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ABSTRACT

Hepatic arterial infusion chemotherapy (HAIC) has been employed as an alternative therapy to sorafenib for the patients with advanced hepatocellular carcinoma (HCC). In this study, we performed a comparative analysis of various immune cell responses including tumor-associated antigen (TAA)-specific T cells, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in advanced HCC patients treated with HAIC. Thirty-six HCC patients were examined in the study. Interferon gamma enzyme-linked immunospot assays were performed to examine the frequency of TAA-specific T cells. The frequencies of Tregs and MDSCs were examined by multicolor fluorescence-activated cell sorting analysis. The treatment with HAIC using interferon (IFN)/ 5-fluorouracil (FU) or IFN/FU + cisplatin modulated the frequencies of various immune cells. In 22.2% of patients, the frequency of TAA-specific T cells increased after HAIC. Although the frequency of Tregs decreased after HAIC, it was not associated with the prognosis of patients. An analysis of prognostic factors for overall survival identified diameter of the tumor (<3.0 cm), absence of major portal vein invasion, absence of distant metastasis, Union Internationale Contre Le Cancer tumor lymph node metastasis stage (I or II), neutrophil lymphocytic ratio (<2.1) and the frequency of MDSCs (<30.5%) as factors that prolonged overall survival time after HAIC. Even in the group adjusted with progressive levels of tumors, patients with a low frequency of MDSCs had a significantly longer overall survival time. In conclusion, the frequency of MDSCs before the treatment is a prognostic factor in HAIC against HCC.

Keywords: immunotherapy, CTL, regulatory T cell, cancer, MDSC

Précis: Hepatic arterial infusion chemotherapy (HAIC) for hepatocellular carcinoma (HCC) modulates the frequencies of various immune cells. The frequency of MDSCs before the treatment is a prognostic factor in patients treated with HAIC against HCC.

Author contributions: Eishiro Mizukoshi, Hidetoshi Nakagawa and Kazumi Fushimi performed and analyzed the experiments. Eishiro Mizukoshi, Tatsuya Yamashita, Kuniaki Arai, Takeshi Terashima, Masaaki Kitahara, Noriho Iida and Shuichi Kaneko designed the clinical trial protocol and experiments. Eishiro Mizukoshi, Tatsuya Yamashita and Takeshi Terashima wrote the manuscript. Eishiro Mizukoshi, Noriho Iida and Shuichi Kaneko edited the manuscript.

Alphabetical list of Abbreviations

AFP, alpha-fetoprotein

BCLC, Barcelona clinic liver cancer

CD, cluster of differentiation

CMV, cytomegalovirus

CR, complete response

CTL, cytotoxic T lymphocyte

ELISPOT, enzyme-linked immunospot

FACS, fluorescence-activated cell sorter/sorting

FCS, fetal calf serum

FOXp3, forkhead box p3

FU, fluorouracil

HAIC, hepatic arterial infusion chemotherapy

HBV, hepatitis B virus

HBsAg, hepatitis B virus surface antigen

HCC, hepatocellular carcinoma

HCV, hepatitis C virus

HCVAb, hepatitis C virus antibody

HIV, human immunodeficiency virus

HLA, human histocompatibility leukocyte antigen

HPLC, high performance liquid chromatography

IFN, interferon

IL, interleukin

MDSC, myeloid-derived suppressor cell

NLR, neutrophil lymphocytic ratio

PBMC, peripheral blood mononuclear cell

PBS, phosphate-buffered saline

PD, progressive disease

PD-L1, programmed death ligand 1

PMA, phorbol myristate acetate

PR, partial response

RFA, radiofrequency ablation

RPMI, Roswell Park Memorial Institute (culture medium)

SD, stable disease

TAA, tumor-associated antigen

TACE, transarterial chemoembolization

TGF, transforming growth factor

TNM, tumor lymph node metastasis stage

Treg, regulatory T cell

UICC, Union Internationale Contre Le Cancer

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and remains an important public health concern because its incidence has continued to increase globally [2]. Many different kinds of approaches, such as surgical resection, liver transplantation, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), chemotherapy, and sorafenib, are performed according to practical guidelines for the treatment of HCC [3]; however, their effects are limited and the recurrence rate of HCC remains very high [4]. Moreover, patients who have already developed HCC are mostly in an advanced stage at the time of diagnosis and, as such, the efficacy of surgical resection, RFA, and TACE is limited for such cases. Although sorafenib has been shown to have a significant survival benefit in several large-scale clinical trials and has been established as the standard treatment for advanced HCC [5], its efficacy and tolerability are also limited [6]. Hepatic arterial infusion chemotherapy (HAIC) has been employed as an alternative therapy to sorafenib in Japan and other Asian countries [7, 8].

The modulation of tumor-associated host immune responses after cancer treatments has recently been reported. For example, HCC treatments with RFA or TACE were found to increase the frequency of tumor-associated antigen (TAA)-specific T cells due to the creation of an antigen source by the destruction of tumor cells [9, 10]. In addition, recent studies demonstrated that beyond their direct cytotoxic effects on cancer cells, several conventional chemotherapeutic drugs promoted the elimination or inactivation of regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), resulting in

enhanced host antitumor immunity [11, 12]. These findings support the immunotherapeutic effects of HAIC for HCC. However, the effects of HAIC on host immune responses and the relationship between the prognosis of patients treated with HAIC and host immune responses remain unclear.

In the present study, we performed a comparative analysis of various immune cell responses including TAA-specific T cells, Tregs, and MDSCs in advanced HCC patients treated with HAIC.

MATERIALS AND METHODS

Patients and laboratory testing

Thirty-six human histocompatibility leukocyte antigen (HLA)-A24-positive patients who had clinically diagnosed HCC were examined in the present study (Table 1). HCC was diagnosed based on underlying chronic liver disease, radiological findings, and elevations in tumor markers. Regarding the 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. 5 or more nodules in the left and/or right lobes as confirmed by radiology). After being diagnosed, all patients were treated with HAIC using interferon (IFN)/ 5-fluorouracil (FU) or IFN/FU + cisplatin at Kanazawa University Hospital between October 2003 and September 2007. All of the patients in this study were first line patients. In the IFN/FU treatment group, 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 1st and 2nd weeks (for 120 h) using an infuser pump (Baxter Infusor SV1, Tokyo, Japan) as described previously [13]. The maximum amount of FU infused over 5 days was 2,500 mg. IFN α-2b (Intron A, Schering-Plough, Osaka, Japan) at a dose of 3,000,000 units was injected intramuscularly 3 times a week for 4 weeks. In the IFN/FU + cisplatin treatment group, cisplatin (Randa, Nippon Kayaku, Tokyo, Japan) at a dose of 20 mg/m^2 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.

The response rate after HAIC was determined using dynamic computed tomography or magnetic resonance imaging performed at the end of each treatment cycle according to the Response Evaluation Criteria in Solid Tumors, version 1.1 [14]. The overall survival time was defined as the period from the time of beginning of treatment until death, and the progression-free survival time was defined as the period from the beginning of the treatment until the confirmation of progression or death.

Blood samples were tested for hepatitis B virus surface antigen (HBsAg) and hepatitis C virus antibody (HCVAb) using commercial immunoassays (Fuji Rebio, Tokyo, Japan). The HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described previously [15]. Serum alpha-fetoprotein (AFP) levels were measured by an enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan).

