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ORIGINAL ARTICLE

Increase in $CD14^{+}HLA\text{-}DR^{-/low}$ myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis

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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 $CD14^{+}HLA\text{-}DR^{-/low}$ 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 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 vascular endothelial growth factor 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. 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.

Keywords Myeloid-derived suppressor cells · Hepatocellular carcinoma · Radiofrequency ablation · Recurrence · Cancer

Abbreviations

MDSCs	Myeloid-derived suppressor cells
HCC	Hepatocellular carcinoma
CLD	Chronic liver disease
RFA	Radiofrequency ablation
TACE	Transcatheter arterial chemoembolization
PBMC	Peripheral blood mononuclear cell
Tregs	Regulatory T cells
HLA	Human leukocyte antigen
FGF	Fibroblast growth factor
CCL	Chemokine C–C motif ligand
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
IP	Interferon gamma-induced protein
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

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JAK Janus kinase
STAT Signal transducer and activator of transcription

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer mortality globally [1, 2]. Current treatment options including surgical resection, radiofrequency ablation (RFA), liver transplantation, chemotherapy, transcatheter arterial chemoembolization (TACE), and sorafenib are reported to improve survival in HCC patients [3–7]. However, despite curative treatments for HCC, tumor recurrence rates remain high and the survival of those who have advanced HCC remains unsatisfactory [3–7]. Therefore, the development of new anti-tumor treatments for HCC remains an urgent and important field of research.

To overcome the limitations of these treatments, several immunotherapies have been developed as attractive strategies for HCC. In several studies of HCC immunotherapy, effective induction of immune-mediated cells by tumor antigen-derived peptides or antigen-presenting cells showed anti-tumor effects, but the population of patients who exhibited such effects was very small [8–12].

In previous studies, it was reported that many kinds of tumor generate a number of immune-suppressive mechanisms [13–15]. Recently, myeloid-derived suppressor cells (MDSCs) have been characterized as key immune regulators in various human cancers [15–24]. They show the capacity to inhibit T cell function and promote tumor development [15, 25]. Human MDSCs are a heterogeneous population that shows $CD11b^+$, $CD33^+$, $HLA\text{-}DR^{-/low}$ and can be divided into granulocytic $CD14^-$ and monocytic $CD14^+$ subtypes [26–28]. In most recent studies, it has been reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were increased in HCC patients and the cells inhibited the function of T cells through the induction of regulatory T cells (Tregs) [24]. Tregs represent 5–10 % of $CD4^+$ T cells and can suppress the activation and proliferation of $CD4^+$ and $CD8^+$ T cells [14, 29]. It was reported that an increased frequency of circulating Tregs was associated with poor survival of HCC patients [30]. Understanding the inhibitory mechanism of MDSCs and controlling their function are very important to develop more effective immunotherapy for HCC.

In this study, we investigate the host factors that are associated with the frequency of MDSCs in HCC patients and the effect of MDSCs on the prognosis of patients and clarify the relationships between a feature of MDSCs and host factors in HCC patients.

Materials and methods

Patients and healthy controls

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

Cell isolation and flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were separated 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 2,000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS, centrifuged at 1,400 rpm for 10 min, 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 1,640 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 FACSAria 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

$CD14^+HLA\text{-}DR^{-/low}$ MDSCs and $CD14^+HLA\text{-}DR^+$ cells were sorted using the Becton–Dickinson FACSAria II

system. 2×10^4 PBMCs were cultured and stimulated with 1 μg/ml plate-bound anti-CD3 (eBioscience) and 1 μg/mL soluble anti-CD28 (eBioscience) in 96-well round-bottomed plates. 24 h later, to determine the suppressive ability of MDSCs, increasing concentrations of MDSCs were added to the stimulated PBMCs. Proliferation was measured by ^{3}Hincorporation after 72 h. [^{3}H] thymidine was added, and cell proliferation was measured by incorporation of radiolabeled thymidine for 24 h.

