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Phase I Trial of Multidrug Resistance-associated Protein 3-derived Peptide in Patients with Hepatocellular Carcinoma

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Abbreviations: MRP3, multidrug resistance-associated protein 3; TAA, tumor-associated antigen; HLA, human leukocyte antigen; IFN, interferon; PBMC, peripheral blood mononuclear cell; HCV, hepatitis C virus; ELISPOT, enzyme-linked immunospot; Treg, regulatory T cell; MDSC, myeloid-derived suppressor cell; CTCAE, Common Terminology Criteria of Adverse Events

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ABSTRACT

Multidrug resistance-associated protein 3 (MRP3) is a carrier-type transport protein belonging to the ABC transporters. In this study, we investigated the safety and immunogenicity of a MRP3-derived peptide ($MRP3_{765}$) as a vaccine and characterized the MRP3-specific T cell responses induced. Twelve hepatocellular carcinoma (HCC) patients treated with hepatic arterial infusion chemotherapy (HAIC) were enrolled. The MRP3-derived peptide was emulsified in incomplete Freund's adjuvant and administered via subcutaneous immunization three times weekly. No serious adverse drug reactions to the peptide vaccine were observed, and the vaccination was well tolerated. The vaccination induced MRP3-specific immunity in 72.7% of the patients. In a phenotypic analysis, the largest post-vaccinated increase in MRP3-specific T cells was due to an increase in cells with the effector memory phenotype. Among the 12 patients, one patient showed a partial response, nine showed a stable disease, and two showed a progressive disease. The median overall survival time was 14.0 months. In conclusion, the safety, effects of immune boosting, and possible prolongation of overall survival by the MRP3-derived peptide demonstrate the potential of the peptide to provide clinical benefit in HCC patients.

Word count: 182 words

Key words: epitope, immunotherapy, cytotoxic T cell, cancer, chemotherapy

1. INTRODUCTION

Hepatocellular carcinoma (HCC), the sixth most frequent type of cancer worldwide, is an important public health concern because its incidence has continued to increase in Western and Asian countries [1; 2]. Advanced HCC is treated with sorafenib as the standard treatment. This is the sole systemic agent available and can prolong survival for 2.3–2.8 months [3; 4]. Hepatic arterial infusion chemotherapy (HAIC) has been used as an alternative therapy to sorafenib in Japan and other Asian countries [5; 6]. However, when no curative treatment is administered at the time of the diagnosis of advanced HCC, the median survival of patients is currently less than 1 year [7]. Thus, there is a continuing need for novel treatment strategies to improve the outcomes of advanced HCC patients. Some recently developed immunotherapies have turned out to be promising strategies for several advanced cancers. As with other cancers, immunotherapy is expected to provide new treatments for HCC.

Multidrug resistance-associated protein 3 (MRP3) is a carrier-type transport protein belonging to the ABC transporters that transport substances against a concentration gradient in an ATP energy-dependent manner [8]. It is expressed at high levels in the

small and large intestines, pancreas, placenta, and adrenal cortex [9], and recent studies have reported that its expression is enhanced in various cancer cells [10; 11; 12].

We previously demonstrated that the MRP3 expression level in HCC tissue was significantly higher than in non-cancerous tissue and that MRP3-specific cytotoxic T cells (CTLs), which showed cytotoxicity against HCC cells overexpressing MRP3, could be induced regardless of liver function, blood α-fetoprotein (AFP) level, and the stage of HCC [13]. These results suggest that MRP3 may be useful as a target antigen in HCC immunotherapy.

Clinical trials using MRP3-derived peptides have been performed in cancer patients, and the results have shown that the peptide vaccine is well tolerated and effective for patients with glioblastoma and prostate cancer [14; 15]. However, the safety and efficacy of MRP3-derived peptide vaccines in HCC patients remain unclear.

The results of recent studies have also suggested that combination therapies of a peptide vaccine and conventional chemotherapy can enhance the anti-tumor effects of peptides [16; 17]. In this study, the safety and immunological effects of a MRP3-derived peptide used as a potential vaccine in HCC patients treated with HAIC were investigated. Furthermore, we characterized the phenotype of MRP3-specific T cells induced by the peptide and monitored the MRP3-specific immune response, the numbers of regulatory T

cells (Tregs) and myeloid-derived suppressor cells (MDSCs), and the anti-tumor effects on HCC after vaccination.

2. MATERIALS AND METHODS

2.1 Patients

Twelve HLA-A24-positive HCC patients were enrolled (Table 1). The inclusion criteria were a histological or radiological diagnosis of primary HCC according to the American Association for the Study of Liver Diseases (AASLD) guidelines for the management of HCC [18], a Karnofsky score of ≥70%, ≥20 years of age, informed consent, and the following normal baseline hematological parameters: hemoglobin ≥8.5 g/dL, white blood cell count ≥2,000/μL, platelet count ≥50,000/μL, creatinine ≤1.5 mg/dL, and Child–Pugh class A or B. Regarding tumor stage, the following patients were included: patients who had (1) severe vascular invasion (i.e., vascular invasion in the main trunk to the secondary branches of the portal vein or invasion in the right, middle, or left hepatic vein) and (2) multiple intrahepatic lesions (i.e., five or more nodules in the left and/or right lobes, as confirmed by radiology).

Exclusion criteria included severe cardiac, renal, pulmonary, hematological, or other systemic diseases associated with a discontinuation risk. Such diseases included human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy, or radiation therapy within 4 weeks of the trial; immunological

disorders, including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; currently lactating or pregnant; history of organ transplantation; or difficulty in follow up.

2.2 Treatment protocol

The present study was designed as a phase I trial. Primary endpoints were the evaluation of the safety and immunological effects of the $MRP3_{765}$ peptide vaccine. The secondary endpoint was the anti-tumor response. After starting treatment, HCC progression was evaluated with dynamic CT or MRI every 2–3 months.