All patients provided 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, No. 5169).

Peptides and preparation of PBMCs

Eleven peptides derived from 5 different TAAs (Supplementary Table 1) [10], a human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$) [16], and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$) [17], which were previously identified as HLA-A24-restricted cytotoxic T lymphocyte (CTL) epitopes were used. Peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be

>90% by analytical high performance liquid chromatography (HPLC). PBMCs were isolated before and 2-4 weeks after starting HAIC as described below; heparinized venous blood was diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque (Sigma, St. Louis, Mo.) in 50-ml tubes. After centrifugation at 2000 rpm for 20 min at room temperature, PBMCs were harvested from interphase, resuspended in PBS, centrifuged at 1400 rpm for 10min, and then resuspended in complete culture medium consisting of Roswell Park Memorial Institute (RPMI) culture medium (GibcoBRL, Grand Island, NY), 10% heat inactivated fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). PBMCs were resuspended in RPMI 1640 medium containing 80% FCS and 10% dimethyl sulfoxide, and cryopreserved until use.

IFN-γ ELISPOT assay

IFN-γ enzyme-linked immunospot (ELISPOT) assays were performed as reported previously [18]. Negative controls consisted of a HIV envelope-derived peptide ($HIVenv_{584}$) [16]. Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) [17]. The amino acid sequences of all peptides are shown in Supplementary Table 1. 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 TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviation of the baseline response detected in 11 normal blood donors (Supplementary Table 1), and if the number of spots in the presence of an

antigen was at least twofold that in its absence. Responses to HIV- and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least twofold that in its absence.

Flow cytometric analysis

A multicolor fluorescence-activated cell sorting analysis was carried out using the Becton Dickinson FACSAria II system to determine the frequency and phenotype of Tregs and MDSCs. The following anti-human monoclonal antibodies were used: anti-cluster of differentiation (CD)4 (Becton Dickinson), anti-CD11b (Becton Dickinson), anti-CD14 (Becton Dickinson), anti-CD15 (Becton Dickinson), anti-CD25 (Becton Dickinson), anti-CD33 (Becton Dickinson), anti-forkhead box p3 (FOXp3) (Becton Dickinson), anti-CD127 (Becton Dickinson), anti-programmed death ligand 1 (PD-L1) (BioLegend) and anti-HLA-DR (Becton Dickinson).

Cytokine profiling

Cytokine levels in serum were measured using the Procarta®Cytokine Assay (Affymetrix, Santa Clara, CA) as previously reported [19]. These included interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10 and transforming growth factor (TGF)-β1. Data acquisition and analysis were carried out using Bio-plex Manager software version 4.1.1.

Statistical analysis

Data are expressed as means ± standard deviation. The chi-squared test with Yates' correction, paired *t*-test, and unpaired *t*-test were used for statistical analyses where

appropriate. The probabilities of overall survival were estimated using the Kaplan-Meier method. The Mantel-Cox log-rank test was used to compare curves between groups. The prognostic factors for overall survival were analyzed for significance by the Kaplan–Meier method (univariate). A level of $p<0.05$ was considered significant.

RESULTS

Patient profiles

The clinical profiles of the 36 patients with advanced HCC who participated in the present study are shown in Table 1. Major portal vein invasion, lymph node metastasis, and distant metastasis were noted in 15, 1, and 3 patients, respectively. Using Barcelona clinic liver cancer (BCLC) TNM staging, 19, 15, and 2 patients were classified as stage B, C, and D, respectively. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th version), 4, 29, and 3 patients were classified as stage II, III and IV, respectively. Thirty patients had liver cirrhosis and 16, 18, and 2 patients were classified as grade A, B, and C according to the Child-Pugh classification. Among the 36 patients, the best study response was a complete response (CR) in 1 (2.8%) patient; a partial response (PR) was observed in 18 (50.0%) patients, stable disease (SD) was observed in 6 (16.7%) patients, and progressive disease (PD) was observed in 11 (30.6%) patients.

Detection of TAA-specific T cells

The detection of TAA-specific T cells was performed by a direct *ex-vivo* analysis (IFN-γ ELISPOT assay). The cut-off of positivity was set at ≥10 specific spots/300,000 PBMCs (see *Materials and Methods*). We examined the frequency of cells that specifically reacted with TAA-derived and control peptides using PBMCs obtained before the treatment with HAIC. Nineteen responses were observed against TAA-derived peptides. Eleven out of 36 (30.6%) patients showed positive responses to

at least one TAA-derived peptide, while most showed responses to 1-3 kinds of TAA-derived peptide. The magnitude of TAA-specific T cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population. The range of the TAA-derived peptide-specific T cell frequency was 10.0-67.0 cells/300,000 PBMCs. The frequencies of T cells specific to CMV-derived peptides were 10.0-299.5 cells/300,000 PBMCs, respectively.

We performed the same analysis using PBMCs obtained 2-4 weeks after starting HAIC. When T cell responses against a single peptide with more than or equal to 10 specific spots and a 2-fold increase were defined as significant, the number of patients who showed a significant increase against at least one TAA-derived peptide after HAIC was 8 (22.2%). However, no significant differences were observed in the frequency of patients who specifically reacted with TAA-derived and control peptides after the treatment (Supplementary Table 1).

Detection of Tregs and MDSCs

We examined the frequency of Tregs and MDSCs in peripheral blood in order to identify the effects of HAIC on immune suppressor cells. The population of Tregs was detected as $CD4^+$ $CD25^+$ $CD127^{-/low}$ cells as previously reported (Fig. 1a) [20]. We also confirmed that this population was highly positive for CD25 and FOXp3. The frequency of Tregs varied greatly (1.9 to 12.1%) in HCC patients before HAIC and was significantly decreased after the treatment ($p=0.043$) (Fig. 1b).

Human MDSCs are classified to $CD14^-CD11b^+CD33^+CD15^+$ and $CD14^+HLA\text{-}DR^-$ cells [21]. In the present study, we identified both fractions in PBMCs of HCC patients and focused in $CD14^+HLA\text{-}DR^-$ phenotype because the recent studies have shown that

the frequency of MDSCs with $CD14^{+}HLA\text{-}DR^{-}$ phenotype is increased in HCC patients (Fig. 1c) [20]. $CD14^{+}HLA\text{-}DR^{-}$ and $CD14^{-}CD11b^{+}CD33^{+}CD15^{+}$ MDSCs showed low expression of PD-L1 before treatment. The fraction of $CD14^{+}HLA\text{-}DR^{-/low}$ detected in this study showed 2 cell populations with low and high PD-L1 expression. After HAIC treatment, the expression levels of PD-L1 in $CD14^{+}HLA\text{-}DR^{-}$ MDSCs were increased in most of the patients examined (Supplementary Fig. 1b and c). The $CD14^{+}HLA\text{-}DR^{-/low}$ population in the PBMCs of HCC patients represented 10.5% to 71.3% of $CD14^{+}$cells before HAIC. In contrast to Tregs, no significant differences were observed in the frequency of MDSCs after HAIC (p=0.609) (Fig. 1d). We observed a decrease in serum TGF-β1 concentration after HAIC (Supplementary Fig. 1a). The concentrations of other cytokines did not change with statistical significance before and after the treatment.

