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Enhancement of tumor-associated antigen-specific T cell responses by radiofrequency ablation of hepatocellular carcinoma

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Abbreviations: TAA, tumor-associated antigen; HLA, human leukocyte antigen; IFN, interferon; PBMC, peripheral blood mononuclear cell; HCV, hepatitis C virus; ELISPOT, enzyme-linked immunospot; MDSC, myeloid-derived suppressor cell

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ABSTRACT

Radiofrequency ablation (RFA) is one of the treatments for hepatocellular carcinoma (HCC) and known to enhance host immune response. However, the epitopes, to which enhanced immune responses occur, the impact for the patient's prognosis and the functions and phenotype of T cells induced are still unclear. To address these issues, we analyzed immune responses before and after RFA in 69 HCC patients using 11 tumor-associated antigen (TAA)-derived peptides that we previously identified to be appropriate to analyze HCC-specific immune responses. The immune responses were analyzed by enzyme-linked immunospot (ELISPOT) and tetramer assays using peripheral blood mononuclear cells.

An increase in the number of TAA-specific T cells detected by IFN-γ ELISPOT assays occurred in 62.3% of patients after RFA. The antigens and their epitope to which enhanced T cell responses occur were diverse, and some of them were newly induced. The number of TAA-specific T cells after RFA was associated with the prevention of HCC recurrence and it was clarified to be predictive of HCC recurrence after RFA by univariate and multivariate analyses.

The number of TAA-specific T cells after RFA was inversely correlated with the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs. The modification of T cell phenotype was observed after RFA. The number of TAA-specific T cells at 24 weeks after RFA was decreased.

Conclusion: Although RFA can enhance various TAA-specific T cell responses and the T cells induced contribute to the HCC recurrence-free survival of patients, besides immunosuppression by MDSCs, the memory phenotype and lifetime of TAA-specific T cells are not sufficient to prevent HCC recurrence completely. Additional treatments by vaccine or immune-modulatory drugs might be useful to improve the immunological effect of RFA.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th most frequent type of cancer in the world and becoming an important public health concern because the incidence of HCC has been increasing in Western and Asian countries (1, 2). Although many kinds of treatments have been performed for HCC, the recurrence rate of HCC after these treatments is very high (3). To inhibit HCC recurrence and improve the prognosis of patients, an immunotherapeutic approach is considered to be an attractive strategy.

Radiofrequency ablation (RFA) is one of the treatments for HCC and it is now widely used for curative strategies (4). In recent studies, it has been reported that RFA creates a tumor antigen source for the generation of antitumor immunity and enhances host immune responses (5). Our previous mouse study also showed that RFA induced anti-tumor immune responses with massive T cell infiltration into a tumor and the effect was enhanced by an active variant of CC chemokine ligand 3 (6). These studies suggest that additional immunological approaches to RFA have the possibility to reduce HCC recurrence after the treatment. However, in human studies, the following important data needed to develop a new immunotherapeutic approach have been lacking. First, the

types of TAAs and their epitopes, to which these enhanced immune responses occur, have not been fully identified. Second, the proportion of patients with enhanced antitumor immune responses and the effect of antitumor immunity for the patient's prognosis after RFA are still unclear. Third, the factors to affect TAA-specific immune responses, the functions and phenotype of T cells induced by RFA have not been identified.

In the present study, to address these issues, we analyzed immune responses in peripheral blood mononuclear cells (PBMCs) before and after RFA in 69 HCC patients using 11 tumor-associated antigen (TAA)-derived peptides that we previously identified to be appropriate to analyze HCC-specific immune responses. This approach offers useful information to develop a new strategy for HCC immunotherapy and improve the prognosis of patients treated by RFA.

MATERIALS AND METHODS

Patients and laboratory testing

In this study, we examined 69 HLA-A24-positive HCC patients with RFA. The diagnosis of HCC was histologically confirmed in 11 patients. For the remaining 58 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 (7).

RFA was performed with a cool-tip RFA system consisting of an 18-gauge, cooled-tip electrode with a 2- or 3-cm exposed tip (Radionics, Burlington, MA) and radiofrequency generator (CC-1 Cosman Coagulator, Radionics). After local anesthesia, the electrode was inserted through a guide needle under ultrasound guidance. Radiofrequency energy was delivered for 6 to 12 min for each session. The energy was increased from 40 watts to 120 watts in a stepwise fashion. During ablation, the electrode was cooled by circulating ice-cooled saline in the electrode lumen to maintain the tip temperature below 20°C. During each treatment, the electrode tip was inserted into the tumor 1-3 times until the target tumor was surrounded by a high-echoic area.

Complete necrosis after RFA was confirmed by dynamic CT or MRI. RFA was repeated in some cases until complete necrosis was confirmed. Thirty-nine and 30 patients received RFA 1 and 2-4 times, respectively. After treatments, HCC recurrence was evaluated with dynamic CT or MRI every 3-4 months.

All patients gave written informed consent to participate in the study in accordance with the Helsinki declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University).

Blood samples were tested for HbsAg and HCVAb using commercial immunoassays (Fuji Rebio, Tokyo, Japan). The patients with HCVAb were tested for serum HCV RNA by real-time PCR (Roche, Tokyo, Japan) and 49 of 52 patients with HCVAb were HCV RNA-positive. HLA-based typing of PBMCs from patients and normal blood donors was performed by reverse sequence-specific oligonucleotide with polymerase chain reaction (PCR-RSSO). The serum 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 pathological study of primary liver cancer (8). The severity of liver disease was evaluated according to the criteria of Desmet et al. using biopsy specimens of liver tissue, where F4 was defined as cirrhosis (9). Fifty-five patients who participated in the

present study received liver biopsy with RFA. Another 14 patients received liver biopsy 1-3 years before RFA.

Peptides and preparation of PBMCs

Eleven peptides that we previously identified as being useful for analysis of immune response in HLA-A24-positive HCC patients were selected (10-13). HIV envelope-derived peptide ($HIVenv_{584}$) (14) and CMV pp65-derived peptide ($CMVpp65_{328}$) (15) were also selected as control peptides. Peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be >90% by analytical HPLC. PBMCs were isolated before and 2-4 weeks after HCC treatments as described previously (11). In the patients who received RFA 2-4 times, PBMCs were obtained 2-4 weeks after the final treatment. In some patients, PBMCs were also obtained at 24 weeks after RFA. PBMCs were resuspended in RPMI 1640 medium containing 80% FCS and 10% dimethyl sulfoxide and cryopreserved until use.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays were performed as reported previously (11). Negative

controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$) (14). Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) (15). The colored spots were counted with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. Responses to peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3SD of the baseline response detected in 11 HLA-A24-positive normal blood donors against TAA-derived peptides, and the number of spots in the presence of an antigen was at least twofold that in its absence. The results of ELISPOT assay with more than 25 spots in the wells without peptides (control wells) were excluded from the analysis.

IFN-γ ELISPOT assays were also performed using PBMC-depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cell is responsive to the peptides. 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 using the MACS separation system with CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions.

Detection of myeloid-derived suppressor cells

For the detection of myeloid-derived suppressor cells (MDSCs), PBMCs were isolated from 20 randomly selected patients 2-4 weeks after HCC treatments. To determine the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs, two-color fluorescence-activated cell sorting analysis was performed using the following antibodies: anti-CD14 and anti-HLA-DR (Becton Dickinson). Flow cytometry was done using Becton Dickinson FACSAria II system. The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was calculated as a percentage of $HLA\text{-}DR^{-/low}$ cells in $CD14^{+}$ cells.