After diagnosis, all patients were treated with HAIC using pegylated (PEG) interferon (IFN)-α-2b/5-fluorouracil (FU) + cisplatin at Kanazawa University Hospital between April 2011 and December 2012. The patients received a continuous hepatic arterial infusion of FU (5-FU, Kyowa Hakko, Tokyo, Japan) at a dose of 300 mg/m^2/day for 5 days in the first and second weeks (for 120 h) using an infusion pump (Baxter Infusor SV1, Tokyo, Japan), as described previously [19]. The maximum amount of FU infused over 5 days was 2,500 mg. PEG IFN α-2b (Schering-Plough, Osaka, Japan) at a dose of 1.0 μg/kg was administered s.c. on days 1, 8, 15, and 22. A dose of 20 mg/m^2 Cisplatin (Nippon Kayaku, Tokyo, Japan) was given by a hepatic arterial infusion over 1.5 h on

days 1 and 8 prior to the administration of FU and after appropriate hydration and antiemetic medication. A treatment cycle comprised 4 weeks of drug administration, including the administration of IFN, and a subsequent 2-week rest period.

Patients also received 0.03–3.0 mg MRP3-derived peptide ($MRP3_{765}$) vaccine on days 1, 8, and 15 during HAIC. The peptide was administered emulsified with incomplete Freund's adjuvant (Montanide ISA-51 VG; SEPPIC, Paris, France) by subcutaneous immunization. Vaccination was also performed during the second course of HAIC. Adverse events/toxicities were categorized and graded using the Common Terminology Criteria of Adverse Events (CTCAE; ver. 3.0). Criteria for discontinuation included unacceptable toxicity and disease progression, defined as progressive disease (PD) according to the RECIST criteria.

All patients provided written informed consent for their participation in the study in accordance with the Helsinki declaration. This study (trial registration: UMIN000005678) was approved by the regional ethics committee (Medical Ethics Committee, Kanazawa University, No. 1018).

2.3 Peptides and preparation of PBMCs

The HCC patients were immunized with the $MRP3_{765}$ peptide, which was synthesized

to GMP grade (Neo MPS, Inc., San Diego, CA, USA). For the immunological analysis, the peptide derived from MRP3 (Peptide 1, $MRP3_{765}$), the HIV envelope-derived peptide (Peptide 2, $HIVenv_{584}$) [20], and the CMV pp65-derived peptide (Peptide 3, $CMVpp65_{328}$) [21] were used (Sumitomo Pharmaceuticals, Osaka, Japan). PBMCs were isolated prior to and 2 weeks after final vaccination in every course, as described previously [22]. In some available patients, PBMCs were also isolated 24 weeks after starting HAIC. PBMCs were resuspended in RPMI 1640 medium containing 80% fetal calf serum (FCS) and 10% dimethyl sulfoxide and then cryopreserved until use.

2.4 IFN-γ ELISPOT assay

IFN-γ ELISPOT assays were performed, as reported previously [23]. The number of spots in control wells was fewer than 10 in all ELISPOT assays. Responses to the MRP3-derived peptides were considered positive if more than the mean + 2 SD specific spots were detected in healthy normal donors and the number of spots detected in the presence of an antigen was at least two-fold that detected in its absence.

2.5 Flow cytometric analysis

Peptide $MRP3_{765}$-specific tetramer was used for the detection of peptide

vaccine-induced CTLs. Peptide HIVenv$_{584}$-specific tetramer was used as a negative control of tetramer assay. All tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). PBMCs were stained with anti-CD8-APC (Becton Dickinson, Tokyo, Japan), anti-CCR7-FITC (eBioscience, Tokyo, Japan), anti-CD45RA-PerCP-Cy5.5 antibodies (eBioscience, Tokyo, Japan), and with tetramer-PE for 30 min at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/PBS, and analyzed using a Becton Dickinson FACSAria II system. In the tetramer assays with the negative control tetramer, we did not observe more than 0.03% tetramer-positive cells in any assay. Based on the results of the negative control, responses to MRP$_{765}$-specific tetramer were considered positive if more than 0.03% tetramer positive cells were detected. At least 1,000,000 PBMCs were acquired for each tetramer assay. For the detection of Tregs and MDSCs, the following anti-human monoclonal antibodies were used: anti-CD4 (Becton Dickinson), anti-CD14 (Becton Dickinson), anti-CD25 (Becton Dickinson), anti-CD127 (Becton Dickinson), and anti-HLA-DR (Becton Dickinson).

2.6 Statistical analysis

Data are expressed as means ± SD. Paired *t*-tests were used for statistical analyses of

the frequency of $MRP3_{765}$ peptide-specific T cells, Tregs, and MDSCs before and after HAIC with the $MRP3_{765}$ peptide vaccine. We used the Kaplan–Meier method to estimate the distribution of progression-free survival (PFS) and overall survival (OS) rates. PFS was calculated from the first day of HAIC to the date of radiographic disease progression using the RECIST criteria. OS was calculated from the first day of HAIC to the date of death from any cause. A per-protocol statistical analysis was performed using the SPSS software (SPSS, Inc., Chicago, IL, USA). A p value < 0.05 was considered to indicate statistical significance.

3. RESULTS

3.1 Patient profiles

Clinical profiles of the patients are shown in Table 1. All patients but one (patient C2) received 3–6 vaccinations and were evaluated with immunological analyses. The etiology of liver diseases consisted of HCV, HBV, and other for 7, 0, and 5 patients, respectively. Ten patients had liver cirrhosis, proven by liver biopsy. The TNM stage was classified according to the Liver Cancer Study Group of Japan (LCSGJ): 4, 3, and 5 patients had stages II, III, and IV, respectively.

3.2 Toxicity of the MRP3-derived peptide vaccine

Adverse events/toxicities were categorized and graded using the CTCAE. As shown in Table 2, three (25.0%) patients showed grade 1 fever. One (8.3%), three (25.0%), two (16.7%), and one (8.3%) patient developed dizziness, ascites, gastric ulcer, and arthralgia, respectively. Blood examinations revealed reductions in neutrophil or platelet counts in each patient. These adverse events were considered to be related to HAIC, as reported previously [5; 24]. No severe adverse event related to the $MRP3_{765}$ peptide vaccine was observed.