Identification of host factors related to the frequencies of Tregs and MDSCs

To identify host factors related to the frequencies of Tregs and MDSCs, we compared the frequencies of these immune suppressor cells with various clinical factors. Although the frequency of circulating Tregs is known to correlate with disease progression in cancer patients, the frequency of $CD4^{+}$ $CD25^{+}$ $CD127^{-/low}$ Tregs did not correlate with the status of tumor progression, which consisted of the UICC stage, BCLC stage, diameter of the tumor, major portal vein invasion, and distant metastasis as well as other clinical factors (Fig. 2a).

In contrast to Tregs, the frequency of MDSCs was associated with these tumor factors. Patients with the advanced UICC stage, a large tumor diameter, major portal vein invasion, and distant metastasis of HCC showed a high frequency of MDSCs in peripheral blood (Fig. 2b). Patients with a high neutrophil to lymphocyte ratio (NLR),

which has been suggested to be associated with the outcomes of patients with various malignancies [22, 23], also showed a high frequency of MDSCs.

Patient outcomes and frequencies of Tregs and MDSCs

We examined the relationship between patient outcomes and the frequencies of Tregs and MDSCs. Patients were divided into two groups: patients with CR or PR and those with SD or PD, and the frequencies of Tregs and MDSCs were compared between these groups before and after the treatment. No significant differences were observed in the frequency of Tregs between these groups before and after the treatment (Fig. 3a and b). In contrast, the frequency of MDSCs was significantly lower in the group with CR or PR before the treatment than in the group with SD or PD ($p=0.006$) (Fig. 3c). Similar results were also obtained in the analysis of MDSCs after the treatment ($p=0.043$) (Fig. 3d).

Effects of immune cells on the prognosis of patients treated with HAIC

To determine the effects of various immune cells on the prognosis of HCC patients treated with HAIC, we analyzed the relationship between the frequencies of immune cells, consisting of TAA-specific T cells, Tregs, and MDSCs, before the treatment and overall survival after HAIC. To analyze TAA-specific T cells, we divided patients into two groups: those with high (above median) and low (below median) specific spots as detected by the ELISPOT assay. We did not find any associations between the frequency of TAA-specific T cells, which was calculated as a total number of T cells detected by IFN-γ ELISPOT assay using 11 TAA-derived peptides (Supplementary Table 1), and overall survival after HAIC (Fig. 4a). The frequency of Tregs before the treatment was

also not associated with the overall survival of patients treated with HAIC (Fig. 4b). In contrast, the overall survival of patients with a high frequency of MDSCs before the treatment was significantly shortened (p=0.003) (Fig. 4c). The difference between the groups was emphasized when 40% was defined as high (p<0.001) (Fig. 4d).

A univariate analysis of prognostic factors for overall survival identified NLR (<2.1), diameter of the tumor (<3.0 cm), absence of major portal vein invasion, absence of distant metastasis, BCLC TNM stage (A or B), UICC TNM stage (I or II), and the frequency of MDSCs (<30.5%) as factors that prolonged the overall survival time after HAIC (Table 2). Since the number of patients was insufficient for a multivariate analysis of prognostic factors for overall survival and the frequency of MDSCs was associated with the progression of tumors, as shown in Figure 4e, we examined the relationship between the frequency of MDSCs and overall survival after HAIC in a limited patient group with only UICC TNM stage II or III. Even in the group adjusted with progressive levels of tumors, patients with a low frequency of MDSCs had a significantly longer overall survival time (p=0.010) (Fig. 4e).

DISCUSSION

Increases have been reported in the frequency of TAA-specific T cells after treatments for HCC such as RFA and TACE [9, 10]. In the present study, we found an increase in the frequency of TAA-specific T cells after HAIC in a very limited number of patients, which was unexpected. The 36 patients evaluated in this study included 19 who showed CR or PR to the treatment and decreases in the tumor size. However, an analysis of changes in the frequency of T cells specific to each epitope revealed increases in only 4 out of the 19 patients. This result suggested that HAIC using cisplatin and 5FU induced necrosis or apoptosis in tumor tissue, but was not appropriate for inducing anti-tumor immunity, unlike RFA and TACE.

Recent studies reported that anti-tumor chemotherapy produced its anti-tumor effects by not only direct cytotoxic effects against tumor cells, but also the elimination or inactivation of cells with suppressive effects on tumor immunity such as Tregs and MDSCs [11, 12]. In the present study, the frequency of Tregs also decreased after the treatment, which was consistent with the previously reported responses of Tregs to chemotherapy against colon cancer using cyclophosphamide and non-small cell lung cancer using paclitaxel [24, 25]. The decrease was more prominent in patients with the higher frequency of Tregs pretreatment, suggesting the treatment might be more effective in such patients. However, the decrease observed in the number of Tregs did not appear to contribute to the increase in the frequency of TAA-specific T cells.

No significant changes were observed in the frequency of MDSCs after the treatment. The chemotherapy evaluated in this study included 5FU, which has been

reported to reduce the frequency of MDSCs in peripheral blood in mouse thymoma and colorectal cancer models and humans with colorectal cancer [26, 27]. Similar results were not obtained in the present study using 5FU presumably because of the difference in its administration route, which was via the hepatic artery. Previous studies on the pharmacokinetics of 5FU reported that the concentration of 5FU in peripheral blood was lower by an administration route via the hepatic artery than by an intravenous administration route [28], and this is considered to have been a reason for the failure in this study to reduce the frequency of MDSCs. There was no statistically significant difference between the patients treated with IFN/5FU and IFN/5FU + cisplatin regarding the clinical data and the frequency of TAA-specific T cells, Tregs and MDSCs before and after the treatment.

We previously reported that the frequency of MDSCs decreased after RFA, a standard treatment for HCC, and also that the number of TAA-specific T cells after this treatment negatively correlated with the frequency of MDSCs [10]. In this study, in 8 patients showing increased TAA-specific T cell responses to at least one TAA-derived peptide after HAIC, 3 and 2 patients showed a decreasing of number of Tregs and MDSCs, respectively. Although the absence of an increase in the number of TAA-specific T cells after chemotherapy in the present study might be related to a failure in the induction of a decrease in the frequency of MDSCs by the treatment, the relationship should be further studied. In addition, one of the limitations of this study is that the function of Tregs and MDSCs has not been investigated. It also should be further studied.

An analysis of the relationships between TAA-specific T cells, Tregs, and MDSCs and the therapeutic effects observed revealed that the frequency of MDSCs before the treatment was significantly lower in the patients that showed CR or PR to HAIC than in

those showed SD or PD. No significant difference was observed in the frequency of TAA-specific T cells or Tregs before the treatment and the frequency of these 3 kinds of cells after the beginning of the treatment. Furthermore, an analysis of patient outcomes after the treatment showed a longer overall survival period in patients with a lower frequency of MDSCs before the treatment. These results were consistent with previous findings in many cancer types [29] and suggested that the frequency of MDSCs is an important prognostic factor in HCC patients undergoing HAIC.