Tetramer staining and flow cytometry

Peptide $MRP3_{765}$-, AFP_{357}-, AFP_{403}- and $hTERT_{461}$-specific tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). PBMCs were stained with anti-CD8-APCAb (Becton Dickinson, Tokyo, Japan), anti-CCR7-FITCAb (eBioscience, Tokyo, Japan), anti-CD45RA-PerCP-Cy5.5Ab (eBioscience, Tokyo, Japan) and tetramer-PE for 30 min at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/PBS and analyzed on a Becton Dickinson FACSAria II system.

Statistical analysis

Data are expressed as the mean ± SD. The estimated probability of tumor recurrence-free survival was determined using the Kaplan–Meier method. The Mantel-Cox log-rank test was used to compare curves between groups. The prognostic factors for tumor recurrence-free survival were analyzed for statistical significance by the Kaplan–Meier method (univariate) and the Cox proportional hazard model (multivariate). Linear regression lines for the relationship between the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and the number of TAA-specific T cells were calculated using Pearson's correlation coefficient. A level of $p<0.05$ was considered significant.

RESULTS

Patient profile

The clinical profiles of the 69 patients analyzed in the present study are shown in Table 1. HCC was histologically classified as well, moderately and poorly differentiated in 7, 3 and 1 cases, respectively. In the other cases, HCC was diagnosed on the basis of typical CT findings and elevated AFP levels. In terms of the size and number, the tumor was classified as large (>2 cm) in 28 cases, small (≤2 cm) in 41, multiple in 29 and solitary in 40. Vascular invasion was noted in 1 patient. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th version) (16), 39, 29, 0, 0, 1 and 0 patients were classified as having stage I, II, IIIA, IIIB, IIIC and IV tumors, respectively.

Detection of TAA-specific T cells before and after RFA

Detection of TAA-specific T cells was performed by direct *ex vivo* analysis (IFN-γ ELISPOT assay). Positive T cell responses against each TAA-derived peptide were observed in 0 to 11 (0.0-17.2%) patients before RFA (Table 2). The same responses

against HIV- and CMV-derived peptides were observed in 1 (1.6%) and 43 (62.3%) patients, respectively. After HCC treatments with RFA, positive T cell responses against TAA-, HIV- and CMV-derived peptide were observed in 8-24 (11.6-35.3%), 2 (2.9%) and 39 (58.2%) patients, respectively. The increase of the frequency of TAA-specific T cells after RFA observed in 7 of 11 peptides ($SART2_{899}$, $SART3_{109}$, $MRP3_{503}$, $MRP3_{765}$, AFP_{357}, AFP_{403}, and $hTERT_{461}$) was statistically significant (Table 2).

The magnitude of TAA-specific T cell responses determined by the frequency of T cells and the proportion of the patients who showed a significant increase of TAA-specific T cells are shown in Fig. 1. When the T cell responses against a single peptide with more than or equal to 10 specific spots and 2-fold increase were defined as significant, a significant increase was observed in 4 to 16 (6.5%-24.6%) patients for each TAA-derived peptide and in 24 (39.3%) patients for total of TAA-derived peptides. On the other hand, the numbers of patients who showed a significant increase against HIV- and CMV-derived peptide were 1 (1.6%) and 8 (11.9%), respectively. The number of patients who showed a significant increase against at least one TAA-derived peptide after RFA was 43 (62.3%).

To determine what kind of T cell is responsive to the peptides, TAA-derived peptide-specific IFN-γ-producing T cells were also analyzed by ELISPOT assay using PBMC-depleted $CD4^+$ or $CD8^+$ cells. The assay showed that IFN-γ-producing T cells against the peptides ($SART2_{899}$, $SART3_{109}$, $MRP3_{503}$, $MRP3_{692}$, $MRP3_{765}$, AFP_{357}, AFP_{403}, AFP_{434}, $hTERT_{167}$, $hTERT_{324}$ and $hTERT_{461}$) mainly consisted of $CD8^+$ cells (Supplementary Fig. 1).

Effect of increase of TAA-specific T cells after RFA for the prognosis of patients

To examine the effect of increase of TAA-specific T cells after RFA for the prognosis of patients, we analyzed the relationship between the number of TAA-specific T cells and the HCC recurrence-free survival after RFA. First, we divided the patients into two groups with high (above median) and low (below median) specific spots detected in ELISPOT assay. In the analysis, we found that a high number of TAA-specific T cells after HCC treatment was statistically significantly correlated with the length of HCC recurrence-free survival (p=0.044) (Fig. 2A). The difference between the groups was emphasized when 50 spots were defined as highly specific spots (p=0.006) (Fig. 2B). On the other hand, there was no correlation between the number of TAA-specific T cells before HCC treatment and the length of HCC recurrence-free survival (p=0.758) (Fig.

2C). Furthermore, the magnitude of enhancement of TAA-specific immune responses was not statistically significantly correlated with the length of HCC recurrence-free survival (p=0.267) (Fig. 2D).

When univariate analysis of prognostic factors for HCC recurrence-free survival was performed, γ-GTP (<30), AFP (<400), Okuda stage (1), and number of TAA-specific T cells after RFA (50≤) were detected as factors that decrease HCC recurrence rate after RFA (Table 3). When multivariate analysis including these three factors was performed, only the number of TAA-specific T cells after RFA (50≤) was found to be a factor that decreases HCC recurrence rate after RFA.

Analysis of the factors that affect the number of TAA-specific T cells after RFA

To identify the factors that affect the number of TAA-specific T cells after RFA, we analyzed clinical parameters of patients and the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs after HCC treatment. We could not find any clinical parameters correlated with the number of TAA-specific T cells after RFA.

The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs after RFA showed various levels and it depended on the patient (Fig. 3A and B). It was significantly decreased after RFA (p =

0.022) except in 3 patients (Fig. 3B) and inversely correlated with the number of TAA-specific T cells after RFA but not with that of CMV-specific T cells (Fig. 3C).

Phenotypic analysis of TAA-specific T cells before and after RFA

Next, we examined the naïve/effector/memory phenotype of increased TAA-specific T cells after RFA using a tetramer assay. The memory phenotype was investigated by the criterion of CD45RA/CCR7 expression (17). In tetramer analysis, the frequency of TAA-derived peptide-specific $CD8^+$ T cells before RFA was 0.00-0.03% of $CD8^+$ cells (Fig. 4A). On the other hand, the frequency was increased after RFA in 10/12 (83.3%) patients and the range was 0.00-0.10% of $CD8^+$ cells. The frequency of $CD45RA^-/CCR7^+$(central memory), $CD45RA^-/CCR7^-$(effector memory) and $CD45RA^+/CCR7^-$(effector) T cells in tetramer-positive cells depended on the patients, and the ratio of these cells changed after RFA (Fig. 4B). The frequency of tetramer positive cells with $CD45RA^-/CCR7^+$ and $CD45RA^-/CCR7^-$ in $CD8^+$ cells was increased in 6/7 (85.7%) and 6/7 (85.7%) patients, respectively, whose samples were available for the assay before and after RFA. Interestingly, the tetramer positive cells with $CD45RA^-/CCR7^+$ were newly induced after RFA in 5/7 (71.4%) patients.