3.3 CTL response

CTL responses before and 2 weeks after final vaccination were evaluated using an IFN-γ ELISPOT assay. Comparisons of the frequency of each peptide-specific CTL before and 2 weeks after vaccination in all patients except one (patient C2) are shown in Figure 1A. The frequencies of $MRP3_{765}$ peptide (peptide 1)-specific CTLs before vaccination were 0-2 per 3×10^5 PBMCs. The frequencies of $HIVenv_{584}$ (peptide 2)- and $CMVpp65_{328}$ peptide (peptide 3)-specific CTLs before vaccination were 0-2.5 and 1-230 per 3×10^5 PBMCs, respectively.

When T cell responses against a single peptide with >2.9 specific spots (i.e., > mean + 2 SDs of the frequency of $MRP3_{765}$-specific T cells in healthy controls) and a two-fold increase were defined as significant, a significant increase in the $MRP3_{765}$ peptide was observed in 8 of 11 (72.7%) patients (patients A1, A3, B3, C1, C3, C4, C5, and C6). In contrast, no increase in the frequency of $HIVenv_{584}$ peptide-specific CTLs was observed in any patient. The frequency of $CMVpp65_{328}$ peptide-specific CTLs increased in 3 of 11 (27.2%) patients (patients A2, B3, and C6).

3.4 Phenotypic analysis of CTLs induced by vaccination

The frequency of $MRP3_{765}$ peptide-specific CTLs was also examined using tetramer analysis, and the memory phenotype of tetramer-positive cells was analyzed using CD45RA/CCR7 expression criteria [25]. In one patient (patient C6), $MRP3_{765}$ tetramer$^+$ $CD8^+$ T cells were readily detectable after vaccination without *in vitro* stimulation with the peptide. Figure 1B presents representative results for patient C6 with respect to changes in the frequency of $MRP3_{765}$ peptide-specific CTLs evaluated by tetramer analysis before and 2 weeks after vaccination. The frequencies of $MRP3_{765}$ peptide-specific CTLs were 0.01% and 0.19% of $CD8^+$ T cells pre- and post-vaccination, respectively. According to phenotypic analysis of $MRP3_{765}$ peptide-specific $CD8^+$ T cells after vaccination, the frequencies of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells were 0.4%, 74.4%, and 24.8% of the $MRP3_{765}$ tetramer$^+$ $CD8^+$ T cells, respectively. In contrast, phenotypic analysis of total $CD8^+$ T cells after vaccination showed that the frequencies of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells were 10.7%, 39.4%, and 38.6% of $CD8^+$ T cells, respectively (Fig. 1C).

3.5 Detection of Tregs and MDSCs

In addition to the analysis of $MRP3_{765}$ peptide-specific $CD8^+$ T cells, we examined the frequency of Tregs and MDSCs in peripheral blood to identify the effects of HAIC with the peptide vaccine on immune suppressor cells. The population of Tregs was detected as $CD4^+$ $CD25^+$ $CD127^{-/low}$ cells, as reported previously (Fig. 2A) [26]. The population of MDSCs was detected as $CD14^+HLA\text{-}DR^{-/low}$ cells, as reported previously (Fig. 2B) [26]. The frequencies of $MRP3_{765}$ peptide-specific $CD8^+$ T cells (Fig. 2C), Tregs (Fig. 2D) and of MDSCs (Fig. 2E) in peripheral blood before and after HAIC with peptide vaccine are shown for each patient.

The frequency of $MRP3_{765}$ peptide-specific $CD8^+$ T cells detected in the IFN-γ ELISPOT assay increased significantly after the treatment ($p = 0.007$; Fig. 2C). The frequency of Tregs varied greatly (4.5–12.9%) in the HCC patients before treatment and decreased significantly after treatment ($p = 0.040$; Fig. 2D). The frequency of MDSCs in the PBMCs of HCC patients represented 8.0–32.6% of $CD14^+$ cells before HAIC. In contrast to Tregs, this increased significantly after treatment ($p = 0.022$; Fig. 2E).

3.6 Kinetics of peptide-specific CTLs after vaccination

To evaluate the kinetics of $MRP3_{765}$ peptide-specific CTLs after vaccination, the

frequency of CTLs 24 weeks after vaccination was examined in six patients (A3, B3, C1, C4, C5, and C6) whose PBMCs were available for ELISPOT assays. The frequency of $MRP3_{765}$ peptide-specific CTLs, evaluated using an IFN-γ ELISPOT assay 24 weeks after vaccination was 0–3 cells per 3×10^5 PBMCs, and no patient maintained a level of >10 cells per 3×10^5 PBMCs (Fig. 3A). With the exception of three patients (patients A3, B3, and C6), the frequencies of CMV- and HIV-derived peptide-specific CTLs did not decrease during the study course,

3.7 Clinical outcomes

A summary of the immune and tumor responses in all patients after vaccination with the $MRP3_{765}$ peptide is shown in Table 3. According to the RECIST criteria, among the 12 patients, one patient showed a partial response (PR), nine showed a stable disease (SD), and two showed a progressive disease (PD). The overall survival time for each patient is presented in Table 3. At the time of writing, all patients have shown disease progression with death, and the median overall survival time was 14.0 months (95% CI = 9.6–18.5 months)(Fig. 3B), which is longer than those in previous studies including patients treated with HAIC without peptide vaccination (median OS = 12.0–12.6 months) [24; 27].

The representative clinical course of the patient with PR is shown in Figure 4. The patient with PR (Patient B3) had a large HCC in the right lobe of the liver and was vaccinated with the $MRP3_{765}$ peptide weekly, for a total of six times. After vaccination, elevated serum AFP and DCP values normalized within 100 days (Fig. 4A), and the size of the HCC (Fig. 4B), evaluated by CT, was getting smaller. During the vaccination, ALT was elevated to the abnormal limit, but it normalized after the final vaccination (Fig. 4A). The frequencies of $MRP3_{765}$ peptide-specific CTLs before and after vaccination are shown in Figure 4C. The frequency of $MRP3_{765}$ peptide-specific CTLs increased after vaccination. However, the frequencies of HIVenv peptide-specific CTLs were unchanged during vaccination, and those of CMVpp65 peptide-specific CTLs fluctuated.