Regarding host factors that affect the frequency of MDSCs, the degree of tumor progression indicated by the UICC stage, BCLC stage, diameter of the tumor, major portal vein invasion, and distant metastasis correlated with a high frequency of MDSCs, i.e., the frequency of MDSCs increased in patients with more advanced disease. Furthermore, a univariate analysis of factors contributing to overall survival identified a low frequency of MDSCs, low UICC or BCLC stage, tumors with small diameters, and the absence of major portal vein invasion and distant metastasis as significant factors. Since a multivariate analysis was not performed in this study due to the lack of a sufficient number of patients, it currently remains unknown whether the frequency of MDSCs influenced patient outcomes directly via immunological mechanisms or indirectly by reflecting the degree of tumor progression. Therefore, in order to elucidate the relationship between the frequency of MDSCs and patient outcomes in greater detail, we examined the relationship between the frequency of MDSCs and overall survival in UICC stage II or III patients. In this analysis, the overall survival was significantly longer in the group with a lower frequency of MDSCs even with standardization of the disease stage, suggesting that the frequency of MDSCs was an independent factor contributing to overall survival. Moreover, we recently reported a higher recurrence-free

survival rate and longer overall survival in patients with a low NLR before the treatment and suggested that the NLR was a prognostic factor in HAIC [30]. The frequency of MDSCs was lower in patients with a low NLR, suggesting that they are related. A review of the literature revealed that the relationship between MDSCs and the NLR has not yet been directly demonstrated, the NLR in HCC has been shown to reflect systemic or intratumoral micro-environmental inflammatory responses and correlated with intratumoral or systemic IL-17 concentrations [31]. Since MDSCs are induced by systemic inflammatory responses and are related to Th17 cell differentiation [32], an increase in the NLR has been suggested to be caused by an increase in MDSCs or the cytokines produced by them. These results also indicate that MDSCs are related to patient outcomes by some immunological mechanism.

In conclusion, this study showed that the frequency of MDSCs before the treatment was a prognostic factor in HAIC against HCC. The results of this study, which suggest that the frequency of MDSCs is a good clinical marker for the selection of patients treated with HAIC and the effectiveness of HAIC could be enhanced by controlling the frequency of MDSCs before the treatment, provide a useful insight into devising therapeutic strategies against HCC. The patients with high frequency of MDSCs before treatment might be better to be treated with another chemotherapeutic agents or sorafenib.

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REFERENCES

1. Mizukoshi E, Yamashita T, Arai K, Terashima T, Kitahara M, Nakagawa H, Iida N, Fushimi K, Kaneko S (2015) Myeloid-derived suppressor cells correlate with patient outcomes in hepatic arterial infusion chemotherapy for hepatocellular carcinoma. Hepatology. 62(S1): 408A. Abstract No. 392. doi: 10.1002/hep.28212

2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 127: 2893-917.

3. Bruix J, Sherman M (2011) Management of hepatocellular carcinoma: an update. Hepatology. 53: 1020-2.

4. Lencioni R (2010) Loco-regional treatment of hepatocellular carcinoma. Hepatology. 52: 762-73.

5. Llovet JM, Ricci S, Mazzaferro V et al. (2008) Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 359: 378-90.

6. Kaneko S, Furuse J, Kudo M et al. (2012) Guideline on the use of new anticancer drugs for the treatment of Hepatocellular Carcinoma 2010 update. Hepatol Res. 42: 523-42.

7. Terashima T, Yamashita T, Arai K et al. (2014) Feasibility and efficacy of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma after sorafenib. Hepatol Res. 44: 1179-85.

8. Song DS, Song MJ, Bae SH et al. (2015) A comparative study between sorafenib and hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma with portal vein tumor thrombosis. J Gastroenterol. 50: 445-54.

9. Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, Burroughs AK, Meyer T, Behboudi S (2007) Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. J Immunol. 178: 1914-22.

10. Mizukoshi E, Yamashita T, Arai K, Sunagozaka H, Ueda T, Arihara F, Kagaya T, Fushimi K, Kaneko S (2013) Enhancement of tumor-associated antigen-specific T cell responses by radiofrequency ablation of hepatocellular carcinoma. Hepatology. 57: 1448-57.

11. Alizadeh D, Larmonier N (2014) Chemotherapeutic targeting of cancer-induced immunosuppressive cells. Cancer Res. 74: 2663-8.

12. Zheng Y, Dou Y, Duan L, Cong C, Gao A, Lai Q, Sun Y (2015) Using chemo-drugs or irradiation to break immune tolerance and facilitate immunotherapy in solid cancer. Cell Immunol. 294: 54-9.

13. Ota H, Nagano H, Sakon M et al. (2005) Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br J Cancer. 93: 557-64.

14. Eisenhauer EA, Therasse P, Bogaerts J et al. (2009) New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer. 45: 228-47.

15. Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T, Kaneko S (2006) Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma. Int J Cancer. 118: 1194-204.

16. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M (1997) Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol. 159: 6242-52.

17. Kuzushima K, Hayashi N, Kimura H, Tsurumi T (2001) Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood. 98: 1872-81.

18. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, Kuzushima K, Takiguchi M, Kaneko S (2006) Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. Hepatology. 43: 1284-94.

19. Mizukoshi E, Nakagawa H, Kitahara M et al. (2015) Immunological features of T cells induced by human telomerase reverse transcriptase-derived peptides in patients with hepatocellular carcinoma. Cancer Lett. 364: 98-105.

20. Arihara F, Mizukoshi E, Kitahara M, Takata Y, Arai K, Yamashita T, Nakamoto Y, Kaneko S (2013) Increase in CD14+HLA-DR -/low myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. Cancer Immunol Immunother. 62: 1421-30.

21. Marvel D, Gabrilovich DI (2015) Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. J Clin Invest. 125: 3356-64.

22. Luo G, Guo M, Liu Z et al. (2015) Blood neutrophil-lymphocyte ratio predicts survival in patients with advanced pancreatic cancer treated with chemotherapy. Ann Surg Oncol. 22: 670-6.

23. Tohme S, Sukato D, Chalhoub D et al. (2014) Neutrophil-Lymphocyte Ratio is

a Simple and Novel Biomarker for Prediction of Survival after Radioembolization for Metastatic Colorectal Cancer. Ann Surg Oncol. 22:1701-7.

24. Ghiringhelli F, Larmonier N, Schmitt E et al. (2004) CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol. 34: 336-44.

25. Zhang L, Dermawan K, Jin M et al. (2008) Differential impairment of regulatory T cells rather than effector T cells by paclitaxel-based chemotherapy. Clin Immunol. 129: 219-29.

26. Vincent J, Mignot G, Chalmin F et al. (2010) 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res. 70: 3052-61.

27. Kanterman J, Sade-Feldman M, Biton M, Ish-Shalom E, Lasry A, Goldshtein A, Hubert A, Baniyash M (2014) Adverse immunoregulatory effects of 5FU and CPT11 chemotherapy on myeloid-derived suppressor cells and colorectal cancer outcomes. Cancer Res. 74: 6022-35.

28. Wagner JG, Gyves JW, Stetson PL, Walker-Andrews SC, Wollner IS, Cochran MK, Ensminger WD (1986) Steady-state nonlinear pharmacokinetics of 5-fluorouracil during hepatic arterial and intravenous infusions in cancer patients. Cancer Res. 46: 1499-506.