Cytokine and chemokine profiling

Blood samples were collected from patients at the same time of PBMC isolation. After centrifugation at 3,000 rpm for 10 min at 4 °C, serum fractions were obtained and stored at −20 °C until use. Serum levels of various cytokines and chemokines were measured using the Bio-Plex Protein Array System. Briefly, frozen serum samples were thawed at room temperature and diluted 1:4 in sample diluents; 50 μl aliquots of the diluted sample was added in duplicate to the wells of 96-well microtiter plates containing the coated beads for a validated panel of human cytokines and chemokines according to the manufacturer's instructions. The following 27 cytokines and chemokines were targeted: IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin (chemokine C–C motif ligand (CCL) 11), G-CSF, GM-CSF, IFN-γ, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF). Nine standards (ranging from 0.5 to 32,000 pg/ml) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1.

Statistical analysis

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

Results

$CD14^+HLA\text{-}DR^{-/low}$ MDSCs are increased in the peripheral blood of HCC patients

We analyzed the peripheral blood of 123 patients with HCC, 26 CLD patients without HCC, and 13 healthy donors for the prevalence of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs. Because the PBMCs are tested after Ficoll, some cells may be lost. Therefore, we examined the population of MDSCs as a percentage of total $CD14^+$ cells by flow cytometry after cell surface labeling for the expression of HLA-DR (Fig. 1a). $CD14^+HLA\text{-}DR^{-/low}$ population in PBMCs of HCC patients represented 3.2–56.8 % of the $CD14^+$ cells. The frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs/$CD14^+$ cells in cryopreserved PBMCs correlated with that in fresh PBMCs (Fig. 1b). Therefore, we analyzed the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs/$CD14^+$ cells using cryopreserved PBMCs.

To confirm the function of these cells, sorted $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and $CD14^+HLA\text{-}DR^+$ (control) cells were added at different ratios to autologous anti-CD3/CD28-stimulated PBMCs, and the proliferation was measured by ^{3}Hincorporation. $CD14^+HLA\text{-}DR^{-/low}$ MDSCs of HCC patients significantly decreased autologous PBMC proliferation (Fig. 1c). On the other hand, $CD14^+HLA\text{-}DR^+$ (control) cells could not suppress the autologous PBMC proliferation.

As shown in Fig. 1d, the frequency of MDSCs was significantly higher in HCC patients (19.0 %) than in healthy donors (9.4 %) ($p < 0.01$). Overall frequencies of $CD14^+$ cells did not differ significantly between the groups (Fig. 1e). Individual frequencies of MDSCs of all the patients and healthy donors are represented as scatter plots (Fig. 2a). The frequency of MDSCs was correlated with the stage of HCC (stage III and IV: 22.3 % ($n = 46$) vs. stage I and II: 17.0 % ($n = 77$), $p < 0.01$) and was significantly higher in HCC patients than CLD patients without HCC and healthy donors. Interestingly, there was no difference between CLD patients without HCC and healthy donors. Moreover, these numbers did not change depending on the degree of fibrosis or inflammatory activity of the liver (Fig. 2b, c).

In previous reports, granulocytic MDSCs were defined in combination with several surface markers including CD14, CD15, CD11b, CD33, CD66b, and HLA-DR in several cancers. Therefore, we examined the frequency of $CD15^+CD14^-CD11b^+CD33^+$ cells in 37 HCC patients and 11 healthy donors (Suppl. figure 1A). Although there was no statistical significant difference, the frequency of