4. DISCUSSION

In previous studies, we performed a simultaneous and comparative analysis of immune responses to 27 different CTL epitopes derived from 14 TAAs using PBMCs from HCC patients to identify suitable TAA-derived epitopes for HCC immunotherapy [28]. We found that MRP3-derived peptides were frequently recognized by T cells and were capable of generating peptide-specific CTLs in HCC patients, suggesting that these peptides were immunogenic.

In this phase I study, immunotherapy using the $MRP3_{765}$ peptide in a formulation with the Montanide ISA-51 adjuvant was shown to be safe and immunogenic. The maximum toxicity observed was grade 3, according to the common terminology criteria, and it was actually considered to be due to the HAIC. Adverse events observed frequently were fever, ascites, and gastric ulcer, and these are also known common adverse events of HAIC [5; 24]. IFN-γ ELISPOT assays revealed that vaccination with the $MRP3_{765}$ peptide induced MRP3-specific immunity in 8 of 11 (72.7%) vaccinated patients. In previous studies of peptide vaccines for HCC, AFP, hTERT, and glypican-3 have been targeted as tumor-associated antigens for the treatment of advanced HCC [29; 30; 31; 32; 33]. In these studies, peptide-specific T cells were reported to be induced in 0–80% of the

patients vaccinated. Thus, the induction rate of TAA-derived peptide-specific T cell responses in $MRP3_{765}$ peptide vaccination was similar to those of AFP- or glypican-3-derived peptides, suggesting that the $MRP3_{765}$ peptide was immunogenic.

In phenotypic analyses of CTLs induced by the $MRP3_{765}$ peptide vaccine, the post-vaccination increase in $MRP3_{765}$ peptide-specific CTLs was due to increases in cells with the $CD45RA^-/CCR7^-$ (effector memory) and $CD45RA^+/CCR7^-$ (effector) phenotypes. A similar phenotypic analysis was reported by Butterfield et al. [34], who identified AFP-specific CTLs induced by AFP-derived peptide-pulsed DCs that were both naïve and of the central memory phenotype and failed to complete differentiation into effector and effector memory T cells. Differences between the present study and the previous one involved the administration method of the peptide vaccine and the use of combined therapy. In the previous study, they used dendritic cells pulsed with multiple peptides and with no additional treatment. All the patients in this study simultaneously received HAIC with the peptide vaccination. Recent studies reported that anti-tumor chemotherapy produced its anti-tumor effects by not only direct cytotoxic effects against tumor cells but also by the elimination or inactivation of cells with suppressive effects on tumor immunity, such as Tregs and MDSCs [35; 36]. In fact, the frequency of Tregs decreased after the treatment, which was consistent with the previously reported

responses of Tregs to chemotherapy against colon cancer using cyclophosphamide and against non-small cell lung cancer using paclitaxel [37; 38]. This may indicate more favorable conditions for the induction of peptide-specific T cells with effector memory or effector phenotypes.

Although peptide-specific CTLs and the effector memory phenotype could be induced in this study, the anti-tumor effects for HCC were limited, which is consistent with previous studies of peptide vaccines for HCC [31; 33]. One reason might be the slow speed and weakness of the anti-tumor effects induced by the peptide vaccine. In this study, the induction rate of $MRP3_{765}$ peptide-specific T cells and their frequencies during the first course of vaccination were low. Another reason may be the short life of $MRP3_{765}$ peptide-specific T cells. The frequency of $MRP3_{765}$ peptide-specific T cells decreased after 6 months in most patients, suggesting 3–6 injections of the $MRP3_{765}$ peptide was not sufficient to induce long-lived T cells. Furthermore, the advanced HCC stages of the patients or continued HAIC may affect the short life of $MRP3_{765}$ peptide-specific T cells. The frequency of MDSCs increased significantly after the treatment in some of the patients. This phenomenon is considered to be unfavorable to anti-tumor immunity. In fact, among the 8 patients who showed $MRP3_{765}$ peptide-specific CTLs in PBMCs after 1 or 2 course vaccination, 5 patients (patients B3, C1, C3, C4 and C5) did not show a

significant increase (more than 10%) of MDSCs (Table 3). In addition, all of the patients without a significant increase of MDSCs after vaccination showed PR or SD as a best study response. Although it is difficult to clearly state the relationship between the increase of MDSCs and the anti-tumor effect of vaccination because of small number of patients in this study, these results suggest that the small number of MDSCs in PBMCs might be favorable conditions for the induction of peptide-specific CTLs and contribute to the anti-tumor effect of peptide vaccination.

Regarding the effects of $MRP3_{765}$ peptide on the prognosis of HCC patients, obviously this is limited because our study was designed as a phase I study and did not involve a large population. However, the results of the present study suggest that the overall survival times of the patients treated with HAIC with the peptide vaccine was longer than those in previous studies including patients treated with HAIC with no peptide vaccination. To confirm these results, larger, later-stage clinical trials are necessary. Specifically, HAIC with more than 6 times injection of MRP3-derived peptide or the combination with the inhibitors of MDSCs such as low dose gemcitabine, cisplatin or 5-FU should be tried as a next clinical trial. Furthermore, previous studies have demonstrated that Sorafenib decreases the number of MDSCs [39; 40], and therefore the vaccination of MRP3-derived peptide with Sorafenib might be effective for patients with

HCC.

In conclusion, the feasibility of a MRP3-derived peptide vaccine has been demonstrated based on its safety, its effect on immune boosting, and even the possible prolongation of overall survival time. The potential to eradicate advanced HCC using a combination therapy of a peptide vaccine and chemotherapy should be further evaluated in additional studies.

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suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 34 (2004) 336-344.

