

1 **Increase of CD14⁺HLA-DR^{-low} Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma**
2 **Patients and Its Impact on Prognosis**
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44 **Abbreviations:** MDSCs, myeloid-derived suppressor cells; HCC, hepatocellular carcinoma; CLD,
45 chronic liver disease; RFA, radiofrequency ablation; TACE, transcatheter arterial
46 chemoembolization; PBMC, peripheral blood mononuclear cell; Tregs, regulatory T cells; HLA,
47 human leukocyte antigen; FGF, fibroblast growth factor; CCL, chemokine C-C motif ligand; G-CSF,
48 granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor;
49 IP, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage
50 inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated upon activation,
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1 normal T cell-expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial
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3 growth factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription;
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Abstract

Myeloid-derived suppressor cells (MDSCs) are known as key immune regulators in various human malignancies, and it is reported that CD14⁺HLA-DR^{-/low} MDSCs are increased in hepatocellular carcinoma (HCC) patients. However, the host factors that regulate the frequency and the effect on the prognosis of HCC patients are still unclear. We investigated these issues and clarified the relationships between a feature of MDSCs and host factors in HCC patients. We examined the frequency of MDSCs in 123 HCC patients, 30 chronic liver disease (CLD) patients without HCC and 13 healthy controls by flow cytometric analysis. The relationships between the clinical features and the frequency of MDSCs were analyzed. In 33 patients who received curative radiofrequency ablation (RFA) therapy, we examined the impact of MDSCs on HCC recurrence. The frequency of MDSCs in HCC patients was significantly increased. It was correlated with tumor progression, but not with the degree of liver fibrosis and inflammation. In terms of serum cytokines, the concentrations of IL-10, IL-13 and VEGF were significantly correlated with the frequency of MDSCs. In HCC patients who received curative RFA therapy, the frequency of MDSCs after treatment showed various changes and was inversely correlated with recurrence-free survival time.

Conclusion: The frequency of MDSCs is correlated with tumor progression and this frequency after RFA is inversely correlated with the prognosis of HCC patients. Patients with a high frequency of MDSCs after RFA should be closely followed and the inhibition of MDSCs may improve the prognosis of patients.

1 **Keywords:** myeloid-derived suppressor cells, hepatocellular carcinoma, radiofrequency ablation,
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4 recurrence, cancer
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10 **Precis:** The frequency of CD14+HLA-DR-/low MDSCs in HCC patients was increased and
11 correlated with tumor progression. In the patients treated by radiofrequency ablation, the frequency
12 of MDSCs after treatment was inversely correlated with their prognosis.
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15 16 **Introduction** 17 18 19 20 21 22

23 Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading
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25 cause of cancer mortality globally [1, 2]. Current treatment options including surgical resection,
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27 radiofrequency ablation (RFA), liver transplantation, chemotherapy, transcatheter arterial
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29 chemoembolization (TACE) and sorafenib are reported to improve survival in HCC patients [3-7].
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33 However, despite curative treatments for HCC, tumor recurrence rates remain high and the survival
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36 of those who have advanced HCC remains unsatisfactory [3-7]. Therefore, the development of new
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39 anti-tumor treatments for HCC remains an urgent and important field of research.
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45 To overcome the limitations of these treatments, several immunotherapies have been developed as
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47 attractive strategies for HCC. In several studies of HCC immunotherapy, effective induction of
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49 immune-mediated cells by tumor antigen-derived peptides or antigen-presenting cells showed
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52 anti-tumor effects, but the population of patients who exhibited such effects was very small [8-12].
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58 In previous studies, it was reported that many kinds of tumor generate a number of
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1 immune-suppressive mechanisms [13-15]. Recently, myeloid-derived suppressor cells (MDSCs)
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4 have been characterized as key immune regulators in various human cancers [15-24]. They show the
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7 capacity to inhibit T-cell function and promote tumor development [15, 25]. Human MDSCs are a
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10 heterogeneous population that shows CD11b⁺, CD33⁺, HLA-DR^{-/low} and can be divided into
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13 granulocytic CD14⁻ and monocytic CD14⁺ subtypes [26-28]. In most recent studies, it has been
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16 reported that CD14⁺HLA-DR^{-/low} MDSCs were increased in HCC patients and the cells inhibited the
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19 function of T cells through the induction of regulatory T cells (Tregs) [24]. Tregs represent 5% to
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22 10% of CD4⁺T cells, and can suppress the activation and proliferation of CD4⁺and CD8⁺T cells [14,
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25 29]. It was reported that an increased frequency of circulating Tregs were associated with poor
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28 survival of HCC patients [30]. Understanding the inhibitory mechanism of MDSCs and controlling
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31 their function are very important to develop more effective immunotherapy for HCC.
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36 In this study, we investigate the host factors that are associated with the frequency of MDSCs in
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39 HCC patients and the effect of MDSCs on the prognosis of patients, and clarify the relationships
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42 between a feature of MDSCs and host factors in HCC patients.
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1 **Materials and methods**

7 **Patients and healthy controls**

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13 Blood samples were obtained from a total of 123 HCC patients, 26 chronic liver disease (CLD)
14 patients without HCC and 13 healthy controls. The diagnosis of HCC was histologically confirmed
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17 in 68 patients. For the remaining 55 patients, diagnosis was made by dynamic CT or MRI. Patient
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20 characteristics and disease classification are shown in Suppl. table 1. All CLD patients without HCC
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23 underwent percutaneous liver biopsy to evaluate the disease severity according to the Metavir
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26 scoring system. In 33 patients treated with curative percutaneous RFA, blood samples were obtained
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29 on the day of treatment and 2 to 4 weeks after treatment and we observed recurrence of these patients
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32 with periodic imaging studies. All subjects provided written informed consent to participate in this
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35 study in accordance with the Declaration of Helsinki. This study was approved by the regional ethics
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38 committee (Medical Ethics Committee of Kanazawa University).
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48 **Cell isolation and flow cytometric analysis**

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55 Peripheral blood mononuclear cells (PBMCs) were separated as described below; heparinized
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58 venous blood were diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque
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(Sigma, St. Louis, Mo.) in 50ml tubes. After centrifugation at 2000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS and centrifuged at 1400 rpm for 10min and finally resuspended in complete culture medium consisting of RPMI (GibcoBRL, Grand Island, NY), 10% heat inactivated 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. The viability of cryopreserved PBMCs was 60-70%. In some patients, fresh and cryopreserved PBMCs were obtained from the same sample. To determine the frequency and phenotype of MDSCs and Tregs, multicolor fluorescence-activated cell sorting analysis was carried out using the Becton Dickinson FACS Aria II system. The following anti-human monoclonal antibodies were used: anti-CD4 (Becton Dickinson), anti-CD11b (Becton Dickinson), anti-CD14 (Becton Dickinson), anti-CD15 (Becton Dickinson), anti-CD25 (Becton Dickinson), anti-CD33 (Becton Dickinson), anti-CD127 (Becton Dickinson) and anti-HLA-DR (Becton Dickinson).

Suppression assay

CD14⁺HLA-DR^{-/low} MDSCs and CD14⁺HLA-DR⁺ cells were sorted using the Becton Dickinson FACS Aria II system. 2X10⁴ PBMCs were cultured and stimulated with 1µg/mL plate-bound anti-CD3 (eBioscience) and 1µg/mL soluble anti-CD28 (eBioscience) in 96-well round-bottomed

1 plates. 24 hours later, to determine the suppressive ability of MDSCs, increasing concentrations of
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4 MDSCs were added to the stimulated PBMCs. Proliferation was measured by ^3H incorporation after 72
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7 hours. [^3H] thymidine was added and cell proliferation was measured by incorporation of
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10 radiolabeled thymidine for 24 hours.
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12 13 14 15 16 Cytokine and chemokine profiling 17

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23 Blood samples were collected from patients at the same time of PBMC isolation. After
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26 centrifugation at 3000 rpm for 10 min at 4°C, serum fractions were obtained and stored at -20°C
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29 until use. Serum levels of various cytokines and chemokines were measured using the Bio-Plex
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32 Protein Array System. Briefly, frozen serum samples were thawed at room temperature, diluted 1:4
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35 in sample diluents, and 50 μl aliquots of diluted sample were added in duplicate to the wells of
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38 96-well microtiter plates containing the coated beads for a validated panel of human cytokines and
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41 chemokines according to the manufacturer's instructions. The following 27 cytokines and
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44 chemokines were targeted: IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7,
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47 IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin
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50 (chemokine C-C motif ligand (CCL) 11), G-CSF, GM-CSF, IFN- γ , interferon gamma-induced protein
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53 (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α ,
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56 MIP-1 β , platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T
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1 cell-expressed and secreted(RANTES), TNF- α and vascular endothelial growth factor(VEGF). Nine
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4 standards (ranging from 0.5 to 32,000 pg/ml) were used to generate calibration curves for each
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7 cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version
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16 Statistical analysis 17 18 19 20 21 22