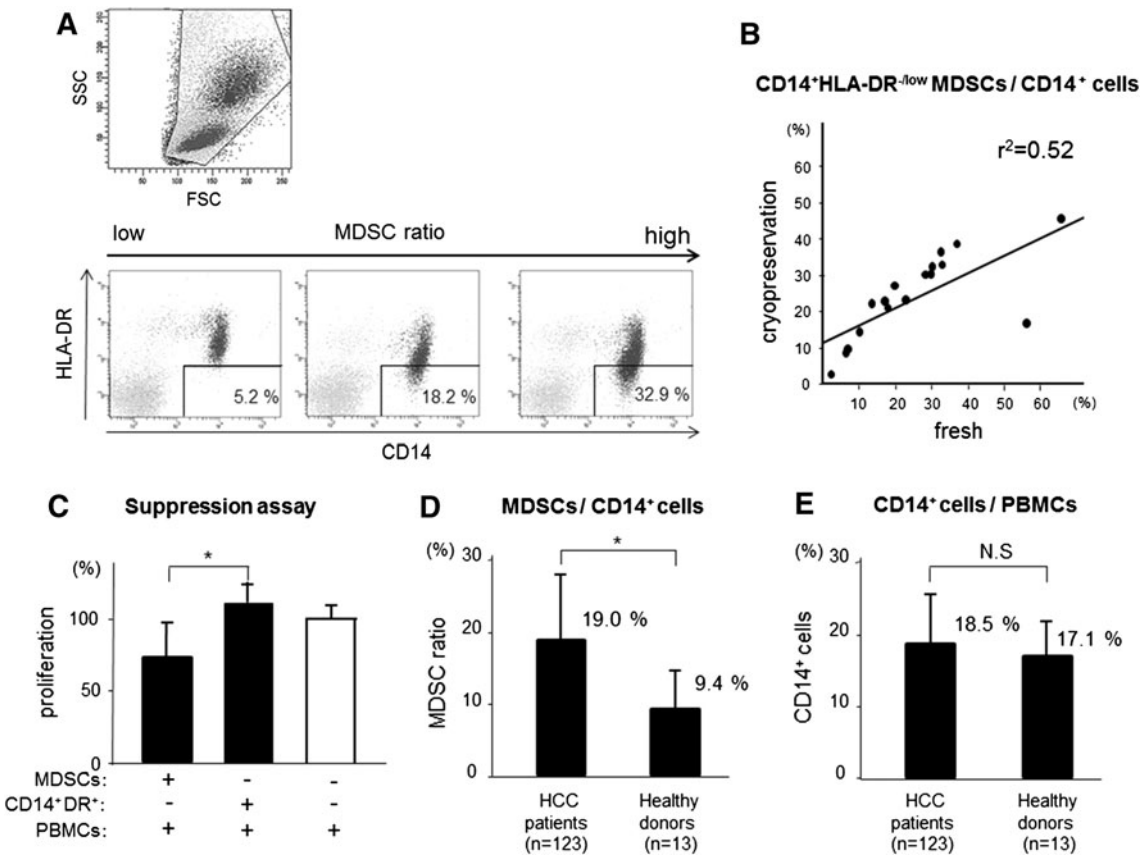

Fig. 1 **a** Flow cytometry shows $CD14^{+}HLA\text{-}DR^{-/low+}$ MDSCs. PBMCs from patients and healthy donors were labeled with anti-CD14 and HLA-DR. Three staining examples of HCC patients are shown in the order from a small number (*left*) to a large number (*right*). **b** The increase in $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs/$CD14^{+}$ cells in cryopreserved PBMC correlated with that in fresh PBMC ($r^2 = 0.52$). **c** Proliferation of PBMCs stimulated by anti-CD3/28 in the presence or absence of MDSCs was measured by ^{3}Hincorporation assay. $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs significantly decreased autologous PBMC proliferation ($n = 4$; *, $p < 0.05$). **d** The frequency of MDSCs was significantly higher in HCC patients than healthy donors (*, $p < 0.01$). **e** Overall frequencies of $CD14^{+}$ cells did not differ significantly

$CD15^{+}CD14^{-}CD11b^{+}CD33^{+}$ cells in HCC patients was higher than that in healthy donors (2.84 vs. 2.06 %, $p = 0.073$) (Suppl. figure 1B). The frequency was correlated with the stage of HCC (stage III and IV: 3.69 % ($n = 13$) vs. stage I and II: 2.39 % ($n = 24$), $p = 0.022$) (Suppl. figure 1C).

Relationship between the frequency of Tregs and MDSCs

It is well known that the frequency of circulating Tregs is increased and correlated with disease progression in HCC patients. The frequency of $CD4^{+}$ $CD25^{+}$ $CD127^{-/low}$ Tregs was significantly increased in HCC patients (Suppl. figure 2A) and associated with tumor progression (Suppl. figure 2B). However, there was not a strong correlation between the frequency of MDSCs and Tregs in our 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, 28.3 vs. 24.4 mm; tumor multiplicity (multiple/solitary), 27/12 vs. 42/42; TNM stage (I and II vs. III and IV), 17/22 vs. 60/24, $p < 0.05$) (Table 1). Moreover, 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).