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FIGURE LEGENDS

Figure 1. Immune responses of PBMCs to MRP3-derived peptide (Peptide 1, $MRP3_{765}$) or control peptides (Peptide 2, $HIVenv_{584}$ and Peptide 3, $CMVpp65_{328}$) in vaccinated HCC patients. (A) IFN-γ ELISPOT assays using PBMCs from vaccinated HCC patients and MRP3-derived or control peptides. White and black bars show the T cell responses before and after vaccination, respectively. * denotes more than 30 specific spots. (B) Peptide $MRP3_{765}$-specific tetramer was used for the detection of peptide vaccine-induced CTLs. The Representative results are shown (patient C6). The frequencies of $MRP3_{765}$ peptide-specific CTLs were 0.01% and 0.19% of $CD8^+$ T cells pre- and post-vaccination, respectively. (C) Phenotypic analysis of CTLs induced by vaccine. The memory phenotype of tetramer-positive cells was analyzed using CD45RA/CCR7 expression criteria. Phenotypic analysis of $MRP3_{765}$ peptide-specific $CD8^+$ T cells after vaccination showed that the frequencies of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells were 0.4%, 74.4%, and 24.8% of $MRP3_{765}$ $CD8^+$ $tetramer^+$ T cells, respectively. In contrast, phenotypic analysis of total $CD8^+$ T cells after vaccination showed that the frequencies of $CD45RA^-/CCR7^+$

(central memory), $CD45RA^{-}/CCR7^{-}$ (effector memory), and $CD45RA^{+}/CCR7^{-}$ (effector) T cells were 10.7%, 39.4%, and 38.6%, respectively.

Figure 2. Frequencies of Tregs and MDSCs in peripheral blood of HCC patients before and after HAIC with MRP3-derived peptide vaccination. (A) Gating strategy of $CD4^{+}$ $CD25^{+}$ $CD127^{-/low}$ Tregs. Percentages represent the proportions of $CD4^{+}$ $CD25^{+}$ $CD127^{-/low}$ Tregs among $CD4^{+}$ cells. The representative results are shown (patient A3). (B) Gating strategy of $CD14^{+}$HLA-$DR^{-/low}$ MDSCs. Percentages represent the proportions of $CD14^{+}$HLA-$DR^{-/low}$ MDSCs among $CD14^{+}$ cells. The representative results are shown (patient B2). (C) The frequency of MRP3-derived peptide-specific T cells before treatment was compared with that 2 weeks after final vaccination. The frequency of MRP3-derived peptide-specific T cells significantly increased after vaccination ($p = 0.007$). (D) The frequency of Tregs before treatment was compared with that 2 weeks after final vaccination. The frequency of Tregs decreased significantly after vaccination ($p = 0.040$). (E) The frequency of MDSCs increased significantly after vaccination ($p = 0.022$).

Figure 3. (A) The kinetics of $MRP3_{765}$ (peptide 1)-, $HIVenv_{584}$ (peptide 2)-, and

CMVpp65$_{328}$ (peptide 3)-specific T cell responses, as determined by IFN-γ ELISPOT assay in vaccinated patients. PBMCs were obtained at three different time points: before and 4 and 24 weeks after vaccination. Each graph indicates the kinetics of peptide-specific T cells in each patient. *1, *2, *3 *4, *5, *6 and *7 denote 231, 237, 232, 255, 99, 139 and 74 specific spots, respectively. (B) Progression-free survival (PFS) versus overall survival (OS) from the starting date of HAIC with peptide vaccination in HCC patients.

Figure 4. Representative clinical course of the patient with PR. (A) Time course of serum ALT, AFP, and DCP in the patient with PR (Patient B3). (B) Dynamic computed tomography imaging of a representative radiological response to MRP3-derived peptide in the patient with PR (Patient B3). Red arrows show the HCC lesion. (C) Immune responses to MRP3-derived peptide (Peptide 1, MRP3$_{765}$) or control peptides (Peptide 2, HIVenv$_{584}$ and Peptide 3, CMVpp65$_{328}$) in the HCC patient with PR (Patient B3). * denotes more than 20 specific spots.

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Table 1 Patient characteristics

Patient	Peptide Dose (mg)	Age	Sex	Etiology	Stage of HCC	ALT (IU/l)	AFP (ng/ml)	Liver cirrhosis (+/-)	Child-Pugh (A/B/C)	Tumor Size (mm) [1]	Number of tumors	Major portal vein invasion (+/-)	Extrahepatic lesion (+/-)	Diff. Degree[2]
A1	0.03	68	M	HCV	IVb	144	350	LC	B	51	10	-	+	mod
A2	0.03	66	M	HCV	III	35	9	LC	B	33	11	-	-	ND
A3	0.03	63	M	HCV	II	72	89	CH	B	16	12	-	-	well
B1	0.3	74	M	HCV	II	50	12	LC	B	20	16	-	-	ND
B2	0.3	73	M	NBNC	IVa	54	4023	LC	A	52	5	+	-	ND
B3	0.3	58	F	NBNC	III	28	181	CH	A	97	1	+	-	por
C1	3.0	72	M	HCV	II	22	86	LC	B	19	8	-	-	ND
C2	3.0	64	M	NBNC	IVa	24	284	LC	A	27	10	+	-	ND
C3	3.0	66	M	HCV	IVa	54	14890	LC	B	42	6	+	-	mod
C4	3.0	58	F	NBNC	III	31	20	LC	B	60	6	-	-	mod
C5	3.0	73	M	HCV	II	42	617	LC	B	16	6	-	-	ND
C6	3.0	71	M	NBNC	IVa	55	26	LC	A	77	10	+	-	por

HCV, hepatitis C virus; NBNC, not hepatitis B and C virus; LC, liver cirrhosis; CH, chronic hepatitis; wel, well differentiated; mod, moderately differentiated; por, poorly differentiated; ND, not determined.

[1]Tumor size was indicated as a diameter of the largest tumor in the liver. [2]Histological degree of HCC.