23 Data are expressed as the mean \pm SD. Chi-squared test with Yates' correction, unpaired *t*-test,
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26 Mann-Whitney's U test and Kruskal-Wallis were used for univariate analysis of two groups that
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29 were classified according to the frequency of MDSCs. The probability of tumor recurrence-free
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32 survival was estimated using the Kaplan-Meier method. The Mantel-Cox log-rank test was used to
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35 compare curves between groups. The prognostic factors for tumor recurrence-free survival were
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38 analyzed for statistical significance by the Kaplan-Meier method (univariate) and the Cox
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41 proportional hazard model (multivariate). Variables with $p < 0.1$ were entered into multivariate
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44 logistic regression analysis. A level of $p < 0.05$ was considered significant.
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51 Results 52 53 54 55 56 57

58 CD14⁺HLA-DR^{-/low} MDSCs are increased in the peripheral blood of HCC patients
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4 We analyzed the peripheral blood of 123 patients with HCC, 26 CLD patients without HCC and 13
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7 healthy donors for the prevalence of CD14⁺HLA-DR^{-low} MDSCs. Because the PBMCs are tested
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10 after Ficoll, where some cells may be lost. Therefore, we examined the population of MDSCs as a
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13 percentage of total CD14⁺ cells by flow cytometry after cell surface labeling for expression of
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16 HLA-DR (Figure 1A). CD14⁺HLA-DR^{-low} population in PBMCs of HCC patients represented 3.2%
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19 to 56.8% of the CD14⁺ cells. The frequency of CD14⁺HLA-DR^{-low} MDSCs / CD14⁺ cells in
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22 cryopreserved PBMCs correlated with that in fresh PBMC (Figure 1B). Therefore, we analyzed the
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25 frequency of CD14⁺HLA-DR^{-low} MDSCs / CD14⁺ cells using cryopreserved PBMCs.
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29 To confirm the function of these cells, sorted CD14⁺HLA-DR^{-low} MDSCs and CD14⁺HLA-DR⁺
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32 (control) cells were added at different ratios to autologous anti-CD3/CD28-stimulated PBMCs and
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35 the proliferation was measured by ³H incorporation. CD14⁺HLA-DR^{-low} MDSCs of HCC patients
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38 significantly decreased autologous PBMC proliferation (Figure 1C). On the other hand,
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41 CD14⁺HLA-DR⁺ (control) cells could not suppress the autologous PBMC proliferation.
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45 As shown in Figure 1D, the frequency of MDSCs was significantly higher in HCC patients
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48 (19.0%) than in healthy donors (9.4%) ($p < 0.01$). Overall frequencies of CD14⁺ cells did not differ
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51 significantly between the groups (Figure 1E). Individual frequencies of MDSCs of all the patients
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54 and healthy donors are represented as scatter plots (Figure 2A). The frequency of MDSCs was
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57 correlated with the stage of HCC (stage III and IV: 22.3% (n=46) vs. stage I and II: 17.0% (n=77),
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1 $p < 0.01$) and was significantly higher in HCC patients than CLD patients without HCC and healthy
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4 donors. Interestingly, there was no difference between CLD patients without HCC and healthy
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7 donors. Moreover, these numbers did not change depending on the degree of fibrosis or
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10 inflammatory activity of the liver (Figures 2B and 2C).

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13 In previous reports, granulocytic MDSCs were defined in combination with several surface
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16 markers including CD14, CD15, CD11b, CD33, CD66b and HLA-DR in several cancers. Therefore,
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19 we examined the frequency of CD15⁺CD14⁻CD11b⁺CD33⁺ cells in 37 HCC patients and 11 healthy
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22 donors (Suppl. figure 1A). Although there was no statistical significant difference, the frequency of
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25 CD15⁺CD14⁻CD11b⁺CD33⁺ cells in HCC patients was higher than that in healthy donors. (2.84% vs.
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28 2.06%, $p = 0.073$) (Suppl. figure 1B). The frequency was correlated with the stage of HCC (stage III
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31 and IV: 3.69% (n=13) vs. stage I and II: 2.39% (n=24), $p = 0.022$) (Suppl. figure 1C).

32 33 34 35 36 37 38 39 Relationship between the frequency of Tregs and MDSCs

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45 It is well known that the frequency of circulating Tregs is increased and correlated with disease
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48 progression in HCC patients. The frequency of CD4⁺ CD25⁺ CD127^{-/low} Tregs was significantly
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51 increased in HCC patients (Suppl. figure 2A) and associated with tumor progression (Suppl. figure
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54 2B). However, there was not a strong correlation between the frequency of MDSCs and Tregs in our
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58 study (Suppl. figure 2C).

Identification of host factors related to the frequency of MDSCs in HCC patients

We divided the HCC patients into two groups using the threshold of an MDSC ratio of 22%. This threshold is the average + 2SD of the MDSC ratio in non-HCC patients. In the group with high frequency, the tumor factors including size, multiplicity and stage were significantly worse (tumor size, 28.3mm vs. 24.4mm; tumor multiplicity (multiple/solitary), 27/12 vs. 42/42; TNM stage (I and II vs. III and IV), 17/22 vs. 60/24, $p < 0.05$) (Table1). In addition, hepatic reserve was also worse in the group with high frequency (Child-Pugh classification (A/B/C), 20/17/2 vs. 64/16/4, $p < 0.05$). In addition, overall survival was significantly shortened in the group with high frequency (hazard ratio 2.67, $p = 0.008$) (Suppl. figure 3A), and recurrence free survival was also significantly shortened (hazard ratio 1.94, $p = 0.010$) (Suppl. figure 3B).

Relationship between serum cytokine levels and the frequency of MDSCs

In previous studies, the balance of circulating cytokines was thought to promote accumulation and activation of MDSCs [18, 36-39]. Therefore, we examined the relationship between serum cytokine levels and the frequency of MDSCs in HCC patients. In 54 HCC patients, serum levels of cytokines and chemokines were measured using the Bio-Plex Protein Array system. Serum concentrations of

1 IL-10, IL-13 and VEGF were significantly increased in the group with a high frequency of MDSCs
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4 (Table 2). In addition, there was a positive correlation between these cytokine levels in serum and the
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7 frequency of MDSCs. We also examined the relationship between serum cytokine levels and the
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10 frequency of Tregs. We divided the HCC patients into two groups using the threshold of 7%, which
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13 is the average + 2SD of the % of Tregs among CD4⁺ cells in non-HCC patients. Serum concentration
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16 of IL-10 was significantly increased in the group with a high frequency of Tregs (Suppl. table 2).
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23 Kinetics of MDSCs before and after curative RFA therapy 24 25 26 27 28

29 We examined the frequency of MDSCs before and after curative RFA therapy in 33 patients. For
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32 this analysis, blood samples were obtained on the day of treatment (before) and 2 to 4 weeks after
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35 treatment (after). The frequency of MDSCs was significantly decreased after RFA therapy (18.0% to
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38 15.5%, $p < 0.05$) (Figure 3A). However, in several patients, the frequency of MDSCs remained at a
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41 high level compared with that in non-HCC patients. The clinical parameters before RFA were not
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44 statistically different between the patients with and without a high frequency of MDSCs after RFA
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47 (Suppl. table 3).
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51 Next, we followed up these patients for recurrence and analyzed the risk factors. If a high
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54 frequency of MDSC was observed after curative RFA therapy, the recurrence-free survival was
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57 significantly shortened (Figure 3B). In contrast, the frequency of MDSCs before treatment did not
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1 affect the recurrence. In univariate analysis for recurrence, post-treatment MDSC ratio $\geq 22\%$ ($p=$
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4 0.023) and tumor multiplicity ($p= 0.010$) were significantly associated with HCC recurrence (Table
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7 3). In multivariable analysis for recurrence, considering the variables in the univariate analysis with
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10 $p < 0.1$, only post-treatment MDSC ratio $\geq 22\%$ (HR 3.906, $p= 0.014$) was extracted as a significant
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13 risk factor for recurrence.
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Discussion

MDSCs are expanded in pathological conditions such as malignancy, infection or trauma and consist of a heterogeneous population of immature myeloid cells [15, 25]. In pathological conditions, immature myeloid cells are blocked to differentiate into mature macrophages, dendritic cells or granulocytes; as a result, MDSCs are accumulated [15, 25]. MDSCs strongly inhibit anti-tumor immune response through a number of mechanisms [15, 25]. As monocytic subsets of MDSCs, CD14⁺HLA-DR^{-/low} MDSCs have been reported in various malignancies, including melanoma, multiple myeloma, prostate cancer and bladder cancer [18, 20, 22, 35]. In the most recent study, Hoechst et al. reported that CD14⁺HLA-DR^{-/low} MDSCs were significantly increased in HCC patients and they suppressed T cell functions through the induction of CD4⁺CD25⁺Foxp3⁺ Treg [24].