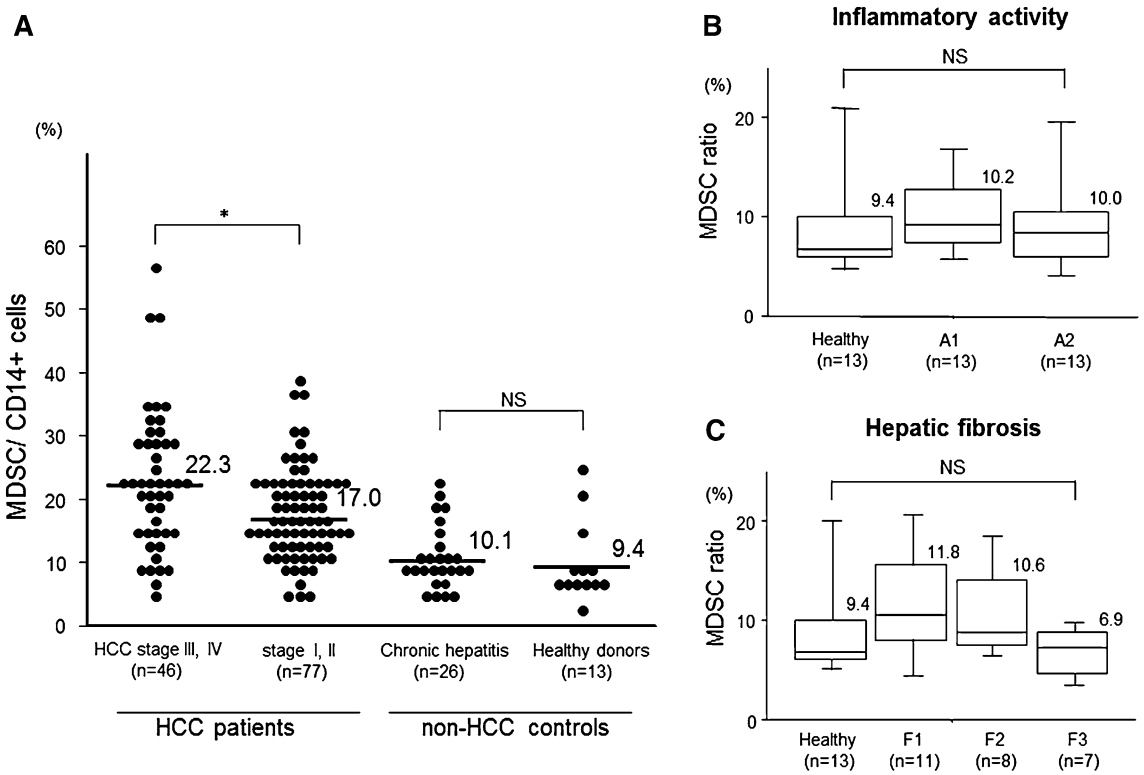

Fig. 2 **a** Scatter plots of MDSC ratio ($CD14^+HLA\text{-}DR^{-/low}$ MDSCs/ $CD14^+$ cells) in patients and healthy donors. The frequency of MDSCs was significantly increased in HCC patients compared with that in non-HCC controls. Moreover, the frequency of MDSCs was correlated with tumor progression (stage III and IV: 22.3 % ($n = 46$) vs. stage I and II: 17.0 % ($n = 77$); *, $p < 0.05$). In non-HCC controls, there was no significant difference in the frequency of MDSCs. **b**, **c** In non-HCC patients, the frequency of MDSCs did not change depending on the degree of fibrosis or inflammatory activity of the liver according to the Metavir scoring system

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, 31–34]. 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 IL-10, IL-13, and VEGF were significantly increased in the group with a high frequency of MDSCs (Table 2). In addition, there was a positive correlation between these cytokine levels in serum and the frequency of MDSCs. We also examined the relationship between serum cytokine levels and the frequency of Tregs. We divided the HCC patients into two groups using the threshold of 7 %, which is the average +2SD of the % of Tregs among $CD4^+$ cells in non-HCC patients. Serum concentration of IL-10 was significantly increased in the group with a high frequency of Tregs (Suppl. table 2).

Kinetics of MDSCs before and after curative RFA therapy

We examined the frequency of MDSCs before and after curative RFA therapy in 33 patients. For this analysis, blood samples were obtained on the day of treatment (before) and 2–4 weeks after treatment (after). The frequency of MDSCs was significantly decreased after RFA therapy (18.0 to 15.5 %, $p < 0.05$) (Fig. 3a). However, in several patients, the frequency of MDSCs remained at a high level compared with that in non-HCC patients. The clinical parameters before RFA were not statistically different between the patients with and without a high frequency of MDSCs after RFA (Suppl. table 3).