Kinetics of TAA-specific T cells induced by RFA

Although the number of TAA-specific T cells was a predictive factor of a decrease of HCC recurrence rate after RFA, as shown in Fig. 2A, more than 50% of the patients with a high number of TAA-specific T cells showed HCC recurrence for 25 months after the treatment. To identify the relationship between TAA-specific T cell responses and HCC recurrence more precisely, we examined the kinetics of TAA-specific T cells in 16 patients whose PBMCs were available for analysis at 24 weeks after RFA. The frequencies of TAA-derived peptide-specific T cells decreased in most of the peptides and patients at 24 weeks after RFA (Fig. 5). In the analysis of the total of each type of TAA-derived peptide-specific T cells, the frequency decreased in 14/16 (87.5%) patients analyzed and most of them showed less than 50 specific spots per $3X10^5$ PBMCs, with the exception of only one patient. In contrast, the frequencies of CMV-derived peptide-specific T cells were maintained in most of the patients.

DISCUSSION

In recent years, HCC-specific TAAs and their T cell epitopes have been identified, which has made analysis of immunological status in HCC patients possible and shown that TAA-specific T cell responses can be detected in peripheral blood (11, 18-20). The immunological analysis of HCC patients with RFA using 11 TAA-derived peptides in this study showed that the enhancement of TAA-specific T cell responses occurred in 62.3% of patients, the antigens and their epitope to which enhanced T cell responses occurred were diverse and some of them were newly induced. The mechanism of enhancement of tumor-specific immune response by RFA is still unclear. Den Brok et al. showed that RFA created an antigen source for antitumor immunity by destruction of tumor cells using a mouse tumor model (5). The antigens used in this study have been reported to be located in cell membrane (MRP3), cytoplasm (SART2 and AFP) and nucleus (hTERT and SART3) (21-24). The results that the target proteins of enhanced T cells are diverse suggest that the central mechanism of enhancement of tumor-specific immune response by RFA is due to tumor cell destruction, which supports the previously mentioned results (5).

In the present study, we also showed that the number of TAA-specific T cells after RFA was associated with the HCC recurrence-free survival of patients. The univariate and multivariate analyses clearly showed it was a predictive factor for HCC recurrence after RFA. These results suggest that TAA-specific T cells induced by RFA contribute to protection from HCC recurrence and additional immunological approaches should be applied to enhance the protective effect after the treatment.

To understand the precise mechanism that RFA enhances TAA-specific T cell responses, we analyzed the factors that affected the number of TAA-specific T cells after RFA. Among the factors analyzed, the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs after RFA was inversely correlated with the number of TAA-specific T cells, suggesting these MDSCs may have a negative effect on TAA-specific immune responses. Regarding the function of MDSCs in cancer patients, it has been reported that they inhibit T lymphocyte responses (25). In HCC patients, it is reported that the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs in PBMCs is significantly increased in comparison with healthy controls and they exert immunosuppressive function via induction of regulatory T cells (26). Taken together with our results, these reports suggest that an additional immunological approach to inhibit the function of MDSCs after RFA may enhance TAA-specific immune responses.

On the other hand, the patients with a high number of TAA-specific T cells were not completely protected from HCC recurrence. To examine the mechanisms behind the failure to control HCC recurrence completely by RFA-induced TAA-specific immune responses, we performed phenotypic and kinetic analysis of T cells enhanced by RFA. The results showed that the frequency of T cells with each memory phenotype depended on the patients, and the ratio of these cells changed after RFA. The memory phenotype of T cells which showed a more than 2 fold increase was $CD45RA^{-}/CCR7^{+}$ (central memory) T cells that required secondary stimulation by antigen to exert stronger antitumor effects (17). Interestingly, they were newly induced, suggesting RFA may modify not only the frequency but also the phenotype of TAA-specific T cells.

The frequencies of TAA-derived peptide-specific T cells decreased in most of the patients at 24 weeks after RFA, suggesting that RFA could not induce long-lived T cells. In a previous study, it was reported that tumor-specific immune responses induced by RFA could not protect from HCC recurrence completely because of tumor immune escape (27). In addition to this mechanism, our results suggest that one of the reasons that RFA-induced tumor-specific immune response is insufficient for controlling HCC recurrence is the weak induction of long-lived T cells.

Taken together with these results, the present study suggests that the antitumor effect

of TAA-specific T cells induced by RFA should be enhanced by an additional immunological approach. In recent studies of cancer immunology, cancer vaccines consisting of TAA-derived protein or peptide, recombinant virus and engineered tumor cells have been considered as candidates to enhance host immune responses (28). Alternatively, immune-modulating antibodies such as anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) and anti-programmed cell death 1 (PD-1) have been considered to reactivate T cell function (28, 29). These approaches may also be effective to enhance the antitumor effect induced by RFA.

In conclusion, the results of this study show that RFA can enhance various TAA-specific T cell responses and it is associated with the HCC recurrence-free survival of patients with RFA. To maintain the TAA-specific T cell responses induced by RFA and to improve the immunological effect for HCC, additional treatment by vaccine or immune-modulatory drugs might be useful.

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FIGURE LEGENDS

Figure 1: Enhancement of TAA-derived peptide-specific T cell responses after RFA. The magnitude of TAA-specific T cell responses determined by the frequency of T cells responsive to each peptide before (the number of left side) and after (the number of right side) RFA and proportion of the patients with a significant increase are shown. The results with a significant increase are shown as grey boxes. The box numbers show the patients with a significant increase in TAA-specific T cell responses. The T cell responses were examined by IFN-γ ELISPOT assay. The results of ELISPOT assay are shown as a specific spot, which was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. The increase was considered significant if more than or equal to 10 specific spots per 300,000 PBMCs were detected and if the number of spots after RFA was at least twofold that before RFA. Characteristics of patients are described in Table 1 and peptide sequences in Table 2.

Figure 2: Kaplan-Meier curves of HCC recurrence-free survival. (A) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the median number of TAA-specific T cells detected by

IFN-γ ELISPOT assay after RFA. (B) The difference between the groups was emphasized when 50 spots were defined as highly specific spots. (C) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the number of TAA-specific T cells detected by IFN-γ ELISPOT assay before RFA. (D) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the median increased number of TAA-specific T cells after RFA.

Figure 3: Frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and its relationship with the frequency of TAA-specific T cells after RFA. (A) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was measured in 20 randomly selected patients by FACS analysis after RFA. Representative results of 2 patients are shown. (B) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was also measured in 12 of 20 randomly selected patients before RFA and compared with that after RFA. (C) The relationship between the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and the frequency of TAA- and CMV-derived peptide-specific T cells after RFA.

Figure 4: Phenotypic analysis of T cells induced by RFA. (A) Enhancement of

TAA-specific T cell responses was also analyzed by tetramer assay. The results of all patients examined are shown. Peptide $MRP3_{765}$-, AFP_{357}-, AFP_{403}- and $hTERT_{461}$-specific tetramers were used. The frequency of tetramer positive cells is shown as % in $CD8^+$ cells. (B) The memory phenotype of tetramer positive cells was analyzed using the criterion of CD45RA/CCR7 expression. The box numbers show % of cells in tetramer positive cells. ND means that the experiments are not available because of the small number of tetramer positive cells. The results with an increase of the frequency after RFA are shown as a grey box.

Figure 5: Kinetics of TAA-specific T cell responses determined by IFN-γ ELISPOT assay. PBMCs were obtained at 3 different time points consisting of before RFA, at 2-4 weeks after RFA and at 24 weeks after RFA. Each graph indicates the kinetics of T cells specific for each peptide in each patient.

Supplementary figure 1: IFN-γ production of CD4- or CD8-depleted PBMCs against TAA-derived peptides. TAA-derived peptide-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 cell is responsive to the peptides.