29. Gabitass RF, Annels NE, Stocken DD, Pandha HA, Middleton GW (2011) Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. Cancer Immunol Immunother. 60: 1419-30.

30. Terashima T, Yamashita T, Iida N et al. (2014) Blood neutrophil to lymphocyte

ratio as a predictor in patients with advanced hepatocellular carcinoma treated with hepatic arterial infusion chemotherapy. Hepatol Res. in press.

31. Motomura T, Shirabe K, Mano Y et al. (2013) Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. J Hepatol. 58: 58-64.

32. Ma S, Cheng Q, Cai Y et al. (2014) IL-17A produced by gammadelta T cells promotes tumor growth in hepatocellular carcinoma. Cancer Res. 74: 1969-82.

FIGURE LEGENDS

Figure 1: Frequencies of Tregs and MDSCs in peripheral blood of HCC patients before and 2-4 weeks after starting HAIC. **(a)** Gating strategy of $CD4^+$ $CD25^+$ $CD127^{-/low}$ Tregs. $CD4^+$ $CD25^+$ $CD127^{-/low}$ Tregs highly expressed the CD25 molecule and FOXp3 (lower panels). **(b)** The frequency of Tregs before HAIC was compared with that 2-4 weeks after starting HAIC. Percentages represent the proportions of $CD4^+$ $CD25^+$ $CD127^{-/low}$ Tregs among $CD4^+$ cells. The frequency of Tregs varied greatly (1.9 to 12.1%) in HCC patients before HAIC and significantly decreased after the treatment ($p=0.043$). **(c)** Gating strategies of $CD14^+$HLA-DR^- and $CD14^-CD11b^+CD33^+CD15^+$ MDSCs. The expression levels of PD-L1 on $CD14^+$HLA-DR^- and $CD14^-CD11b^+CD33^+CD15^+$ MDSCs (red lines) were examined by FACS analysis. Blue lines show the results using isotype antibody. **(d)** The frequency of MDSCs did not significantly change after HAIC. Percentages represent the proportions of $CD14^+$HLA-$DR^{-/low}$ MDSCs among $CD14^+$ cells.

Figure 2: An analysis of tumor factors related to the frequencies of Tregs and MDSCs. Percentages represent the proportions of Tregs and $CD14^+$HLA-$DR^{-/low}$ MDSCs among $CD4^+$ and $CD14^+$ cells, respectively. **(a)** The frequency of Tregs detected by flow cytometry before HAIC did not change with the status of tumors or any clinical factors. **(b)** The frequency of MDSCs correlated with tumor progression and NLR.

Figure 3: An analysis of patient outcomes and frequencies of Tregs and MDSCs.

Patients were divided into two groups: patients with CR or PR and those with SD or PD, according to the Response Evaluation Criteria in Solid Tumors, version 1.1. **(a)** An analysis of patient outcomes and the frequency of Tregs before HAIC. **(b)** An analysis of patient outcomes and the frequency of Tregs after starting HAIC. **(c)** An analysis of patient outcomes and the frequency of MDSCs before HAIC. **(d)** An analysis of patient outcomes and the frequency of MDSCs after starting HAIC.

Figure 4: Kaplan-Meier curves of overall survival. **(a)** Kaplan-Meier curves indicating the relationship between time after starting HAIC and the overall survival rate were grouped by the median number of TAA-specific T cells (indicated as CTLs) before HAIC. The number of TAA-specific T cells was calculated as a total number of T cells detected by IFN-γ ELISPOT assay using 11 TAA-derived peptides shown in supplementary table 1. **(b)** Kaplan-Meier curves indicating the relationship between time after starting HAIC and the overall survival rate were grouped by the median of the frequency of Tregs detected by flow cytometry before HAIC. **(c)** Kaplan-Meier curves indicating the relationship between time after starting HAIC and the overall survival rate were grouped by the median of the frequency of MDSCs detected by flow cytometry before HAIC. **(d)** The difference between the groups was emphasized when 40 % was defined as a high frequency of MDSCs. **(e)** Kaplan-Meier curves of overall survival in HCC patients with stage 2 or 3 according to the UICC TNM classification. Kaplan-Meier curves indicating the relationship between time after starting HAIC and the overall survival rate were grouped by the frequency of MDSCs detected by flow cytometry before HAIC.

Table 1 Characteristics of patients with HCC

	n=36	Median
Age (years)	62.7±8.5	62.5
Sex (M/F)	32/4	
ECOG PS (0/1/2)	23/12/1	
Treatment (IFN+5FU/IFN+5FU+CDDP)	17/19	
HCV-Ab (positive/negative)	21/15	
HBsAg (positive/negative)	13/23	
Platelet count ($X10^4$/μl)	11.7±6.2	11.1
ALT (IU/L)	46.2±26.3	38.5
Active prothrombin (%)	76.9±16.6	76.0
Albumin (g/dl)	3.5±0.4	3.5
Total bilirubin (mg/dl)	1.3±0.9	0.9
AFP (ng/ml)	13538.7±42742.1	501.0
Diameter of the main tumor (mm)	47.7±44.8	30.0
Major portal vein invasion (+/-)	15/21	
Lymph node metastasis (+/-)	1/35	
Distant metastasis (+/-)	3/33	
BCLC TNM stage (A/B/C/D)	0/19/15/2	
UICC TNM stage (I/II/III/IV)	0/4/29/3	
Liver cirrhosis (+/-)	30/6	
Liver function (Child A/B/C)	16/18/2	
Best study response (CR/PR/SD/PD)	1/18/6/11	

Values represent the numbers of patients or mean ± SD. ECOG = Eastern Cooperative Oncology Group; IFN = interferon; PS = performance status; ALT = alanine aminotransferase; AFP = alpha-fetoprotein; Major portal vein invasion = tumor invasion in the main trunk of the 1^{st} branches of the portal vein; BCLC = Barcelona Clinic Liver Cancer; UICC = Union for International Cancer Control.

Table 2 Univariate analysis of prognostic factors for overall survival

Variable	Univariate analysis *p*-value
Gender (male/female)	0.714
Age (<62.5/62.5≤)	0.339
HCV-Ab (+/-)	0.339
HBsAg (+/-)	0.370
Liver cirrhosis (+/-)	0.819
Child-Pugh class (A/B, C)	0.816
AST (<65.0/65.0≤ IU/L)	0.207
ALT (<38.5/38.5≤ IU/L)	0.952
Alb (<3.4/3.4≤ g/dL)	0.180
T-bil. (<0.9/0.9≤ mg/dL)	0.515
NLR (<2.1/2.1≤)	0.014
Active prothrombin (<76/76≤ %)	0.208
DCP (<241/241≤ mAU/mL)	0.833
AFP (<501/501≤ ng/mL)	0.224
Diameter of the tumor (<3.0/3.0≤ cm)	0.008
Major portal vein invasion (+/-)	0.032
Distant metastasis (+/-)	0.038
BCLC TNM stage (A,B/C,D)	0.011
UICC TNM stage (I,II/III,IV)	0.015
TAA-specific T cells (<18.3/18.3≤)*	0.269
Tregs (<6.4/6.4≤ %)	0.879
MDSCs (<30.5/30.5≤ %)	0.009