Next, we followed up these patients for recurrence and analyzed the risk factors. If a high frequency of MDSC was observed after curative RFA therapy, the recurrence-free survival was significantly shortened (Fig. 3b). In contrast, the frequency of MDSCs before treatment did not affect the recurrence. In univariate analysis for recurrence, post-treatment MDSC ratio ≥22 % ($p = 0.023$) and tumor

Table 1 Clinical findings and MDSCs

Clinical characteristics	MDSC ratio ≥22 ($n = 39$)	MDSC ratio <22 ($n = 84$)	p value
Age (year)	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
γGTP (IU/l)	78.0	76.0	0.252
Platelet (10^4/μl)	10.9	10.6	0.884
Prothrombin time (%)	75.2	82.3	0.045
Serum albumin (g/dl)	3.53	3.68	0.120
Total bilirubin (mg/dl)	1.21	0.94	0.286
WBC (/μl)	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

AST aspirate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactic dehydrogenase, *γGTP* 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 U test, and Kruskal–Wallis test were used for univariate analysis of two groups that were classified according to the frequency of MDSCs

multiplicity ($p = 0.010$) were significantly associated with HCC recurrence (Table 3). In multivariable analysis for recurrence, considering the variables in the univariate analysis with $p < 0.1$, only post-treatment MDSC ratio ≥22 % (HR 3.906, $p = 0.014$) was extracted as a significant risk factor for recurrence.

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, $CD14^+HLA\text{-}DR^{-/low}$ 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. [24] reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were significantly increased in HCC patients and they suppressed T cell functions through the induction of $CD4^+CD25^+Foxp3^+$ Treg.

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 $CD14^+HLA\text{-}DR^{-/low}$ 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 in 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 in inflammatory cell infiltration into liver might result in an increase in MDSCs following the progression of HCC. However, there was no relationship between the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and underlying liver status in our study. From our observations, increase in MDSCs was only correlated with tumor progression, but not with hepatic fibrosis or disease activity of CLD. This finding suggests that the expansion of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was mostly derived from the tumor environment itself, but not from inflammation or fibrosis of liver tissue around the tumor. The finding that a significant decrease in the frequency of circulating $CD14^+HLA\text{-}DR^{-/low}$ MDSCs is observed in most patients with curative treatment in this study supports this hypothesis. On the other hand, Tregs were also increased in HCC patients and associated with the progression of HCC. Though it was reported that MDSCs suppressed T cell function through the induction of Tregs, there was not a strong correlation between the frequencies of these two immunosuppressive cells.

Regarding the mechanism of MDSC expansion, we also analyzed the relationship between the serum cytokine levels and the frequency of MDSCs. We observed that the serum concentrations of IL-10, IL-13, and VEGF were significantly increased in the group with high frequency of MDSCs and there was a positive correlation between these cytokine levels and the frequency of MDSCs. Moreover, although there was no significant difference, the serum

Table 2 Serum cytokines and MDSCs

Cytokine	Healthy donor (mean) ($n = 13$)	MDSC ratio ≥ 22 (mean) ($n = 21$)	Range	MDSC ratio < 22 (mean) ($n = 31$)	Range	p value
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	(1,312–10,136)	4013	(831–13,557)	0.484
RANTES	2978	2890	(1,040–4,826)	3184	(599–6,165)	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

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 *U* test was used for univariate analysis of two groups that were classified according to the frequency of MDSCs