Table 1 Characteristics of patients with HCC

	n=69	Median
Age (years)	67.3±9.4	69.0
Sex (M/F)	51/18	
PLT ($X10^4$/ul)	15.9±26.5	10.9
PLT (>15 $X10^4$/ul/≤15$X10^4$/ul)	19/50	
ALT (IU/L)	46.7±33.8	38.0
ALT (>30 IU/L /≤30 IU/L)	44/25	
PT (%)	78.4±14.6	77.0
PT (>70%/≤70 %)	50/19	
Alb (g/dl)	3.6±0.5	3.6
Alb (>3.5 g/dl /≤3.5 g/dl)	42/27	
T-Bil (mg/dl)	1.1±0.6	0.9
T-Bil (>2.0 mg/dl/≤2.0 mg/dl)	4/65	
AFP (ng/ml)	134.7±468.3	11.0
AFP (>100 ng/ml/≤100 ng/ml)	12/57	
Diff. degree of HCC (well/moderate/poor/ND) [a]	7/3/1/58	
Tumor size (>2 cm/≤2 cm)	28/41	
Tumor multiplicity (multiple/solitary)	29/40	
Vascular invasion (+/-)	1/68	
TNM factor		
(T1/T2-4)	40/29	
(N0/N1)	68/1	
(M0/M1)	69/0	
TNM stage (I/II/IIIA/IIIB/IIIC/IV)	39/29/0/0/1/0	
Histology of non-tumor liver (LC/Chronic hepatitis)	55/14	
Liver function (Child A/B/C)	52/17/0	
Etiology (HCV/HBV/Others)	52/8/9	
Additional treatment (+/-)[b]	14/55	

Data are expressed as the mean ± SD. [a] ND: not determined.

[b] TAE was performed as an additional treatment.

Table 2 Peptides and response frequency

Peptide name	Amino acid sequence	Number of specific spots in normal donors (mean ± SD)	Frequency of T cell response before RFA	Frequency of T cell response after RFA	Chi-square analysis *p*-value
$SART2_{899}$	SYTRLFLIL	1.0±1.4	0/69 (0.0%)	14/69 (20.3%)	<0.001
$SART3_{109}$	VYDYNCHVDL	2.1±1.9	7/69 (10.1%)	20/69 (29.0%)	0.009
$MRP3_{503}$	LYAWEPSFL	0.2±0.5	3/69 (4.3%)	17/69 (24.6%)	0.001
$MRP3_{692}$	AYVPQQAWI	1.5±2.1	4/68 (5.9%)	8/69 (11.6%)	0.366
$MRP3_{765}$	VYSDADIFL	0.9±1.0	3/69 (4.3%)	17/69 (24.6%)	0.001
AFP_{357}	EYSRRHPQL	1.8±2.0	3/68 (4.4%)	14/68 (20.6%)	0.008
AFP_{403}	KYIQESQAL	1.1±1.5	9/66 (13.6%)	24/68 (35.3%)	0.005
AFP_{434}	AYTKKAPQL	0.8±1.1	7/68 (10.3%)	14/68 (20.6%)	0.153
$hTERT_{167}$	AYQVCGPPL	0.8±1.1	9/65 (13.8%)	15/68 (22.1%)	0.263
$hTERT_{324}$	VYAETKHFL	0.5±0.7	6/62 (9.7%)	9/68 (13.2%)	0.591
$hTERT_{461}$	VYGFVRACL	0.7±1.2	11/64 (17.2%)	23/69 (33.3%)	0.046
HIV env_{584}	RYLRDQQLL	1.3±2.0	1/63 (1.6%)	2/68 (2.9%)	>0.999
CMV $pp65_{328}$	QYDPVAALF	13.3±15.7	43/68 (63.2%)	39/67 (58.2%)	0.599

Table 3 Univariate and multivariate analyses of prognostic factors for tumor-free survival

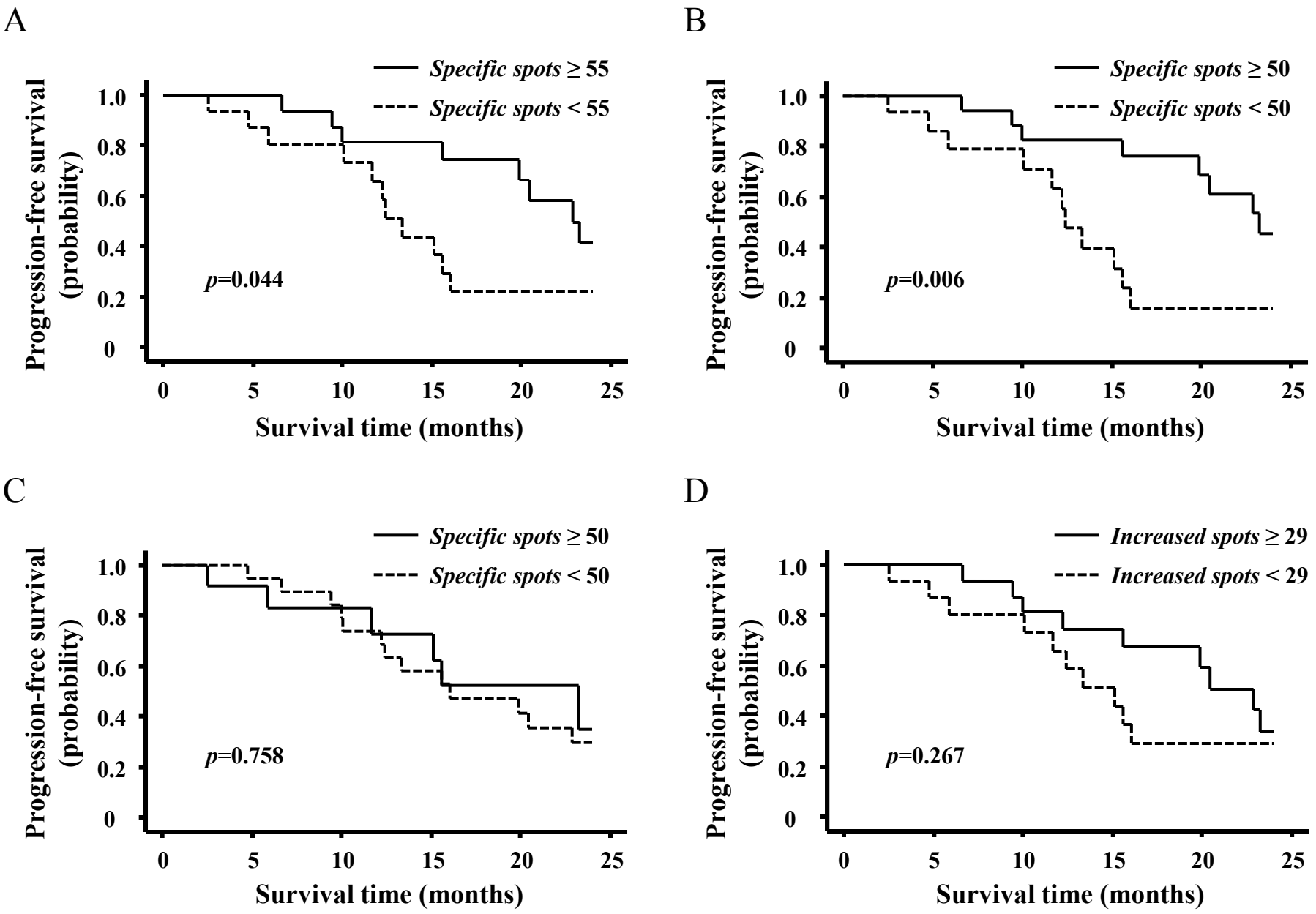
Variable	Univariate analysis *p*-value	Multivariate analysis *p*-value	95% CI	Hazard ratio
Age (<65/65≤)	0.582			
Platelet (<10/10≤ x10^4/μl)	0.570			
AST (<40/40≤ IU/L)	0.298			
ALT (<40/40≤ IU/L)	0.628			
γ-GTP (<30/30≤ IU/L)	0.010	0.223	0.586-9.898	2.408
Alb (<3.5/3.5≤ g/dL)	0.588			
T-bil. (<1/1≤ mg/dL)	0.386			
PT (<60/60≤ %)	0.282			
DCP (<100/100≤ mAU/mL)	0.630			
AFP (<400/400≤ ng/mL)	0.008	0.056	0.045-1.039	0.216
L3 (<10/10≤ %)	0.100			
Child-Pugh (A/B)	0.260			
Diameter of tumor (<2/2≤ cm)	0.706			
Tumor multiplicity (solitary/multiple)	0.686			
Okuda stage (1/2)	0.043	0.103	0.698-48.630	5.828
BCLC (A/BC)	0.190			
CLIP (0,1/2,3)	0.703			
HCV-Ab (-/+)	0.080			
Number of TAA-specific T cells before RFA (<50/50≤)*	0.740			
Number of TAA-specific T cells after RFA (<50/50≤)*	0.006	0.024	1.203-13.664	4.054