* The number of TAA-specific T cells was calculated per $3X10^5$ PBMCs

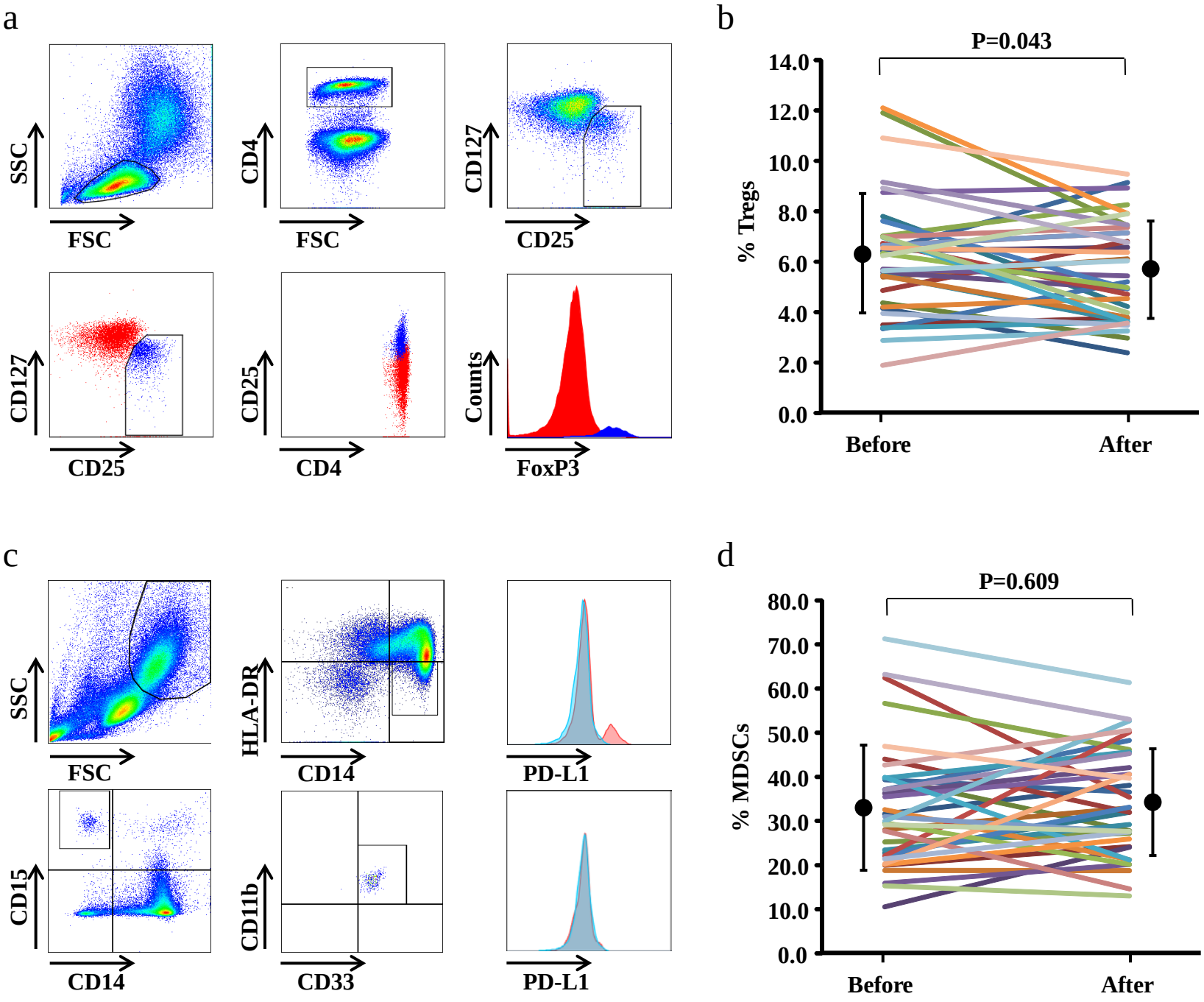

a
SSC
FSC
CD4
FSC
CD127
CD25
CD127
CD25
CD25
CD4
Counts
FoxP3
b
P=0.043
% Tregs
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Before
After
c
SSC
FSC
HLA-DR
CD14
PD-L1
CD15
CD14
CD11b
CD33
PD-L1
d
P=0.609
% MDSCs
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Before
After