In the present study, in addition to an increase in the number of MDSCs in HCC patients, we observed that the frequency was correlated with the progression of HCC. Consistent with our results, it has also been reported that the frequency of CD14⁺HLA-DR^{-/low} MDSCs was correlated with tumor progression in patients with other cancers, such as melanoma, prostate cancer and bladder cancer [22, 35, 36]. However, the mechanisms behind the increase of MDSCs in advanced cancer patients are still unclear. As is well known, there is a close relationship between hepatocarcinogenesis and histological status of underlying liver [37, 38]. Therefore, the advance of hepatic fibrosis and the increase of inflammatory cell infiltration into liver might result in an increase

1 of MDSCs following the progression of HCC. However, there was no relationship between the
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4 frequency of CD14⁺HLA-DR^{-/low} MDSCs and underlying liver status in our study. From our
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7 observations, increase of MDSCs was only correlated with tumor progression, but not with hepatic
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10 fibrosis or disease activity of CLD. This finding suggests that the expansion of CD14⁺HLA-DR^{-/low}
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13 MDSCs was mostly derived from the tumor environment itself, but not from inflammation or fibrosis
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16 of liver tissue around the tumor. The finding that a significant decrease in the frequency of
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19 circulating CD14⁺HLA-DR^{-/low} MDSCs is observed in most patients with curative treatment in this
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22 study supports this hypothesis. On the other hand, Tregs were also increased in HCC patients and
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25 associated with the progression of HCC. Though it was reported that MDSCs suppressed T cell
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28 function through the induction of Tregs, there was not a strong correlation between the frequencies of
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31 these two immunosuppressive cells.
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36 Regarding the mechanism of MDSC expansion, we also analyzed the relationship between the
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39 serum cytokine levels and the frequency of MDSCs. We observed that the serum concentrations of
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42 IL-10, IL-13 and VEGF were significantly increased in the group with high frequency of MDSCs
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45 and there was a positive correlation between these cytokine levels and the frequency of MDSCs.
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48 Moreover, although there was no significant difference, the serum concentrations of IL-1ra, IL-2,
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51 IL-6, IL-12(p70) and G-CSF tended to be increased in the group with high frequency of MDSCs. In
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54 accordance with our results, various cytokines, including IL-6, IL-10, IL-13, G-CSF and VEGF, that
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57 trigger Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling
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1 pathways have been reported to be associated with the frequency of MDSCs [39]. In particular, the
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4 cytokines involved in the JAK2-STAT3 signaling pathway are considered to be the main regulators
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7 of the expansion of MDSCs, which leads to stimulation of myelopoiesis and inhibition of
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10 myeloid-cell differentiation [40-42].
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13 Another important finding of our study is that the frequency of MDSCs showed various changes
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16 after curative RFA and this frequency is an independent risk factor of HCC recurrence. In most of the
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19 patients, the frequency of MDSCs decreased after RFA. A similar phenomenon has also been
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22 reported in other cancer treatments [19, 21, 36]. Liu et al. reported that MDSCs were decreased in
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25 non-small cell lung cancer patients who had clinical benefit from chemotherapy or who received
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28 curative surgery [21]. These results suggest that a decrease of the frequency of MDSCs is due to
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31 tumor eradication.
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36 It is well known that tumor factors including multiplicity, tumor diameter, serum levels of tumor
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39 marker and hepatic reserve are risk factors of HCC recurrence after RFA [43, 44], but it has not been
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42 reported that the frequency of circulating MDSCs is also a risk factor. From our findings, there was a
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45 clear inverse correlation between the frequency of MDSCs after RFA and recurrence-free survival.
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48 Consistent with our results, in the patients with pancreatic, esophageal and gastric cancer, Gabitass et
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51 al. reported that an increase of MDSCs was associated with an increased risk of death and that the
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54 frequency of MDSCs was an independent prognostic factor for patient survival[23]. Taken together
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58 with these findings, our results suggest that the frequency of MDSCs might be one of the prognostic
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1 factors of patients after cancer treatments.
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4 As we showed, the frequency of MDSCs is primarily correlated with tumor progression. However,
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7 between the patients with high and low frequency of MDSCs after RFA, there was no significant
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10 difference in hepatic reserve and tumor factors before treatment. Although an incomplete HCC
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13 eradication at a microscopic level may allow a high frequency of MDSCs after RFA, there may be
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16 other mechanisms such as subsequently tumor-specific immune responses after RFA. In addition,
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19 there is a limitation of the present study because we used cryopreserved PBMCs for phenotypic
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22 analysis of MDSCs. Further studies using fresh PBMCs are needed for precise phenotypic analysis
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25 of MDSCs and elucidation of the mechanism to regulate the frequency of MDSCs after HCC
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28 treatment.
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32 In conclusion, the frequency of MDSCs in HCC patients is correlated with tumor progression and
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35 the frequency after RFA is inversely correlated with the prognosis of HCC patients. HCC patients
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38 who show a high frequency of MDSCs after RFA should be closely followed and the inhibition or
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41 elimination of MDSCs after HCC treatments may improve the prognosis of HCC patients.
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46 **Conflict of interest** The authors declare no conflict of interest
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Figure legends

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4 Figure 1: (A) Flow cytometry shows CD14⁺HLA-DR^{-/low} MDSCs. PBMCs from patients and
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7 healthy donors were labeled with anti-CD14 and HLA-DR. Three staining examples of HCC patients
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10 are shown in the order from a small number (left) to a large number (right). (B) The increase of
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13 CD14⁺HLA-DR^{-/low} MDSCs/CD14⁺ cells in cryopreserve PBMC correlated with that in fresh PBMC
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16 ($r^2=0.52$). (C) Proliferation of PBMCs stimulated by anti-CD3/28 in the presence or absence of
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19 MDSCs was measured by ³Hincorporation assay. CD14⁺HLA-DR^{-/low} MDSCs significantly decreased
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22 autologous PBMC proliferation (n=4; *, $p<0.05$). (D) The frequency of MDSCs was significantly
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25 higher in HCC patients than healthy donors (*, $p<0.01$). (E) Overall frequencies of CD14⁺ cells did
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28 not differ significantly.
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32 Figure 2: (A) Scatter plots of MDSC ratio (CD14⁺HLA-DR^{-/low} MDSCs/CD14⁺ cells) in patients
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35 and healthy donors. The frequency of MDSCs was significantly increased in HCC patients compared
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38 with that in non-HCC controls. Moreover, the frequency of MDSCs was correlated with tumor
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41 progression (stage III and IV: 22.3% (n=46) vs. stage I and II: 17.0% (n=77); *, $p<0.05$). In
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44 non-HCC controls, there was no significant difference in the frequency of MDSCs. (B), (C) In
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47 non-HCC patients, the frequency of MDSCs did not change depending on the degree of fibrosis or
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50 inflammatory activity of the liver according to the Metavir scoring system.
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55 Figure 3: (A) In 33 HCC patients who received curative RFA therapy, the frequency of MDSCs
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58 was significantly decreased after treatment. However, in several patients, the frequencies were
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1 increased after treatment (dotted lines) (*, $p < 0.05$). (B) Kaplan-Meier curve for recurrence-free
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4 survival after RFA therapy. The patients with high frequency of MDSCs (solid line) relapsed.
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Table 1. Clinical findings and MDSCs