* Number of TAA-specific T cells before and after RFA was calculated per 3X10^5 PBMCs

Fig. 1

Peptides

Patients	$SART2_{899}$	$SART3_{109}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	AFP_{357}	AFP_{403}	AFP_{434}	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$	Total of TAA-derived peptides	$HIVenv_{584}$	$CMVpp65_{328}$
1	0/3	0/13	0/9	0/14	0/6	0/0	0/1	7/1	9/12	0/0	2/0	18/59	0/0	34/65
2	0/1	0/2	0/4	0/0	0/2	0/1	1/1	0/1	7/1	3/0	8/0	19/13	3/1	0/2
3	0/1	0/0	0/4	0/2	0/1	0/5	0/3	0/0	0/2	0/2	0/6	0/26	0/3	82/108
4	6/0	8/43	0/1	2/1	5/1	1/0	ND/1	0/0	ND/12	ND/0	ND/0	ND	6/0	2/0
5	0/11	0/31	3/46	8/5	3/25	1/11	4/25	9/19	3/18	3/7	5/20	39/218	5/2	17/19
6	0/0	0/0	0/6	3/0	0/0	0/0	0/9	0/0	2/5	0/6	1/0	6/26	0/0	15/13
7	0/9	0/4	1/7	3/3	0/7	1/0	3/5	2/3	2/4	2/3	4/1	18/46	1/4	6/26
8	9/17	7/3	0/13	0/4	10/22	0/0	0/5	4/17	0/4	5/12	12/14	47/111	0/0	13/7
9	0/1	0/9	0/0	0/0	0/5	0/0	0/6	0/0	0/0	0/1	0/1	0/23	0/2	78/52
10	4/0	0/0	1/3	0/1	1/0	0/0	4/10	6/0	0/5	5/0	4/12	25/31	8/4	13/13
11	7/13	16/16	0/7	0/6	0/27	0/9	5/8	2/2	0/4	11/1	9/6	50/99	8/3	23/4
12	5/0	4/6	4/2	0/4	4/19	2/4	1/1	0/2	0/2	0/6	0/0	20/46	0/0	45/22
13	0/0	1/4	1/1	0/0	1/0	0/12	1/2	0/0	0/2	0/0	2/0	6/21	1/3	24/23
14	4/1	1/2	3/2	12/8	5/6	6/ND	3/ND	19/ND	5/ND	0/0	16/13	ND	2/2	34/7
15	1/2	2/4	0/0	2/1	4/1	1/1	0/2	1/5	0/5	1/0	4/3	16/24	0/0	0/3
16	0/0	1/0	1/2	4/0	1/1	0/2	0/0	2/1	1/0	1/0	5/0	16/6	1/0	32/23
17	1/13	0/11	1/11	0/2	2/4	0/3	6/13	1/10	7/4	ND/9	2/15	ND	ND/8	167/517
18	2/0	5/4	0/3	5/3	3/2	2/0	2/6	2/1	3/2	3/2	2/0	29/23	2/2	33/14
19	0/0	1/2	0/1	0/0	0/0	0/0	0/0	0/0	0/2	0/2	0/0	1/7	1/0	13/14
20	0/3	0/0	4/31	2/0	0/0	7/19	24/35	7/6	6/2	0/1	12/21	62/118	8/0	9/10
21	1/1	3/0	0/3	1/3	1/0	4/2	0/4	3/0	1/8	8/0	5/0	27/21	7/0	61/47
22	6/6	2/0	0/0	0/5	0/0	2/0	5/0	3/6	0/0	0/0	0/0	18/17	6/0	3/3
23	2/1	14/11	2/0	0/1	0/29	5/0	2/0	5/24	2/0	2/0	1/0	35/66	1/1	3/0
24	5/6	8/2	3/6	0/0	0/0	5/3	9/10	0/7	2/0	5/0	0/9	37/43	0/0	0/7
25	0/2	1/3	0/12	2/8	0/5	7/10	12/1	0/3	0/1	0/0	30/14	52/59	2/1	1/18
26	0/0	10/0	3/4	0/5	1/4	1/1	17/24	4/29	23/29	0/23	1/3	60/122	0/0	119/128
27	2/0	4/0	1/4	4/0	2/0	4/2	0/7	1/9	0/5	24/17	9/33	51/77	3/11	84/510
28	0/22	4/0	0/1	0/0	6/0	0/0	0/0	0/0	0/4	0/0	12/10	22/37	0/0	3/0
29	0/1	0/1	0/0	0/0	0/0	0/0	2/2	0/5	0/1	1/0	0/0	3/10	ND/0	9/4
30	0/0	0/0	0/0	0/0	4/12	0/0	0/1	1/10	6/4	0/0	0/10	11/37	8/1	316/ND
31	7/4	14/9	3/5	0/0	6/14	0/0	5/13	2/4	6/4	6/0	7/14	56/67	2/0	385/434
32	7/12	0/0	0/0	2/0	3/0	0/27	7/17	0/0	1/0	15/14	9/9	44/79	8/4	18/24
33	0/0	5/0	10/10	0/0	0/0	0/101	0/0	0/0	0/0	0/1	0/21	15/133	0/0	0/0
34	0/6	2/139	6/12	13/11	8/23	21/19	6/0	2/27	0/1	4/0	27/19	89/257	1/0	10/15