Fig. 2

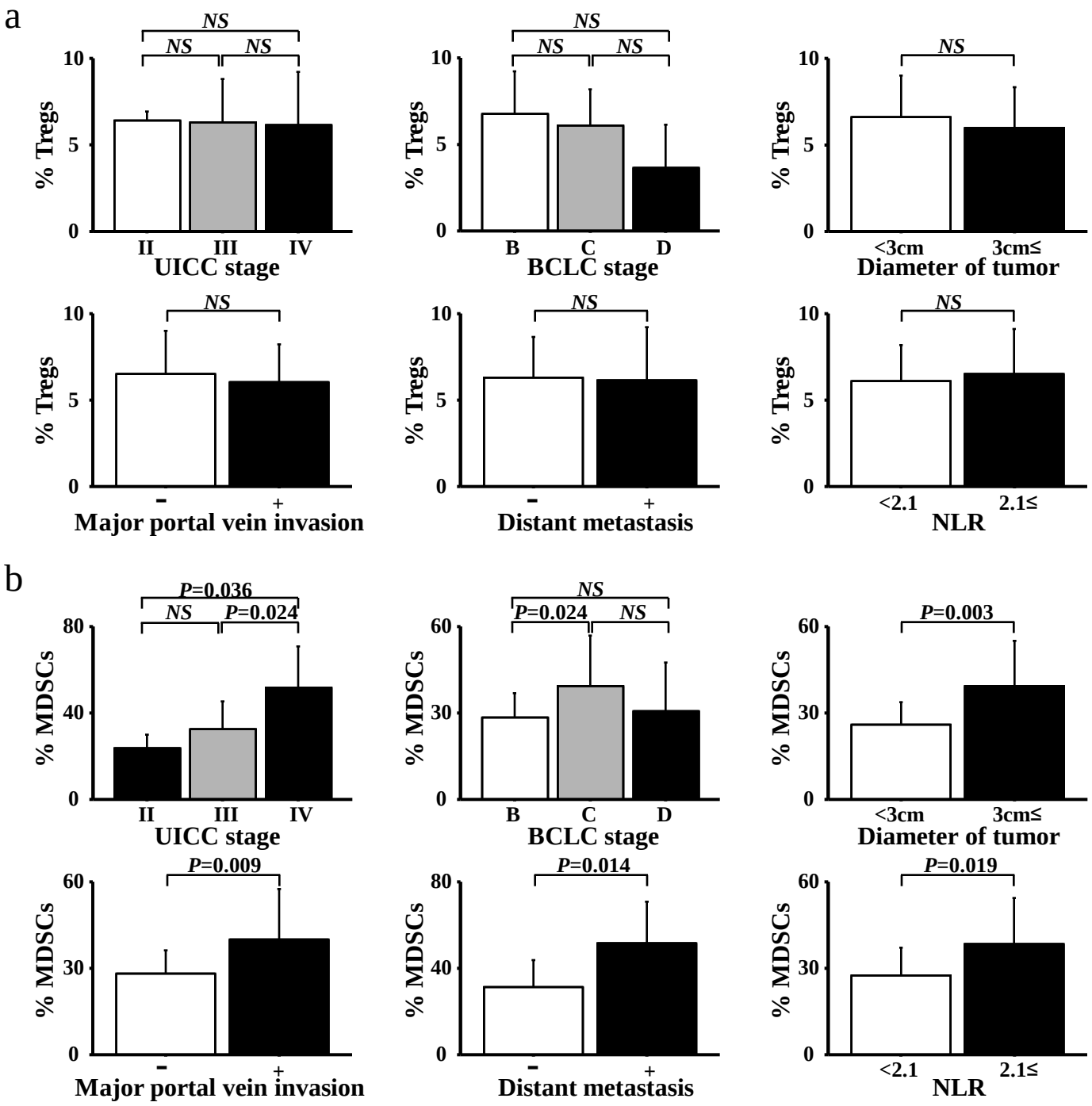
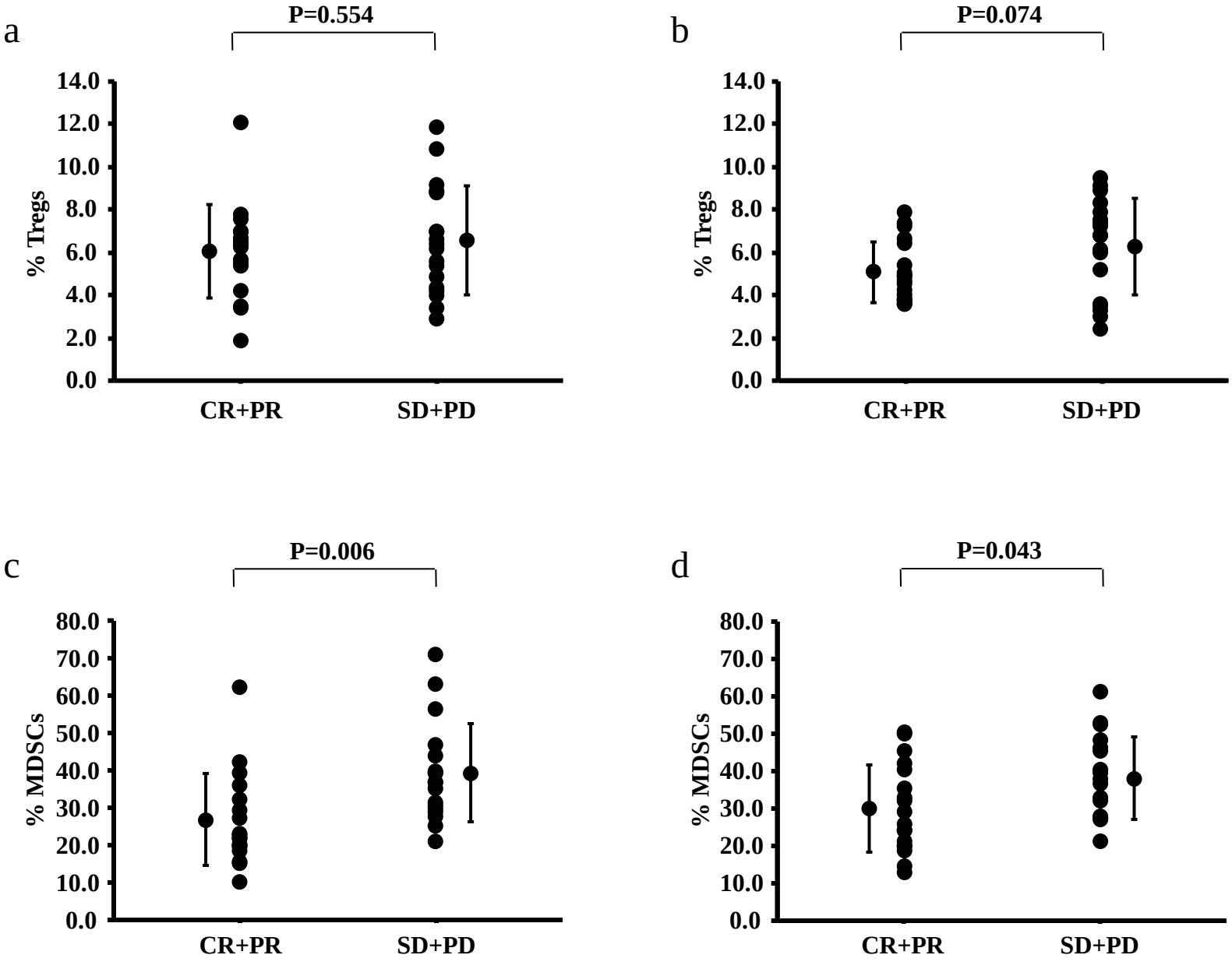
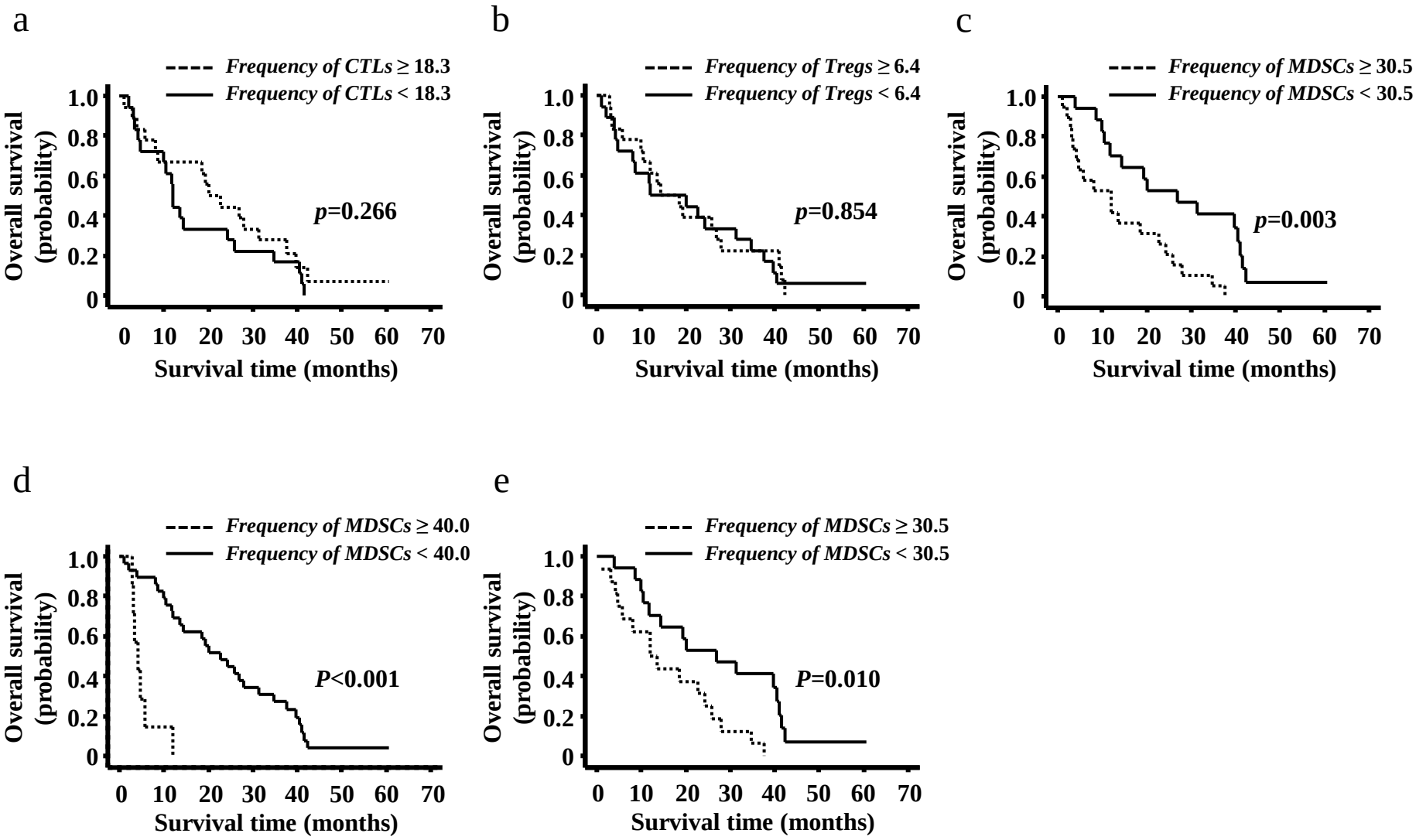