Clinical characteristics	MDSC ratio ≥ 22	MDSC ratio < 22	p value
	(n=39)	(n=84)	
Age (yr)	68.5	70.1	0.646
Sex (M/F)	29/10	54/30	0.267
AST (IU/l)	62.0	61.5	0.543
ALT (IU/l)	47.1	53.9	0.759
LDH (IU/l)	225	218	0.832
γ GTP (IU/l)	78.0	76.0	0.252
Platelet ($10^4/\mu$ l)	10.9	10.6	0.884
Prothrombin time (%)	75.2	82.3	0.045
Serum albmin (g/dl)	3.53	3.68	0.120
Total Bilirubin (mg/dl)	1.21	0.94	0.286
WBC (μ l)	3910	3610	0.235
Neutrophil (%)	63.2	59.4	0.093
Lymphocyte (%)	26.1	29.5	0.047
Total Cholesterol (mg/dl)	151	149	0.926
HbA1c (%)	5.27	5.43	0.197
Type IV collagen 7S (ng/ml)	8.2	7.3	0.086
DCP (mAU/ml)	5157	432	0.561
AFP (ng/ml)	1301	934	0.240
Tumor Size (mm)	28.3	24.4	0.014
Tumor Multiplicity (multiple/solitary)	27/12	42/42	0.046
TNM Stage (I plus II/III plus IV)	17/22	60/24	0.003
Child-Pugh (A/B/C)	20/17/2	64/16/4	0.015
Etiology (HCV/HBV/others)	21/11/7	61/11/12	0.081
CD4 ⁺ CD25 ⁺ CD127 ^{-low} Tregs / CD4 ⁺ cells (%)	7.04	6.70	0.281

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactic dehydrogenase; γ GTP, gamma glutamyltransferase; WBC, white blood cell; Hb, hemoglobin; DCP, des-gamma-prothrombin; AFP, Alpha-fetoprotein; HCV, hepatitis C virus; HBV, hepatitis B virus; Tregs, regulatory T cells

Chi-squared test with Yates' correction, unpaired t-test, Mann-Whitney's U test and Kruskal-Wallis test were used for univariate analysis of two groups that were classified according to the frequency of MDSCs.

Table 2. Serum cytokines and MDSCs

Cytokine	Healthy donor	MDSC ratio ≥ 22	Range	MDSC ratio < 22	Range	p value
	(mean)	(mean)		(mean)		
	(n=13)	(n=21)		(n=31)		
IL-1ra	34.2	97.0	(21.5-600)	40.3	(3.4-151)	0.057
IL-2	10.5	38.1	(4.3-54.3)	11.3	(0.9-49.7)	0.055
IL-4	2.6	5.75	(1.47-11.9)	5.03	(0.71-10.9)	0.159
IL-6	9.9	21.5	(1.2-130)	10.1	(0.2-97.2)	0.065
IL-8	24.5	64.7	(10.9-291)	35.1	(6.2-142)	0.156
IL-10	2.76	6.01	(0.8-11.5)	2.81	(0.1-12.0)	0.003
IL-12(p70)	14.6	33.3	(0.6-140)	17.6	(1.4-57)	0.058
IL-13	7.6	13.1	(1.2-33.6)	8.2	(2.7-22.9)	0.015
IL-17	15.7	23.5	(4.6-70)	20.8	(2.1-119)	0.115
Eotaxin	104	141	(51.9-493)	124	(26.3-331)	0.675
G-CSF	7.9	13.2	(2.7-41.3)	8.7	(0.5-17.9)	0.050
IFN- γ	52.6	95.4	(23.1-417)	69.9	(2.5-238)	0.136
MCP-1	20.2	26.8	(8.4-114)	23.8	(3.5-77)	0.744
MIP-1b	97.6	120	(58.3-490)	108	(39.7-263)	0.508
PDGF	4012	4375	(1312-10136)	4013	(831-13557)	0.484
RANTES	2978	2890	(1040-4826)	3184	(599-6165)	0.186
TNF- α	10.5	34.9	(0.1-175)	27.6	(2.9-105)	0.756
VEGF	34.6	101.7	(22.5-371)	59.5	(9.3-183)	0.045

Abbreviations: IL, interleukin; G-CSF, granulocytic colony stimulating factor; IFN, interferon; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T cell-expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

Mann-Whitney's U test was used for univariate analysis of two groups that were classified according to the frequency of MDSCs.

Table 3. Cox proportional-hazards regression for recurrence

Variant	Univariate HR (95% CI)	p value	Multivariable HR (95% CI)	p value
Sex: female	0.763 (0.446-1.308)	0.326		
Age: ≥ 70 yr	1.111 (0.671-1.840)	0.683		
Pre MDSC ratio: $\geq 22\%$	1.210 (0.698-2.096)	0.497		
Pre neutrophil	0.990 (0.969-1.012)	0.385		
Pre lymphocyte	1.014 (0.987-1.043)	0.311		
Pre neutrophil/lymphocyte	0.978 (0.787-1.216)	0.844		
Pre ALT	1.001 (0.993-1.008)	0.882		
Pre serum albumin: < 3.5 mg/dl	1.143 (0.665-1.982)	0.647		
Pre prothrombin time: $< 70\%$	1.662 (0.961-2.903)	0.073	1.881 (0.522-6.777)	0.101
Post MDSC ratio: $\geq 22\%$	2.795 (1.150-6.792)	0.023	3.906 (1.313-11.616)	0.014
Post neutrophil	1.005 (0.975-1.035)	0.762		
Post lymphocyte	0.993 (0.960-1.027)	0.678		
Post neutrophil/lymphocyte	1.003 (0.810-1.242)	0.980		
Post ALT	0.995 (0.981-1.010)	0.501		
type IV collagen 7S	1.122 (0.992-1.268)	0.067	1.192 (0.907-1.566)	0.207
AFP: ≥ 100 ng/ml	1.357 (0.743-2.480)	0.321		
Tumor size: ≥ 20 mm	1.29 (0.78-2.12)	0.328		
Tumor Multiplicity: multiple	2.00 (1.18-3.40)	0.010	1.851 (0.721-4.753)	0.201

Abbreviations: HR, hazard ratio; CI, confidence interval; ALT, alanine aminotransferase; AFP, Alpha-fetoprotein