Table 2 Summary of toxicity

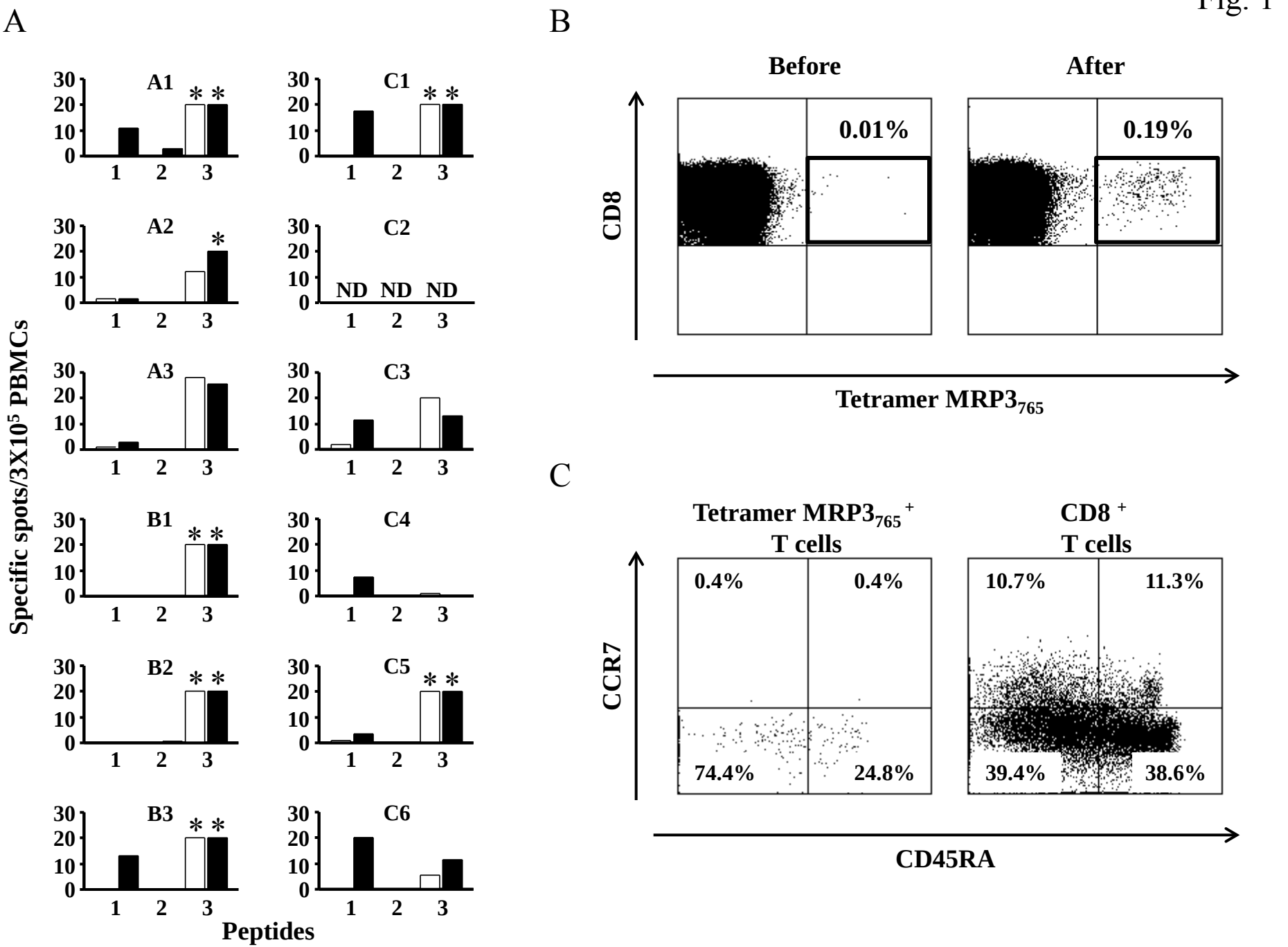
Toxicity	**Grade**			**Total patients**
	1	2	3	(n=12)(%)
Constitutional symptoms				
Fever	3	0	0	3(25.0)
Dizziness	1	0	0	1(8.3)
Gastrointestinal disorders				
Ascites	0	3	0	3(25.0)
Gastric ulcer	0	2	0	2(16.7)
Musculoskeletal and connective tissue disorders				
Arthralgia	1	0	0	1(8.3)
Investigations				
Neutrophil count decreased	0	1	0	1(8.3)
Platelet count decreased	0	0	1	1(8.3)

Table 3 Summary of immune and tumor responses after vaccination with the MRP3-derived peptide

Patient	ELISPOT			**Increase of MDSCs***	Best study response	Overall survival time
	Pre	After 1 course	After 2 course		(CR/PR/SD/PD)	(days)
A1	-	-	+	+	SD	272
A2	-	ND	-	+	SD	416
A3	-	-	+	+	SD	514
B1	-	ND	-	-	SD	938
B2	-	-	ND	+	PD	561
B3	-	+	+	-	PR	552
C1	-	ND	+	-	SD	454
C2	-	ND	ND	ND	PD	47
C3	-	+	ND	-	SD	105
C4	-	-	+	-	SD	340
C5	-	-	+	-	SD	586
C6	-	ND	+	+	SD	333

A significant increased % of MDSCs after vaccination was defined as more than 10%.

ND: not determined.


A
A1
C1
A2
C2
A3
C3
B1
C4
B2
C5
B3
C6
ND ND ND
Specific spots/3X10^5 PBMCs
Peptides
B
Before
After
0.01%
0.19%
CD8
Tetramer MRP3765
C
Tetramer MRP3765 + T cells
CD8 + T cells
0.4%
0.4%
74.4%
24.8%
10.7%
11.3%
39.4%
38.6%
CCR7
CD45RA