35	0/18	0/10	8/39	0/53	0/0	12/19	8/23	28/28	11/24	0/0	0/0	67/214	7/4	38/29
36	1/4	0/9	0/3	0/0	1/4	2/0	1/1	0/2	0/0	0/0	0/1	5/24	0/0	0/1
37	0/4	0/0	1/1	2/0	1/0	2/0	1/0	0/0	0/0	ND/0	ND/0	ND	ND/1	0/0
38	0/4	0/2	0/12	4/0	0/0	2/0	1/11	6/0	17/15	1/7	2/0	33/51	6/0	7/7
39	2/0	0/0	0/0	0/0	1/0	6/18	0/0	2/0	0/0	0/0	2/0	13/18	0/0	1/1
40	3/2	10/6	1/2	3/2	2/0	1/1	3/14	3/15	4/3	4/0	2/0	36/45	1/0	9/0
41	0/28	0/18	0/5	0/3	0/10	0/2	9/6	0/9	0/16	0/10	0/0	9/107	9/0	32/5
42	1/0	3/0	0/0	3/0	2/13	0/0	0/0	1/0	2/0	2/0	6/1	20/14	2/0	14/5
43	3/21	2/32	4/8	3/49	4/18	1/0	2/9	1/4	10/3	0/0	0/0	30/144	5/0	5/1
44	0/1	0/0	0/0	0/0	0/0	3/7	4/3	0/4	0/0	0/1	0/0	7/16	4/0	8/7
45	2/13	5/7	0/8	8/19	7/6	0/0	ND/23	0/5	ND/1	ND/5	ND/10	ND	7/6	14/9
46	0/0	0/0	0/0	0/0	0/3	0/10	0/11	0/2	ND/2	ND/ND	ND/13	ND	ND/0	7/15
47	5/14	8/7	3/4	6/2	5/14	1/0	7/6	2/0	8/5	5/0	2/8	52/60	7/12	4/6
48	0/0	0/2	0/0	2/0	0/0	0/0	0/0	0/1	0/2	0/0	0/4	2/9	0/0	121/60
49	1/0	3/0	7/10	4/0	3/3	1/0	0/8	0/0	0/5	5/4	0/0	24/30	0/0	22/71
50	1/5	0/1	5/1	ND/1	0/1	0/1	3/2	1/0	0/0	ND/0	0/0	ND	ND/1	5/3
51	0/1	9/2	0/57	1/7	1/0	0/2	0/12	1/1	1/4	1/3	1/0	15/89	2/0	38/50
52	3/2	1/0	0/0	1/0	1/0	0/7	3/2	1/2	9/8	0/2	1/4	20/27	0/4	1/8
53	4/0	6/17	2/0	0/0	8/2	1/2	9/0	6/4	6/2	5/0	17/10	64/37	9/0	1/0
54	3/1	3/0	11/19	6/10	2/5	15/18	11/26	11/11	16/10	12/23	14/19	104/142	25/1	97/276
55	1/9	2/3	25/15	6/0	21/15	0/9	36/18	42/26	11/14	5/0	18/15	167/124	8/0	119/94
56	0/0	0/9	0/0	0/0	0/0	0/3	0/0	13/4	2/0	0/0	0/0	15/16	1/0	47/16
57	0/7	8/11	0/2	0/0	3/7	7/0	5/10	4/2	9/11	3/0	0/0	39/50	6/0	25/28
58	0/5	0/10	4/13	8/7	0/6	7/6	9/20	4/14	14/12	9/5	7/4	62/102	1/6	48/47
59	5/0	7/2	3/6	18/4	0/0	3/0	0/10	6/0	8/0	77/0	12/3	139/25	5/0	64/61
60	0/53	0/12	7/13	0/12	1/102	0/0	16/19	6/3	5/18	0/0	4/18	39/250	6/0	22/21
61	2/27	13/14	0/15	24/28	5/0	7/0	6/25	14/18	11/15	3/19	2/23	87/184	0/0	23/2
62	0/0	0/17	9/0	0/0	13/0	4/0	10/0	12/0	7/0	9/0	4/0	68/17	5/0	139/48
63	0/4	0/0	0/3	0/0	4/0	0/0	0/3	0/0	0/0	4/0	0/0	8/10	ND/ND	ND/ND
64	3/0	11/17	3/99	4/0	4/11	2/0	5/0	2/0	12/7	3/2	3/0	52/136	5/0	22/30
65	9/8	3/4	0/0	0/0	4/4	8/13	0/0	9/1	7/0	0/0	1/0	41/30	8/0	107/55
66	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/0	0/0	0/2	0/1	0/1
67	0/14	5/27	0/6	0/9	0/8	ND/15	ND/26	ND/19	ND/19	ND/20	ND/14	ND	1/5	96/30
68	5/5	7/13	6/5	0/0	0/10	0/5	32/26	0/5	5/3	0/7	4/11	59/90	0/5	263/623
69	3/0	0/19	9/5	2/3	5/16	9/10	17/34	8/4	0/16	18/12	11/45	82/164	4/0	32/47
Response frequency	**43**	**11**	**14**	**12**	**5**	**16**	**7**	**16**	**9**	**5**	**4**	**11**	**24**	**1**	**8**
(%)	**62.3**	**15.9**	**20.3**	**17.4**	**7.4**	**23.2**	**10.4**	**24.6**	**13.4**	**7.8**	**6.5**	**17.2**	**39.3**	**1.6**	**11.9**