Fig. 1

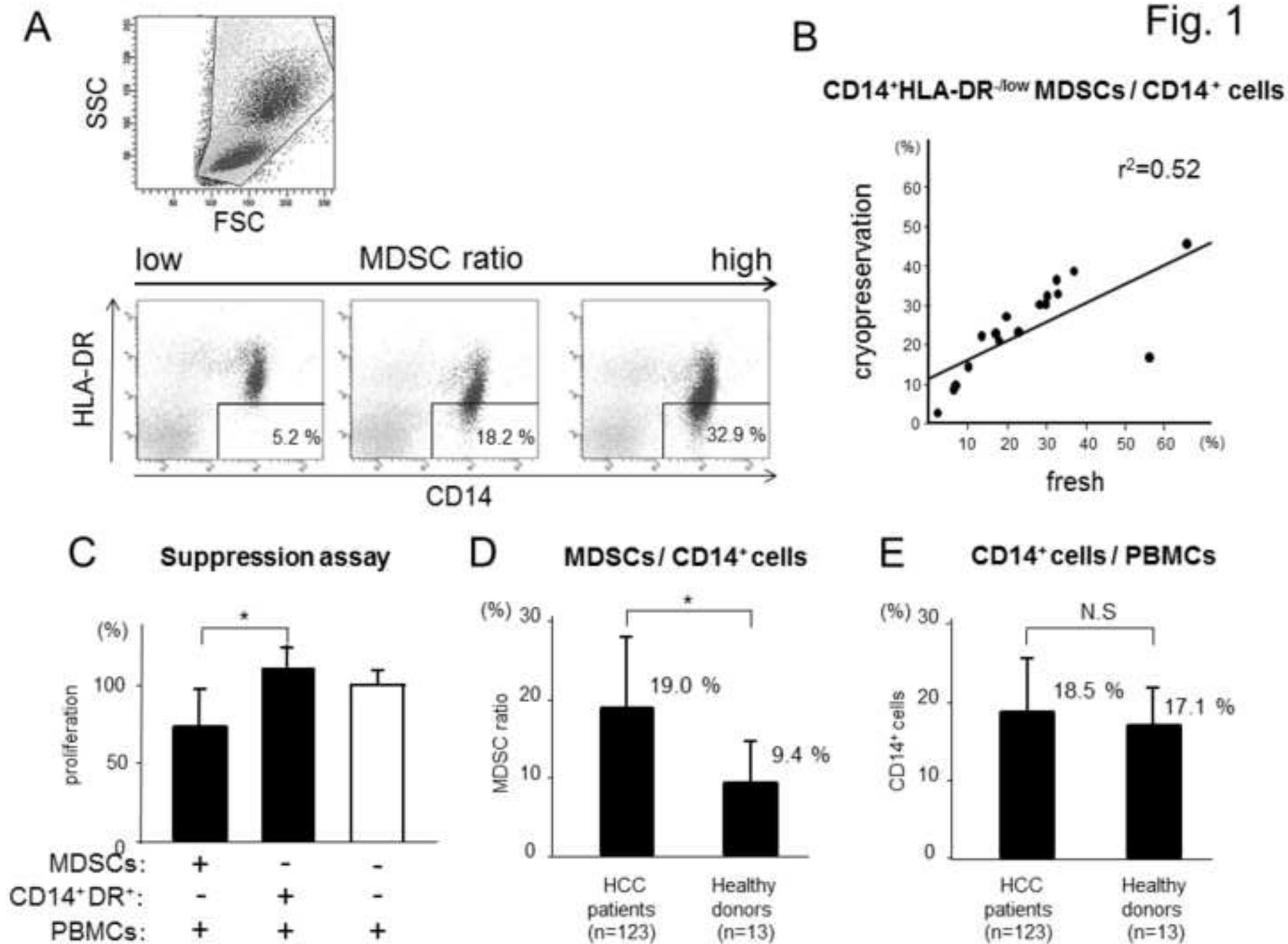


Fig. 2

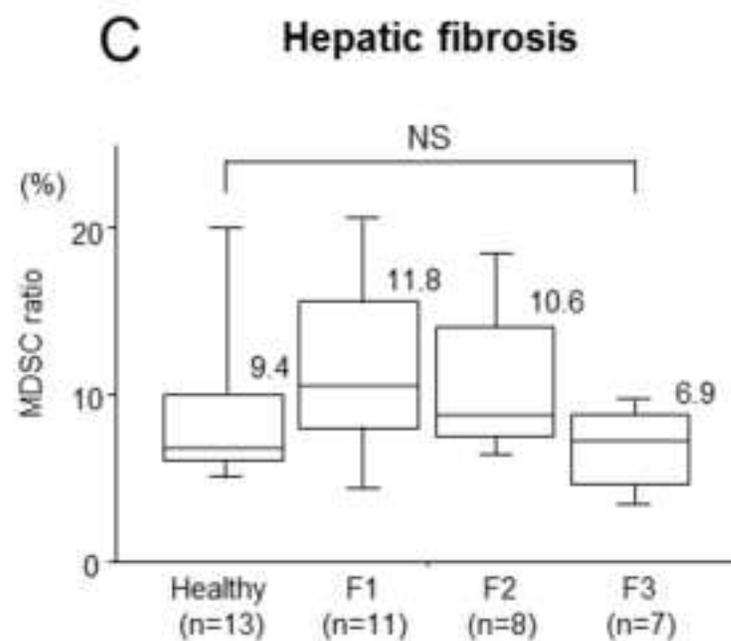
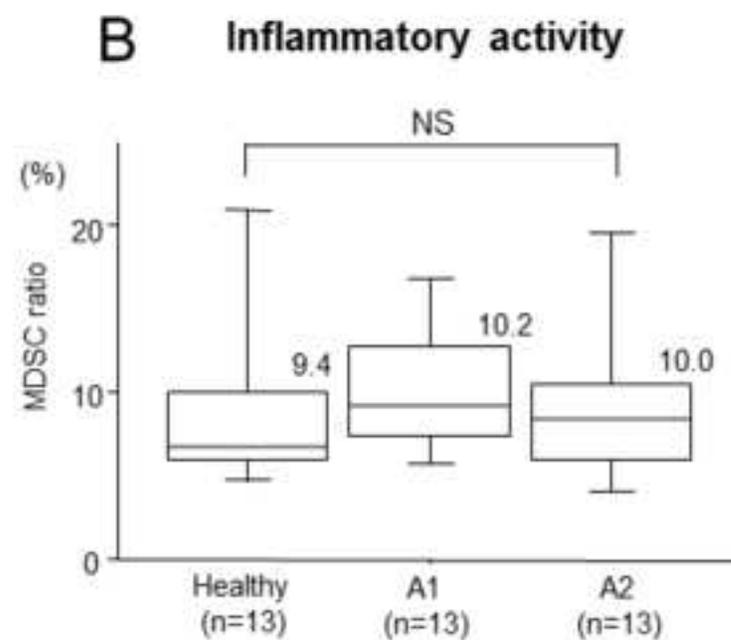
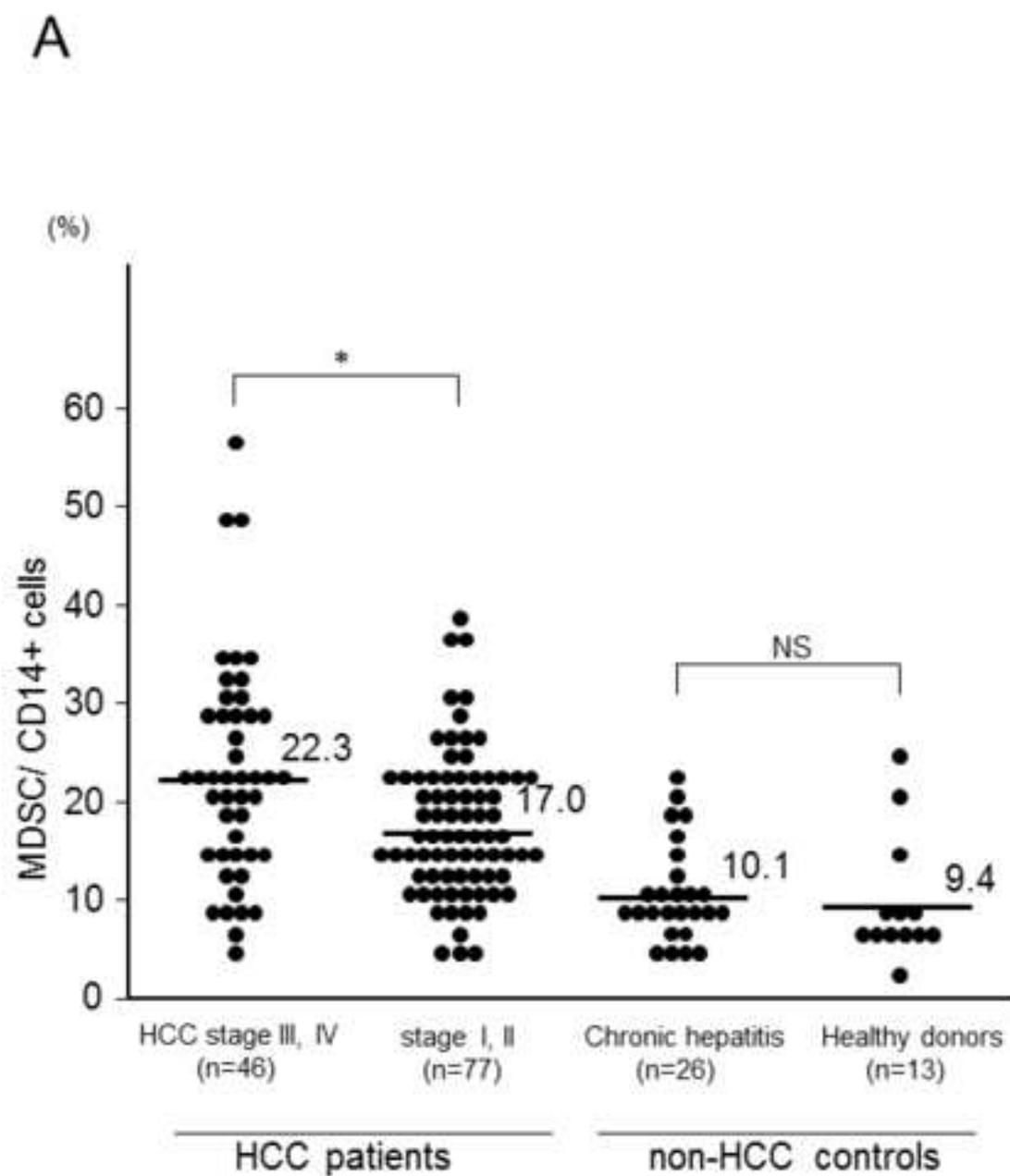
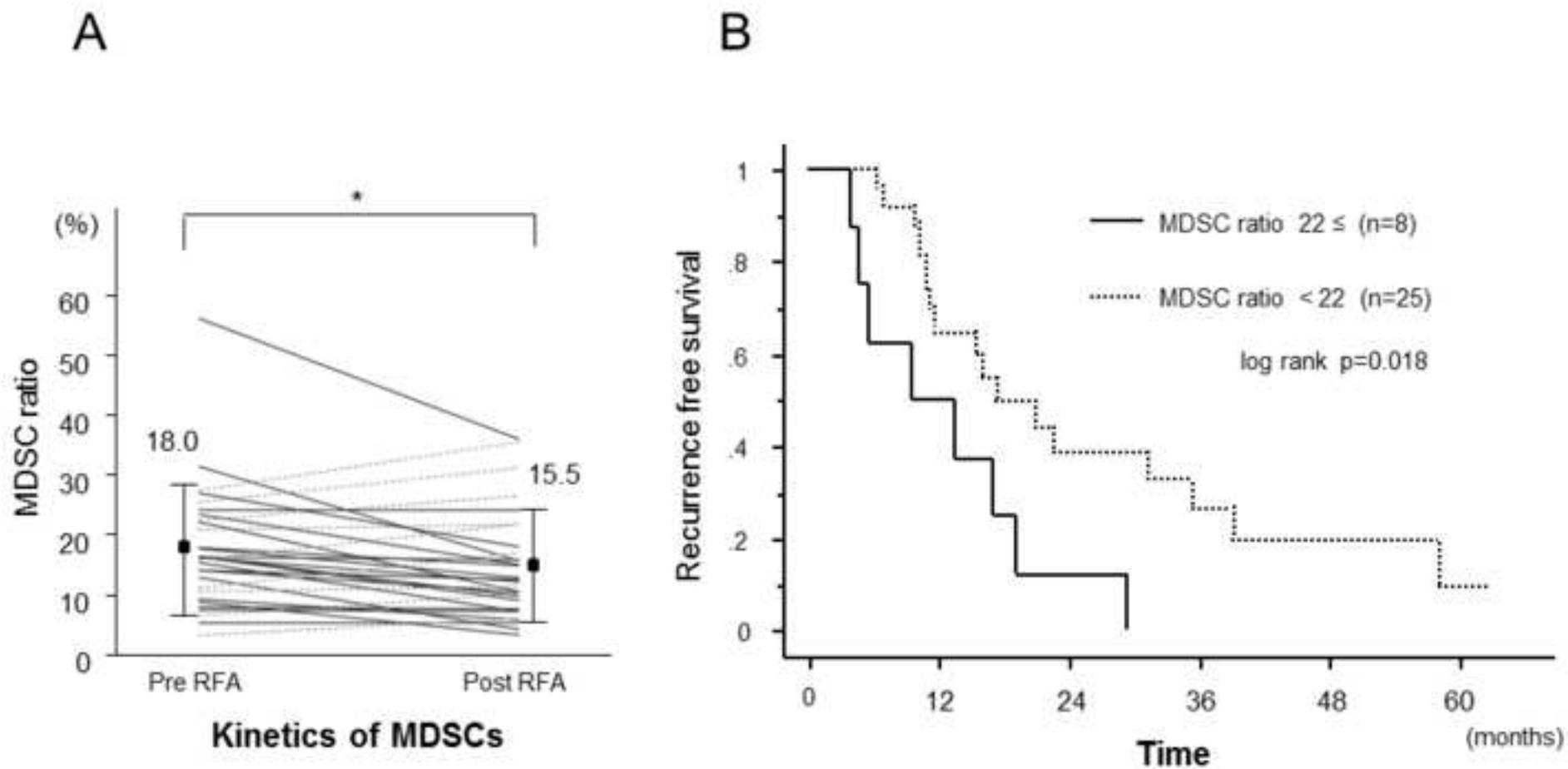