a
NS
NS
NS
% Tregs
10
5
0
II
III
IV
UICC stage
B
C
D
BCLC stage
<3cm
3cm≤
Diameter of tumor
-
+
Major portal vein invasion
Distant metastasis
<2.1
2.1≤
NLR
b
P=0.036
P=0.024
% MDSCs
80
40
30
60
P=0.003
P=0.009
P=0.014
P=0.019

Fig. 3

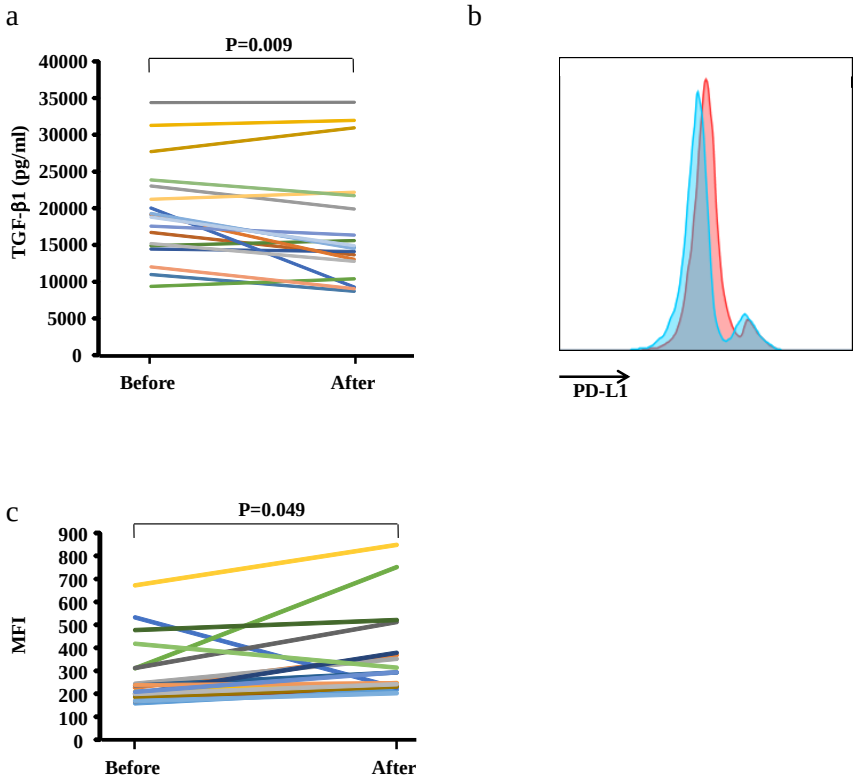

a
P=0.554
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
% Tregs
CR+PR
SD+PD
b
P=0.074
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
% Tregs
CR+PR
SD+PD
c
P=0.006
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% MDSCs
CR+PR
SD+PD
d
P=0.043
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% MDSCs
CR+PR
SD+PD


a
Frequency of CTLs ≥ 18.3
Frequency of CTLs < 18.3
p=0.266
b
Frequency of Tregs ≥ 6.4
Frequency of Tregs < 6.4
p=0.854
c
Frequency of MDSCs ≥ 30.5
Frequency of MDSCs < 30.5
p=0.003
d
Frequency of MDSCs ≥ 40.0
Frequency of MDSCs < 40.0
P<0.001
e
Frequency of MDSCs ≥ 30.5
Frequency of MDSCs < 30.5
P=0.010
Overall survival (probability)
Survival time (months)
0 10 20 30 40 50 60 70
0 0.2 0.4 0.6 0.8 1.0

Supplementary Table 1 Peptides and response frequencies

Peptide name	Amino acid sequences	Number of specific spots in normal donors (mean ± SD)	Frequency of patients with positive T cell responses before chemotherapy	Frequency of patients with positive T cell responses after chemotherapy	Chi-square analysis *p*-value
$SART2_{899}$	SYTRLFLIL	1.0±1.4	1/36 (2.8%)	2/36 (5.6%)	>0.999
$SART3_{109}$	VYDYNCHVDL	2.1±1.9	2/36 (5.6%)	2/36 (5.6%)	>0.999
$MRP3_{503}$	LYAWEPSFL	0.2±0.5	1/36 (2.8%)	0/36 (0%)	>0.999
$MRP3_{692}$	AYVPQQAWI	1.5±2.1	1/36 (2.8%)	0/36 (0%)	>0.999
$MRP3_{765}$	VYSDADIFL	0.9±1.0	2/36 (5.6%)	2/36 (5.6%)	>0.999
AFP_{357}	EYSRRHPQL	1.8±2.0	3/36 (8.3%)	1/36 (2.8%)	0.614
AFP_{403}	KYIQESQAL	1.1±1.5	1/36 (2.8%)	1/36 (2.8%)	>0.999
AFP_{434}	AYTKKAPQL	0.8±1.1	4/36 (11.1%)	3/36 (8.3%)	>0.999
$hTERT_{167}$	AYQVCGPPL	0.8±1.1	1/36 (2.8%)	1/36 (2.8%)	>0.999
$hTERT_{324}$	VYAETKHFL	0.5±0.7	3/36 (8.3%)	5/36 (13.9%)	0.710
$hTERT_{461}$	VYGFVRACL	0.7±1.2	0/36 (0.0%)	3/36 (8.3%)	0.239
HIV env_{584}	RYLRDQQLL	1.3±2.0	1/36 (2.8%)	0/36 (0%)	>0.999
CMV $pp65_{328}$	QYDPVAALF	13.3±15.7	17/36 (47.2%)	16/36 (44.4%)	>0.999

Supplementary Fig. 1



Supplementary Figure 1: (a) Concentrations of TGF-β1 in serum before and after HAIC. **(b)** The representative data of PD-L1 expression on $CD14^{+}HLA\text{-}DR^{-}$ MDSCs before (blue line) and after (red line) HAIC. **(c)** The expression levels of PD-L1 on $CD14^{+}HLA\text{-}DR^{-}$ MDSCs before and after HAIC.