A
Specific spots ≥ 55
Specific spots < 55
p=0.044
Progression-free survival (probability)
Survival time (months)
B
Specific spots ≥ 50
Specific spots < 50
p=0.006
Progression-free survival (probability)
Survival time (months)
C
Specific spots ≥ 50
Specific spots < 50
p=0.758
Progression-free survival (probability)
Survival time (months)
D
Increased spots ≥ 29
Increased spots < 29
p=0.267
Progression-free survival (probability)
Survival time (months)

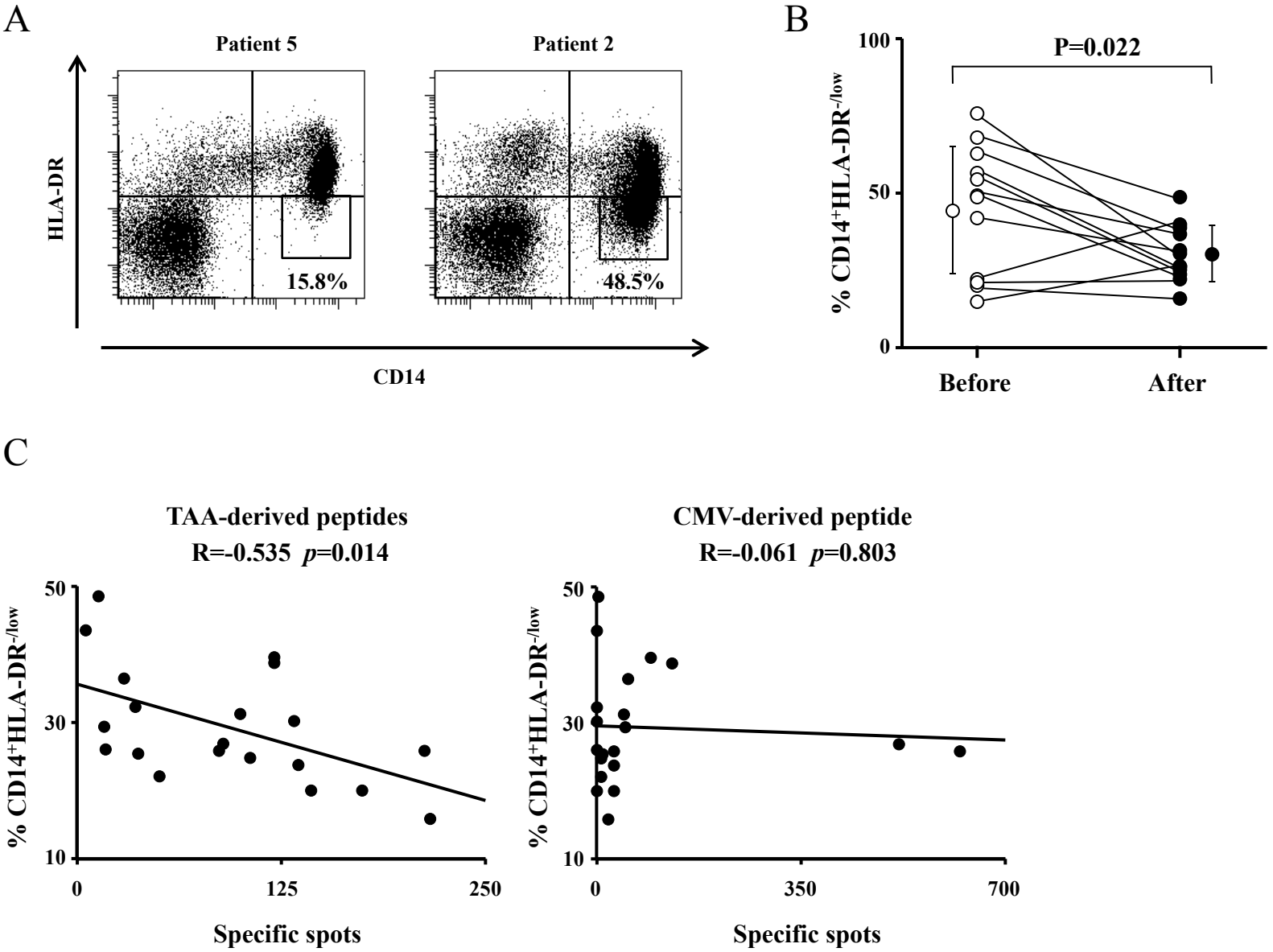
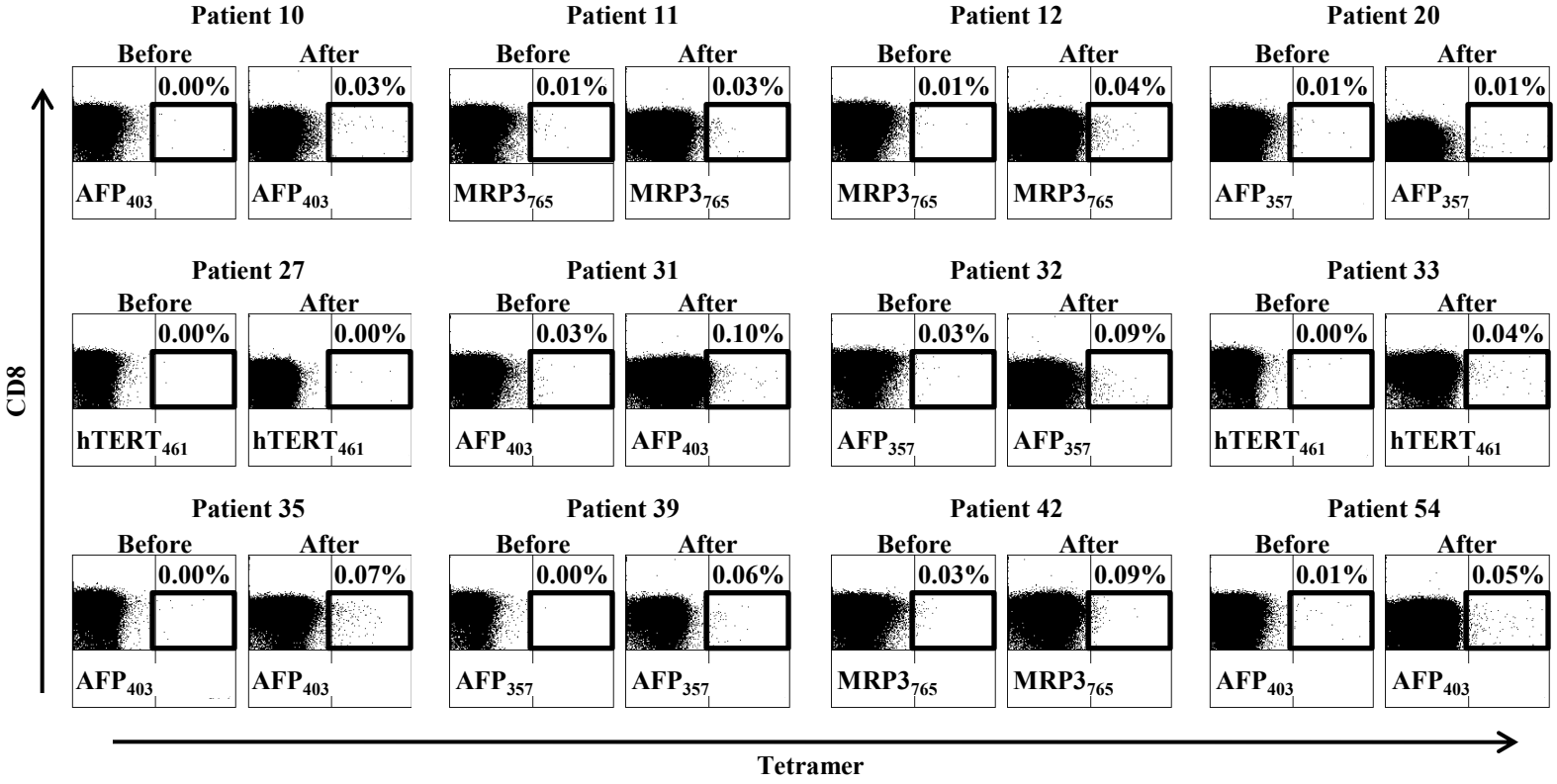

A
Patient 5
Patient 2
HLA-DR
CD14
15.8%
48.5%
B
P=0.022
% CD14+HLA-DR-/low
100
50
0
Before
After
C
TAA-derived peptides
R=-0.535 p=0.014
CMV-derived peptide
R=-0.061 p=0.803
% CD14+HLA-DR-/low
50
30
10
0
125
250
350
700
Specific spots