Fig. 3



Supplemental Table 1. Characteristics of patients and healthy donors

Clinical Diagnosis	No. of Patients	Sex M/F	ALT (IU/L)	Etiology (HCV/HBV/others)	AFP (ng/ml)	Child-Pugh (A/B/C)	Tumor Size (large/small)	Tumor Multiplicity (multiple/solitary)	TNM Stage (I/II/III/IV)
HCC patients	123	83/40	52 ± 36	82/22/19	1051 ± 4391	84/33/6	69/54	69/54	32/45/35/11
CLD patients	26	14/12	46 ± 27	22/4/0	11 ± 7	26/0/0	ND	ND	ND
Healthy donors	13	8/5	17 ± 5	ND	ND	ND	ND	ND	ND

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; HBV hepatitis B virus; AFP, Alpha-fetoprotein; HCC, hepatocellular carcinoma; CLD, chronic liver disease;

SD, standard deviation

All continuous variables were expressed as mean ± SD.

Supplemental Table 2. Serum cytokines and CD4⁺ CD25⁺ CD127^{-/low} Tregs

Cytokine	Healthy donor (mean) (n=13)	Treg ≥7 % (mean) (n=29)	Range	Treg <7% (mean) (n=23)	Range	p value
IL-1ra	34.2	54.2	(11.0-430)	79.7	(5.9-600)	0.383
IL-2	10.5	26.0	(2.1-54.3)	16.0	(0.9-50.6)	0.167
IL-4	2.6	6.17	(1.47-11.9)	4.54	(0.71-10.9)	0.128
IL-6	9.9	14.6	(0.2-97.2)	15.1	(1.6-130)	0.872
IL-8	24.5	49.5	(6.2-291)	40.1	(10.9-256)	0.563
IL-10	2.76	4.99	(0.6-12.1)	2.97	(0.1-10.4)	0.030
IL-12(p70)	14.6	25.8	(0.6-140)	23.2	(2.8-78)	0.715
IL-13	7.6	11.6	(2.7-33.6)	8.8	(1.2-22.9)	0.167
IL-17	15.7	20.1	(4.2-70)	25.4	(2.1-119)	0.340
Eotaxin	104	132	(59.0-493)	119	(26.3-245)	0.906
G-CSF	7.9	11.2	(2.7-41.3)	10.1	(0.5-33.7)	0.424
IFN-γ	52.6	85.9	(23.1-417)	78.6	(2.5-343)	0.319
MCP-1	20.2	27.2	(3.5-114)	21.9	(8.4-71)	0.375
MIP-1b	97.6	123	(56.6-490)	103	(39.7-229)	0.696
PDGF	4012	4179	(1271-10136)	4305	(831-13557)	0.815
RANTES	2978	3041	(1040-4714)	3097	(599-6165)	0.598
TNF-α	10.5	29.1	(0.1-175)	32.7	(2.9-155)	0.747
VEGF	34.6	83.4	(24.5-371)	71.9	(9.3-224)	0.538