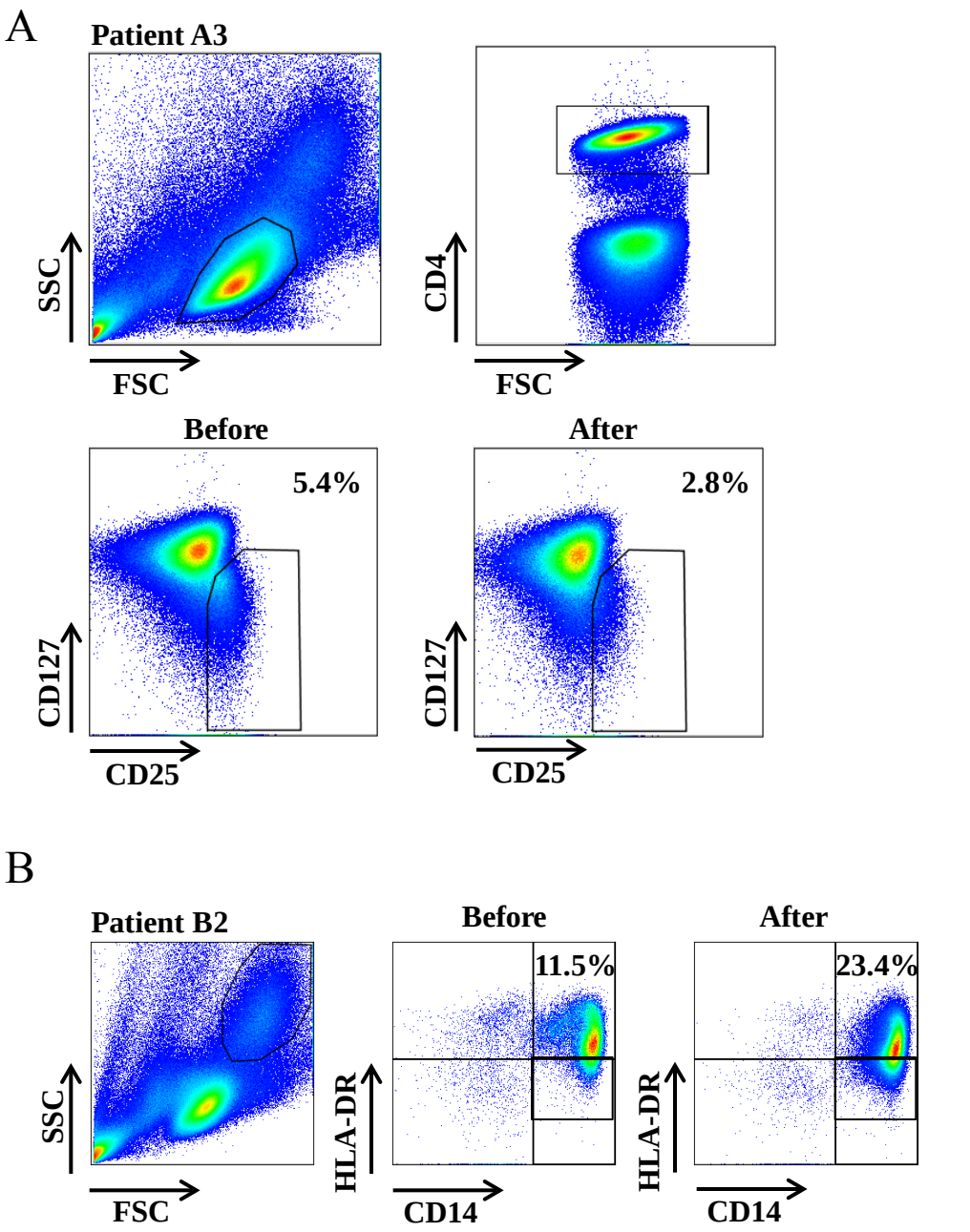
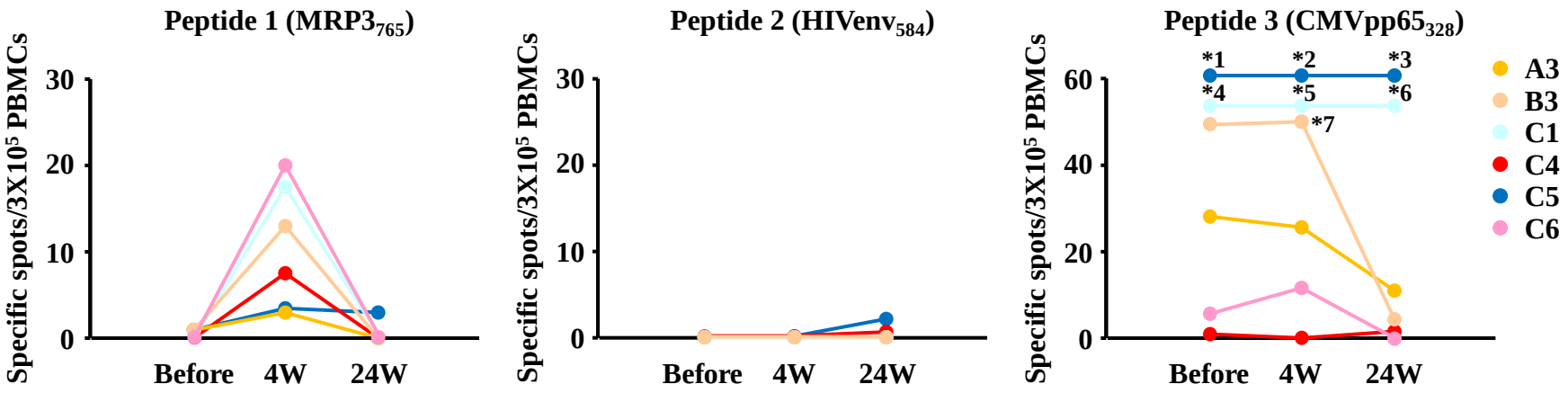

A
Patient A3
SSC
FSC
CD4
FSC
Before
After
5.4%
2.8%
CD127
CD25
CD127
CD25
B
Patient B2
SSC
FSC
Before
After
11.5%
23.4%
HLA-DR
CD14
HLA-DR
CD14
C
P=0.007
Specific spots/3X10⁵ PBMCs
20
10
0
Before
After
A1
A2
A3
B1
B2
B3
C1
C3
C4
C5
C6
D
P=0.040
% Tregs
16
8
0
Before
After
E
P=0.022
% MDSCs
60
30
0
Before
After

Fig. 3

A


Peptide 1 (MRP3765)
Peptide 2 (HIVenv584)
Peptide 3 (CMVpp65328)
Specific spots/3X10^5 PBMCs
Before
4W
24W
*1
*2
*3
*4
*5
*6
*7
A3
B3
C1
C4
C5
C6