Fig. 4

A

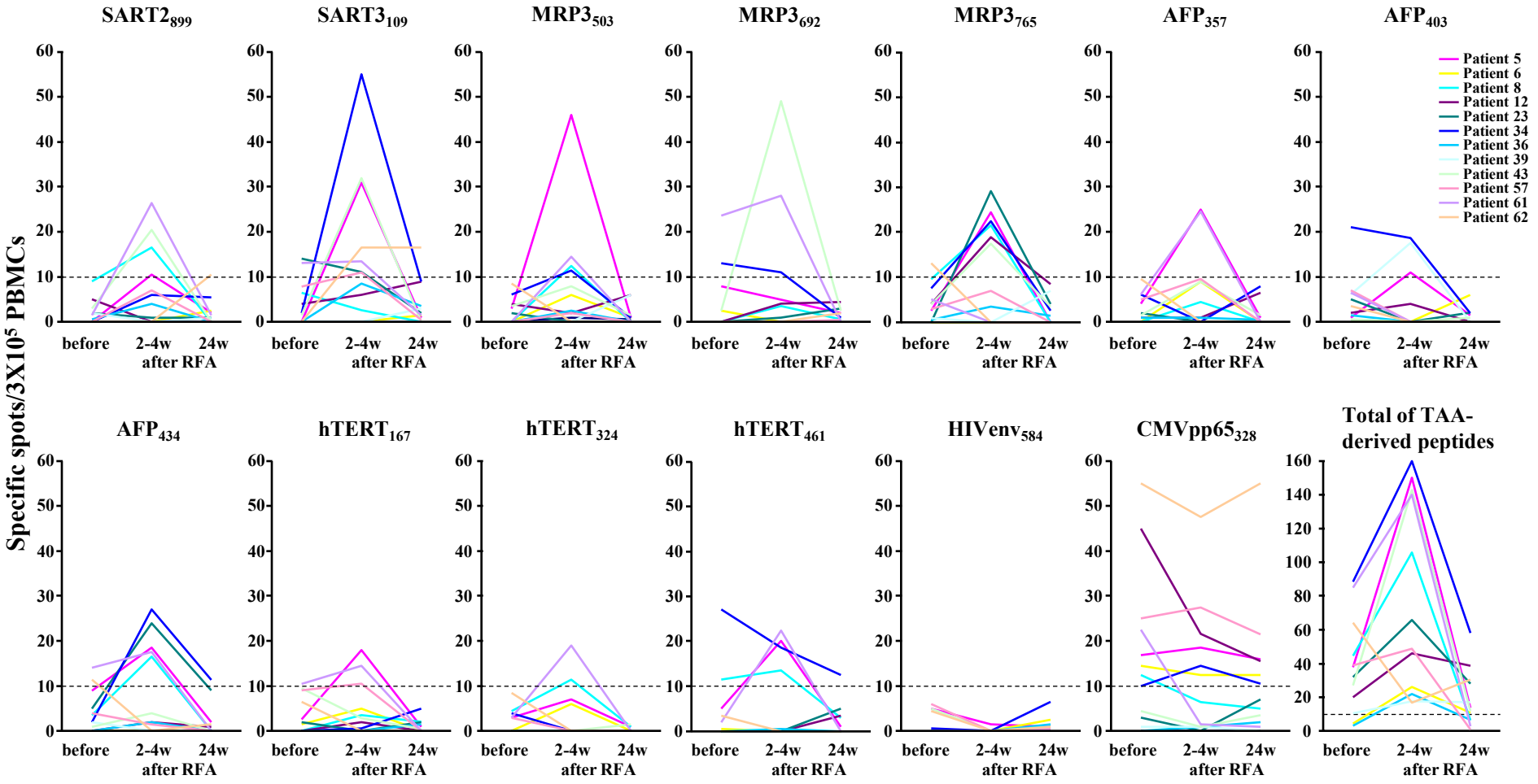


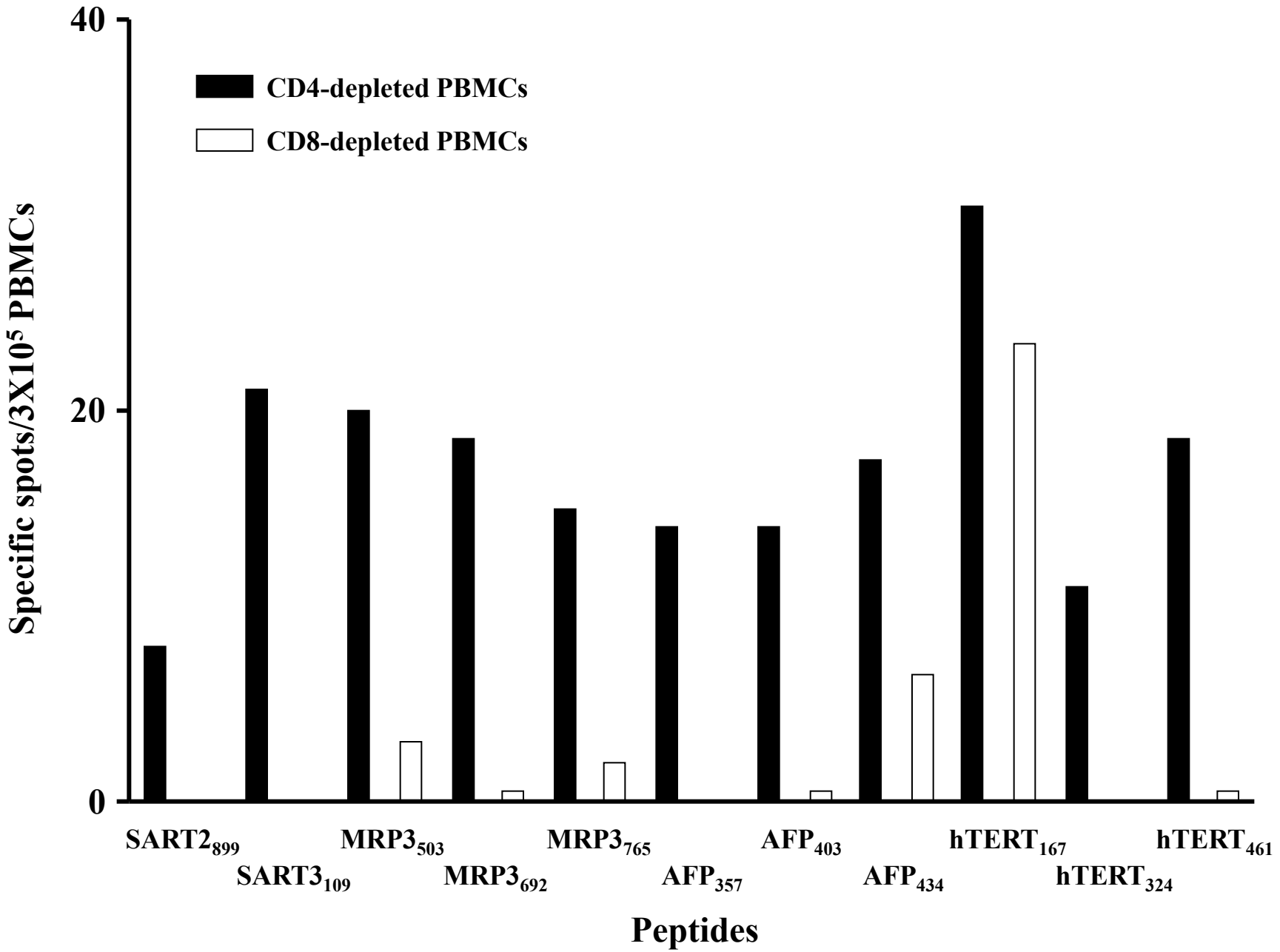
B

	Patient 10		Patient 11		Patient 12		Patient 20		Patient 27		Patient 31	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	ND	2.1	7.4	10.3	0.0	4.2	0.0	2.2	ND	ND	13.5	2.2
CD45RA-CCR7-	ND	27.1	14.8	17.6	47.8	41.7	7.4	13.0	ND	ND	18.9	51.7
CD45RA+CCR7-	ND	22.9	44.4	33.8	21.7	10.1	74.1	69.6	ND	ND	35.1	42.2

	Patient 32		Patient 33		Patient 35		Patient 39		Patient 42		Patient 54	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	0.0	7.6	ND	6.2	ND	3.9	ND	6.2	0.0	6.9	0.0	3.1
CD45RA-CCR7-	29.6	38.6	ND	27.2	ND	4.5	ND	8.3	19.6	22.4	20.0	23.1
CD45RA+CCR7-	44.4	49.7	ND	11.1	ND	67.0	ND	41.7	80.4	63.8	60.0	51.5

Fig. 5


SART2899
SART3109
MRP3503
MRP3692
MRP3765
AFP357
AFP403
AFP434
hTERT167
hTERT324
hTERT461
HIVenv584
CMVpp65328
Total of TAA-derived peptides
Specific spots/3X10^5 PBMCs
before
2-4w
24w
after RFA
Patient 5
Patient 6
Patient 8
Patient 12
Patient 23
Patient 34
Patient 36
Patient 39
Patient 43
Patient 57
Patient 61
Patient 62


40
20
0
CD4-depleted PBMCs
CD8-depleted PBMCs
Specific spots/3X10^5 PBMCs
SART2$_{899}$
SART3$_{109}$
MRP3$_{503}$
MRP3$_{692}$
MRP3$_{765}$
AFP$_{357}$
AFP$_{403}$
AFP$_{434}$
hTERT$_{167}$
hTERT$_{324}$
hTERT$_{461}$
Peptides