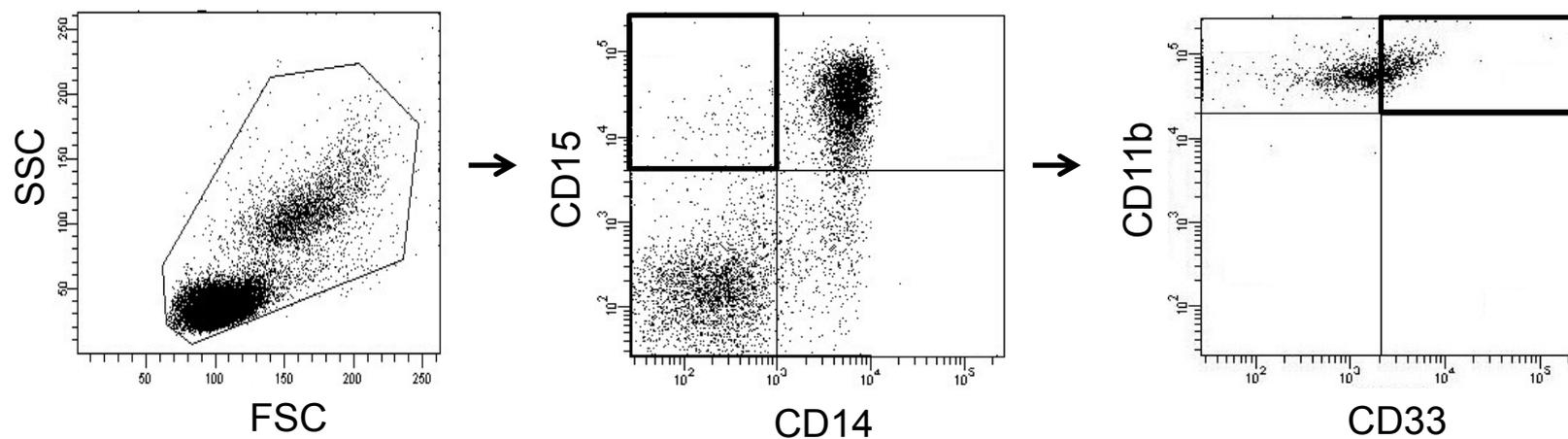
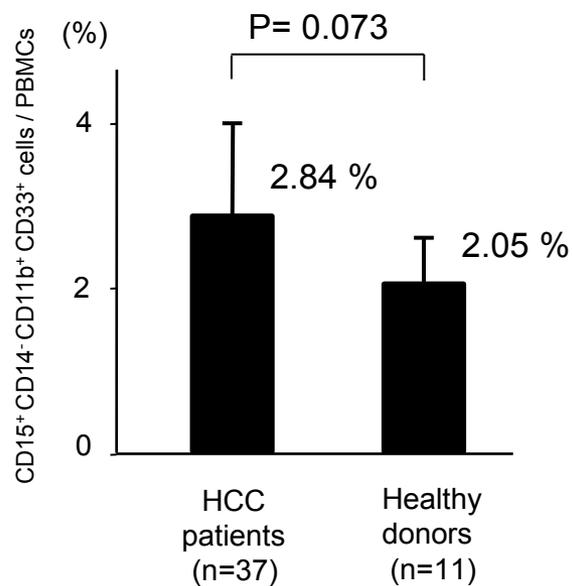
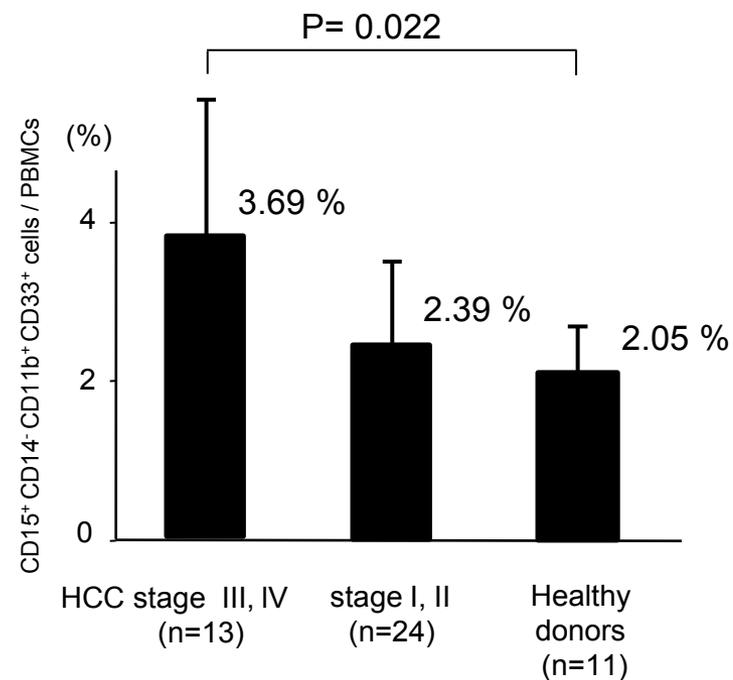
Abbreviations: IL, interleukin; G-CSF, granulocytic colony stimulating factor; IFN, interferon; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T cell-expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

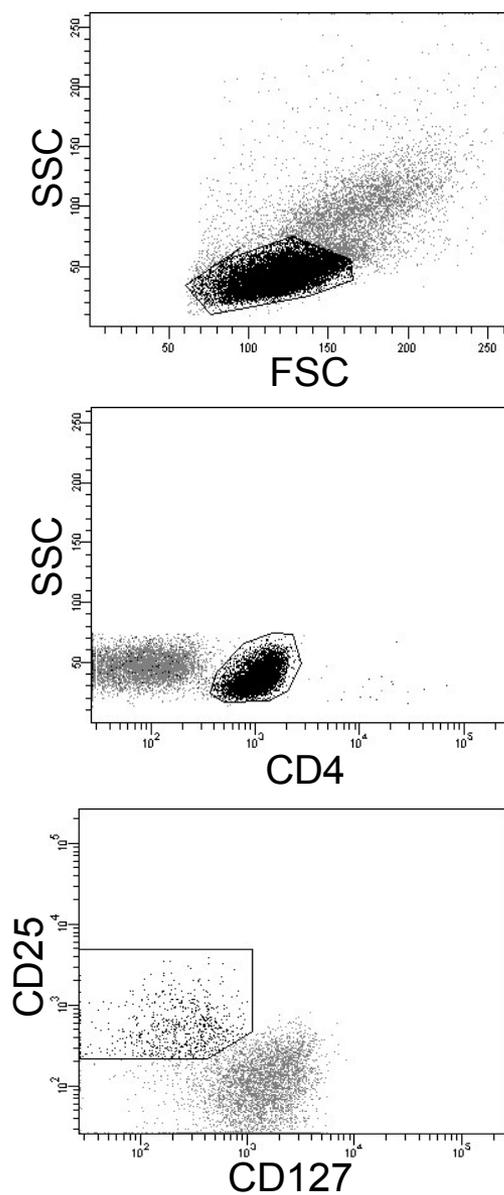
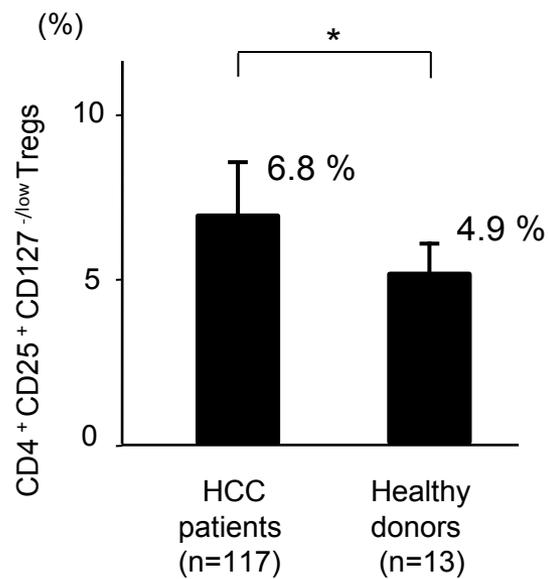
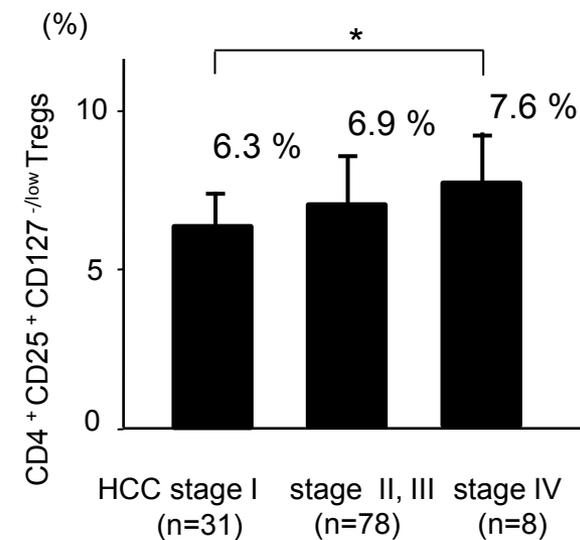
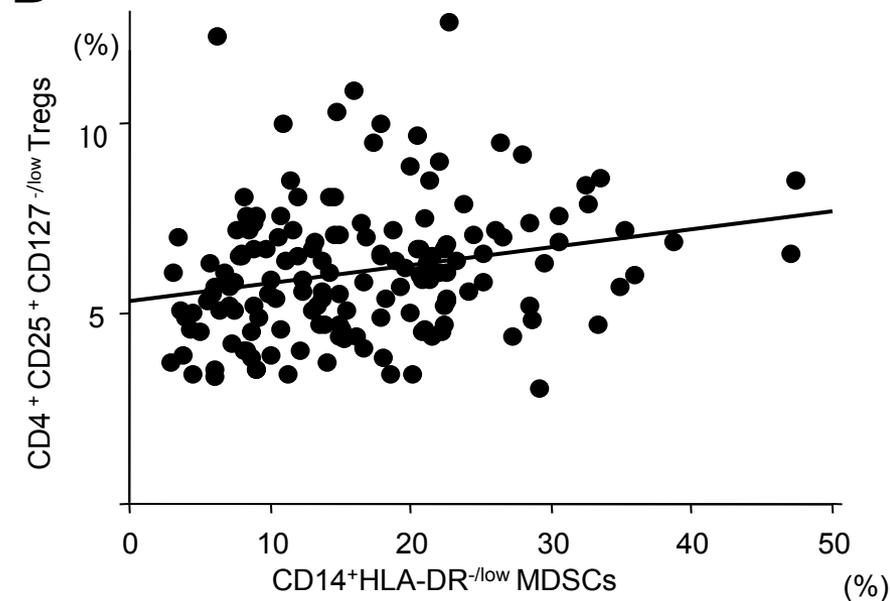
Mann-Whitney's U test was used for univariate analysis of two groups that were classified according to the frequency of MDSCs.

