PH4	HCC (HCV^+)	16.06	15.94

[a]O.D. values read at 492 nm wavelength; [b]Arbitrary unit of Architect system anti-HCV test, cut-off values <1.

respectively. We found an increment of circulating B cells following RFA in metastatic cancer patients, while no significant increase of anti-MUC1 or anti-HCV (in HCC patients) IgG titers was detected. Several reasons could account for this discrepancy between the B cell modulation and IgG titers e.g. release of antigens from the necrotic tumor extracts could consume circulating antibodies or the antibody determination could be too close to the stimulus. Moreover, the immune system of cancer patients undergoing RFA is already impaired by the establisment of immunosuppressive networks that contribute to the progression of liver tumor and to the metastatic process (29). Removal of this immunosuppressive network is needed to potentiate an active anti-tumor immune response (30). In mouse models, RFA

treatment can induce a protective immune response towards an exogenous antigen such ovalbumin only when combined with the removal of Treg through the CTLA-4 blockade (31). In humans, RFA has been shown to be more effective than chemotherapy for the clinical treatment of metastatic cancer patients with minimal liver lesions (32). On the other hand, RFA treatment does not show any advantage in long-term survival compared to surgery in metastatic cancer patients (33).

The *in vivo* evidence suggests that the inflammatory *noxa* engendered by thermal ablation (both RFA and cryo-ablation) appears to be a robust stimulus for the enhancement of a specific immune response, releasing tumor antigens and supplying strong 'danger signals' such as HSPs and release of inflammatory cytokines (21), although not sufficient to overcome tumor immunosuppression.

A key point for the activation and maintenance of an efficacious and long-lasting immune response is the trafficking of immune cells between tumor site, lymphoid organs and systemic circulation. Herein, we describe that RFA exerted immunomodulatory effects on distinct effector immune cells in metastatic cancer patients, in particular in the redistribution of circulating T cells; similar event was observed in pancreatic cancer patients undergoing RFA (data not shown). No modulation effect seems to occur in HCC patients: the chronic condition underlying tumor development could account for an overall weakening of NK and T cell-mediated immunity (34,35). However, it is noteworthy that in all the liver cancer patients studied a significant and stable decrease of circulating $CD62L^+$ T cells of both memory and naïve subsets was observed suggesting the migration of these cells into the tissues. Any strategy for the activation of an efficacious immune response is aimed at enhancing the anti-tumor immunity and to prime and expand the repertoire of *naïve* anti-tumor immune cells that become activated only when the tumor antigen is presented in secondary lymphoid organs over a sufficiently long period of time (36). Transient, but sufficient amount of antigens, antigen presenting cell (APCs) activation, adequate cytokine environment may dictate the delicate balance needed for an efficacious immune response (37,38). Indeed, tumor ablation by radiofrequency provides these conditions *in vivo*. The necrotic/apoptotic events induced by RFA can supply an adequate inflammatory environment to activate APCs and generate a massive and transient release of tumor antigens that could be presented by the activated APC to T cells recruited from the circulation. On the other hand, massive antigen release could overload the immune system thus producing exhaustion of responder T cells. The inflammatory environment could also switch the balance from activation of anti-tumor to regulatory immune cells (30). Clinical parameters, such the number of treated lesions, the total tumor mass, the efficacy of necrosis induced may have great relevance in stirring the balance between activation and suppression of the anti-tumor immune response. Moreover, the baseline immune performance of patients may well be another restriction point for the activation of productive immunity.

In conclusion, we showed that RFA can activate a specific immune response to the MUC1 tumor antigen in metastatic liver patients and analysis of the immune cell repertoire suggests that RFA may activate different immunological responses, implying an important clinical impact for the use of this ablative technique. The bystander biological effects produced by RFA on the immune system (strong activation signal, recruitment of immune cells, release of tumor antigens) and the applicability of this procedure for treatment of solid tumors of different origins (39), make tumor ablation an appealing procedure when designing combined therapeutical approaches for the treatment of cancer disease.

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