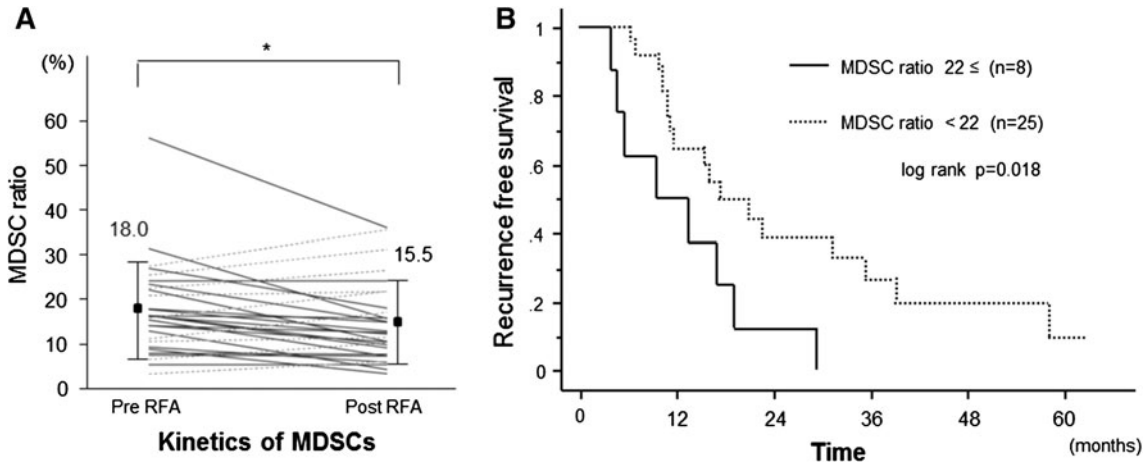

Fig. 3 **a** In 33 HCC patients who received curative RFA therapy, the frequency of MDSCs was significantly decreased after treatment. However, in several patients, the frequencies were increased after treatment (*dotted lines*) (*, $p < 0.05$). **b** Kaplan–Meier curve for recurrence-free survival after RFA therapy. The patients with high frequency of MDSCs (*solid line*) relapsed

concentrations of IL-1ra, IL-2, IL-6, IL-12(p70), and G-CSF tended to be increased in the group with high frequency of MDSCs. In accordance with our results, various cytokines, including IL-6, IL-10, IL-13, G-CSF, and VEGF, that trigger Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways have been reported to be associated with the frequency of MDSCs [39]. In particular, the cytokines involved in the JAK2-STAT3 signaling pathway are considered to be the main regulators of the expansion of MDSCs, which leads to stimulation of myelopoiesis and inhibition of myeloid-cell differentiation [40–42].

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 years	1.111 (0.671–1.840)	0.683		
Pre-MDSC ratio: ≥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 (0993–1.008)	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: ≥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

HR hazard ratio, *CI* confidence interval, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein

Another important finding of our study is that the frequency of MDSCs showed various changes after curative RFA and this frequency is an independent risk factor of HCC recurrence. In most of the patients, the frequency of MDSCs decreased after RFA. A similar phenomenon has also been reported in other cancer treatments [19, 21, 36]. Liu et al. [21] reported that MDSCs were decreased in non-small cell lung cancer patients who had clinical benefit from chemotherapy or who received curative surgery. These results suggest that a decrease in the frequency of MDSCs is due to tumor eradication.

It is well known that tumor factors including multiplicity, tumor diameter, serum levels of tumor marker, and hepatic reserve are risk factors of HCC recurrence after RFA [43, 44], but it has not been reported that the frequency of circulating MDSCs is also a risk factor. From our findings, there was a clear inverse correlation between the frequency of MDSCs after RFA and recurrence-free survival. Consistent with our results, in the patients with pancreatic, esophageal, and gastric cancer, Gabitass et al. [23] reported that an increase in MDSCs was associated with an increased risk of death and that the frequency of MDSCs was an independent prognostic factor for patient survival. Taken together with these findings, our results suggest that the frequency of MDSCs might be one of the prognostic factors of patients after cancer treatments.

As we showed, the frequency of MDSCs is primarily correlated with tumor progression. However, between the patients with high and low frequency of MDSCs after RFA, there was no significant difference in hepatic reserve and tumor factors before treatment. Although an incomplete HCC eradication at a microscopic level may allow a high frequency of MDSCs after RFA, there may be other mechanisms such as subsequently tumor-specific immune responses after RFA. In addition, there is a limitation of the present study because we used cryopreserved PBMCs for phenotypic analysis of MDSCs. Further studies using fresh PBMCs are needed for precise phenotypic analysis of MDSCs and elucidation of the mechanism to regulate the frequency of MDSCs after HCC treatment.

In conclusion, the frequency of MDSCs in HCC patients is correlated with tumor progression, and the frequency after RFA is inversely correlated with the prognosis of HCC patients. HCC patients who show a high frequency of MDSCs after RFA should be closely followed, and the inhibition or elimination of MDSCs after HCC treatments may improve the prognosis of HCC patients.

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Conflict of interest The authors declare no conflict of interest.

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