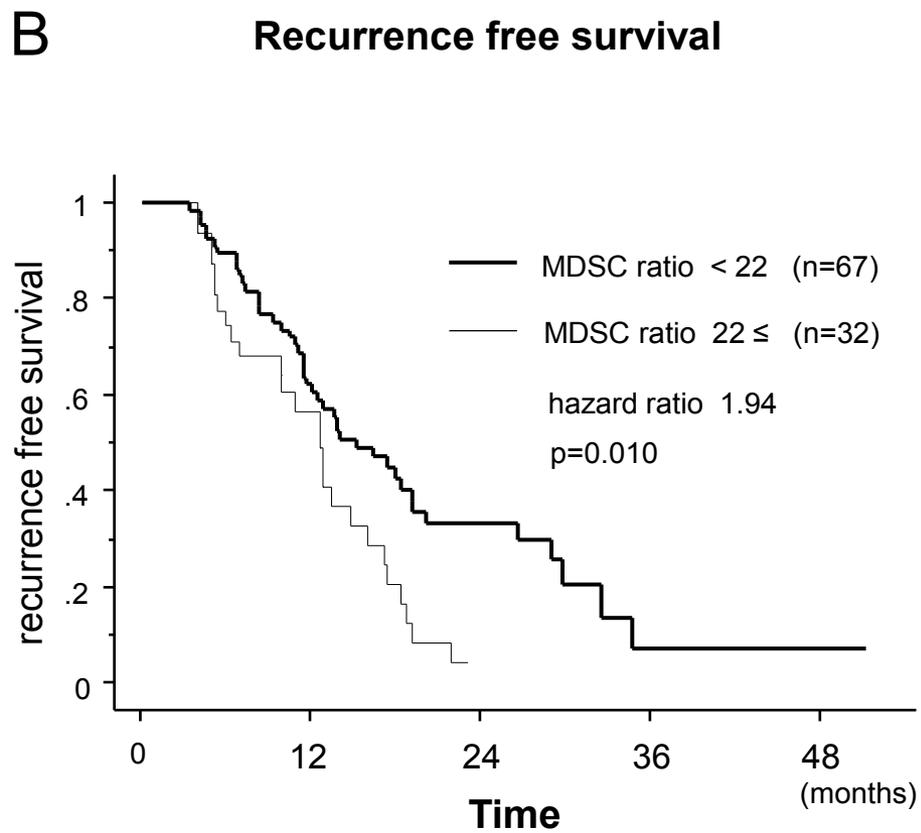
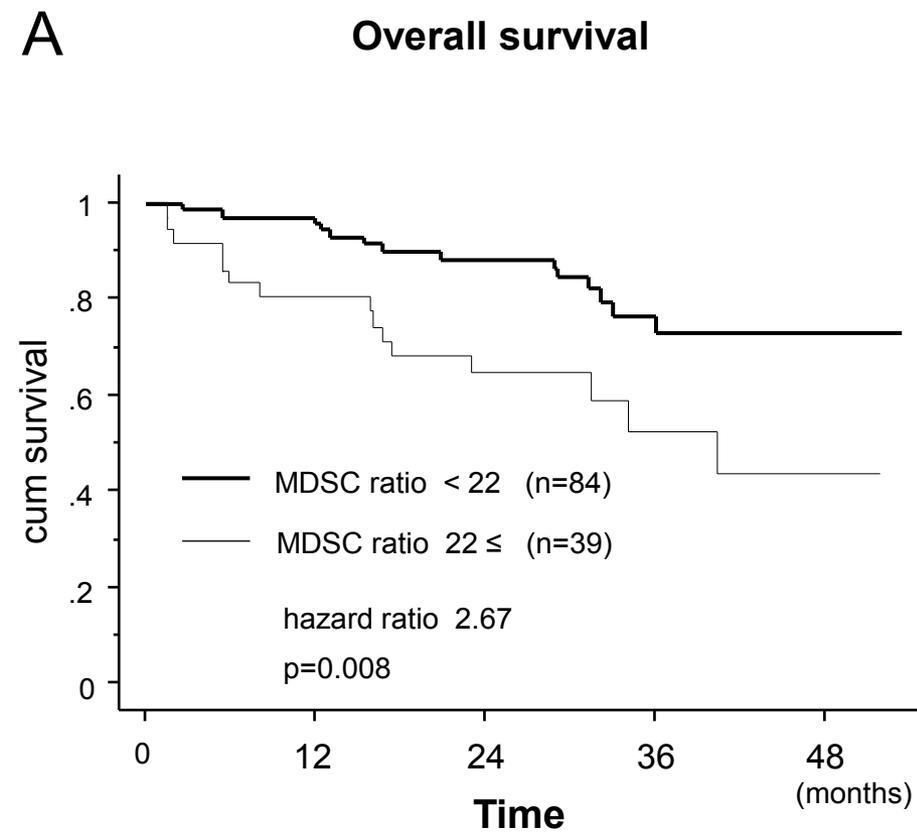
Supplemental table 3. The clinical parameters before RFA

Clinical characteristics	MDSC ratio ≥ 22	MDSC ratio < 22	p value
	(n=8)	(n=25)	
Pre MDSC ratio (%)	28.7	14.8	0.004
Post MDSC ratio (%)	30.2	10.8	<0.001
Sex (M/F)	6/2	13/12	0.252
Prothrombin time (%)	82.8	79.9	0.663
Serum albmin (g/dl)	3.70	3.67	0.794
Total bilirubin	0.83	1.03	0.408
Platelet ($10^4/\mu\text{l}$)	8.6	9.5	0.473
Type IV collagen 7S (ng/ml)	7.5	7.6	0.924
AFP (ng/ml)	146	41.7	0.214
Pre neutrophil/lymphocyte	1.98	2.19	0.486
Post neutrophil/lymphocyte	2.70	2.16	0.097
Pre ALT (IU/l)	45.0	55.3	0.421
Post ALT (IU/l)	42.6	52.2	0.584
Child-Pugh (A/B/C)	7/1/0	20/5/0	0.632
Tumor Size (mm)	21.8	18.2	0.313
Tumor Multiplicity (multiple/solitary)	6/2	13/12	0.252

Abbreviations: AFP, Alpha-fetoprotein; ALT, alanine aminotransferase

A CD15⁺ CD14⁻ CD11b⁺ CD33⁺ cells**B** CD15⁺ CD14⁻ CD11b⁺ CD33⁺ cells / PBMCs**C** CD15⁺ CD14⁻ CD11b⁺ CD33⁺ cells / PBMCs

A CD4⁺ CD25⁺ CD127^{-/low} Tregs**B** Tregs / CD4⁺ cells**C** Tregs / CD4⁺ cells**D**



Suppl. figure 1: (A) Gating strategy of CD15⁺CD14⁻CD11b⁺CD33⁺ cells (B) The frequency of CD15⁺CD14⁻CD11b⁺CD33⁺ cells in PBMCs was higher in HCC patients than healthy donors (2.84% vs. 2.06%, $p = 0.073$). (C) The frequency of CD15⁺CD14⁻CD11b⁺CD33⁺ cells was correlated with tumor progression (stage III and IV: 3.69% (n=13) vs. stage I and II: 2.39% (n=24), $p = 0.022$).

Suppl. Figure 2: (A) Gating strategy of CD4⁺ CD25⁺ CD127^{-/low} Tregs (B) The frequency of CD4⁺ CD25⁺ CD127^{-/low} Tregs was significantly increased in HCC patients than healthy donors (*, $p < 0.01$). (C) The frequency of Tregs was correlated with tumor progression. (D) There was only a weak correlation between the frequencies of CD14⁺HLA-DR^{-/low} MDSCs and CD4⁺ CD25⁺ CD127^{-/low} Tregs ($r = 0.24$).

Suppl. figure 3: (A) Kaplan-Meier curve for overall survival in our cohort. (B) Kaplan-Meier curve for recurrence free survival in our cohort.