B

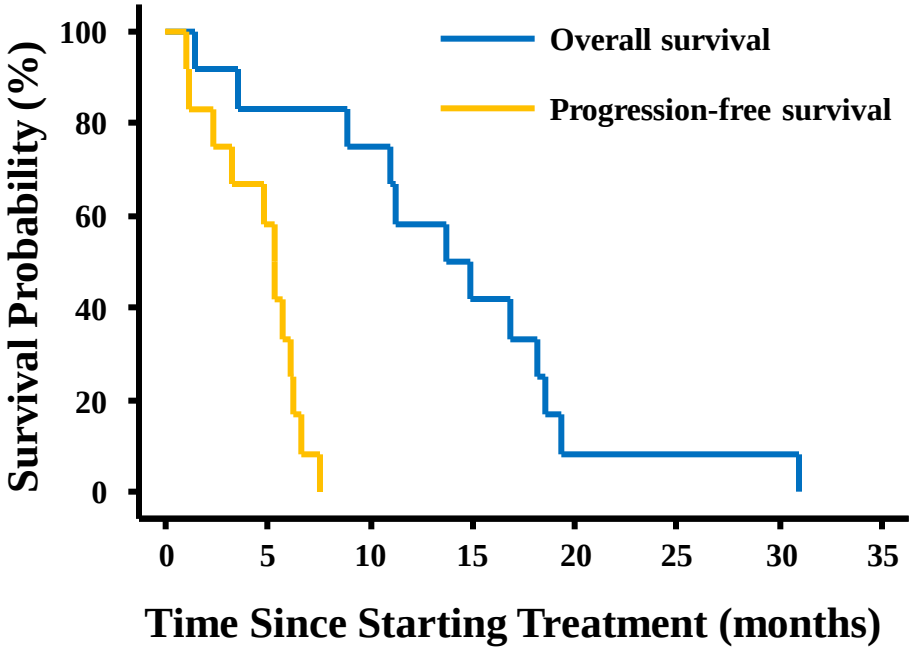
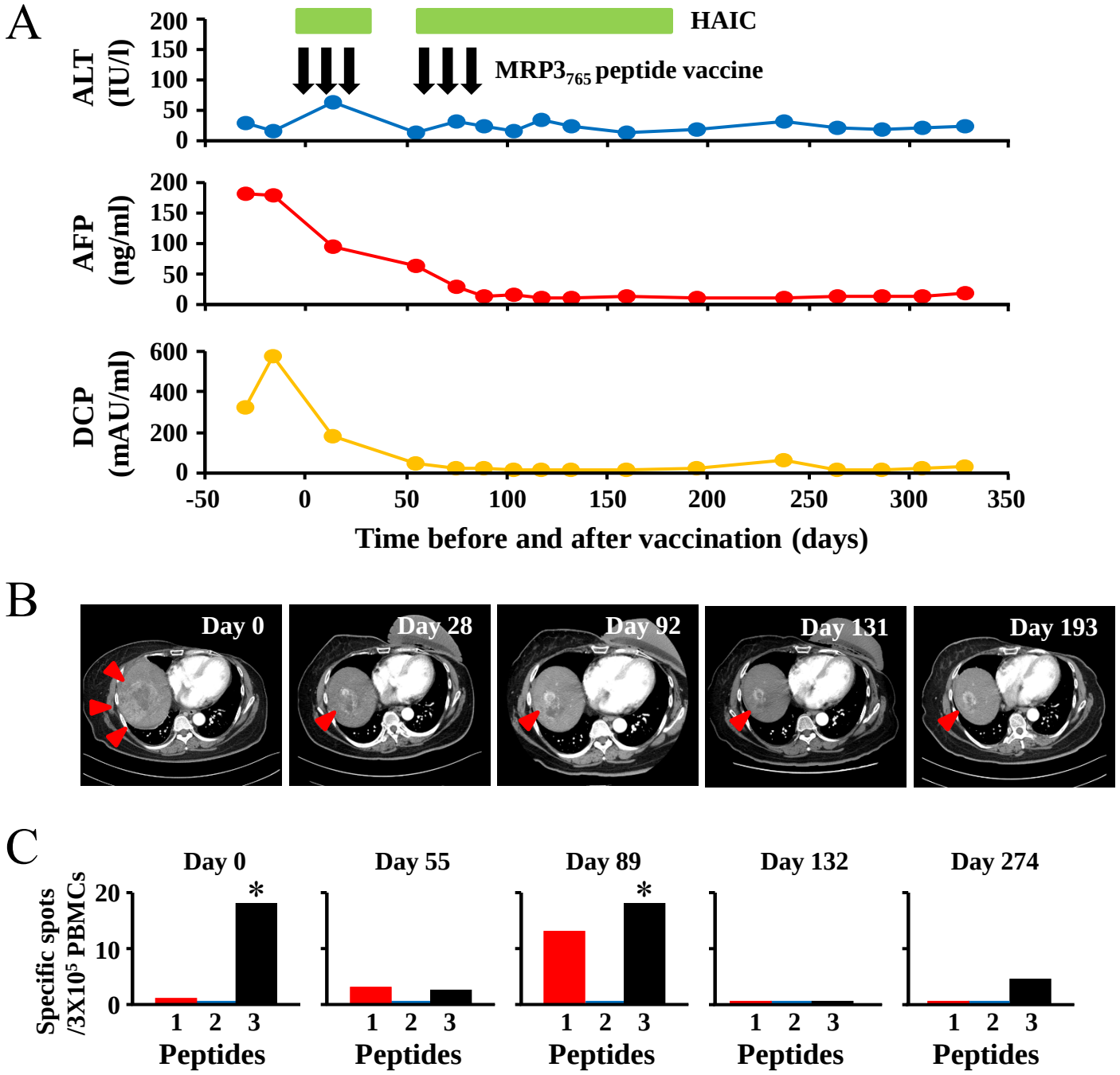

Survival Probability (%)
Overall survival
Progression-free survival
Time Since Starting Treatment (months)

Fig. 4


A
HAIC
MRP3765 peptide vaccine
ALT (IU/l)
AFP (ng/ml)
DCP (mAU/ml)
Time before and after vaccination (days)
B
Day 0
Day 28
Day 92
Day 131
Day 193
C
Day 0
Day 55
Day 89
Day 132
Day 274
Specific spots /3X10⁵ PBMCs
Peptides