staging system. The frequency of TEMs did not differ between the patients with advanced HCC stages (BCLC, C and D) and those with early stages (A and B) (3.6 ± 2.2% vs. 3.3 ± 2.3%). These results show that the increase of TEMs is closely related to the presence of HCC, irrespective of the stage of cancer. Furthermore, in non-HCV-infected HCC patients (NBNC-, alcoholic- and HBV-HCC patients), the same increment of peripheral TEMs was observed (Supplementary figure 1), suggesting that the increase of TEMs is influenced by HCC, not by infection with hepatitis viruses.

We serially examined the frequency of TEMs in HCC patients who underwent RFA therapy or tumor resection. In clinical practice, we assessed the viability of HCC by CT or MRI scanning every 3 to 6 months after the treatment. In patients without HCC recurrence, the frequency of TEMs dramatically decreased after successful HCC ablation and resection (Fig. 2C). By contrast, in patients with subsequent HCC recurrence, TEMs increased again before the apparent radiological identification of HCC (Fig. 2C). Therefore, the TEM frequency dynamically changes in patients in correlation with the presence or absence of HCC.

In order to assess the clinical impact of TEMs, we compared various clinical parameters between patients with higher TEM frequency and those with lesser frequency. We categorized HCC patients according to their peripheral TEM frequency of above ($TEM^{high}$) or below ($TEM^{low}$) the median value (cut off value = 2.75). We found that the patients in the $TEM^{high}$ group displayed a more

advanced Child-Pugh grade (B) and higher the model for endstage liver disease (MELD) score, a lower prothrombin time and a lower albumin level (Table 2). These results imply that an increment of TEMs is associated with a deterioration of liver function in HCC patients. Furthermore, the $TEM^{high}$ group as assessed before the RFA treatment or the resection of HCC shows significantly shorter recurrence-free survival rates than the $TEM^{low}$ group, suggesting that the numerical assessment of TEMs holds some prognostic value (Fig. 2D). The overall survival of the patients with HCC after the treatments was not different between the $TEM^{high}$ and $TEM^{low}$ groups in this observation period (Supplementary figure 2).

***TEMs are located in the perivascular areas of HCC***

Most of the TEMs, identified by $CD14^{+}TIE2^{+}$ cells in situ, are located in the perivascular area of cancer tissues determined under immunofluorescence staining (Fig. 3A-[A], [B], [D]). Most of the $CD16^{+}$ signals are overlapped on $CD14^{+}TIE2^{+}$ cells (Fig. 3A-[C]). Some of the TEMs are localized inside the lumen of vascular cells ($CD14^{-}TIE2^{+}$ cells) in cancer lesions (Fig. 3A-[D]). However, they are scarce in adjacent non-cancerous tissues (not shown).

***TEMs accumulate in HCC tissue***

The frequency of TEMs in tumor-infiltrating leukocytes (TIL) was higher than that in cells

infiltrating non-tumor tissue (NIL) and PBMC (Fig. 3B). Moreover, in patients with HCC, the frequencies of TEMs in PBMC and TIL are positively correlated (Fig. 3C). These results suggest that TEMs tend to accumulate into HCC tissue. Of particular importance is that the peripheral TEM frequency reflects the degree of their infiltration into HCC tissues.

***The TEM frequency is well correlated with the degrees of microvessels in HCCs***

To study whether the frequencies of peripheral or intra-hepatic TEMs are correlated with the degrees of vascular formation in liver cancer, we examined the presence of $CD34^+$ cells in liver tissues obtained from 12 HCC patients. The expression of CD34 was predominantly confined to the cytoplasm of vascular endothelial cells. In general, microvessels are represented by brownish yellow capillaries or small cell clusters. The $CD34^+$ cells were located mainly in tumor cell areas (Fig. 4A). The values of microvessel density (MVD) in HCC ($67.0 \pm 57.8$) tended to be higher than those in non-cirrhotic ($26.7 \pm 7.5$) or cirrhotic non-cancerous tissues ($32.1 \pm 11.6$), respectively. Furthermore, the values of MVD in HCC tissues were positively correlated with the frequencies of peripheral and intra-hepatic TEMs in HCC patients (Fig. 4B). These results suggest that TEMs are involved in the promotion of neo-vascularization in liver cancer.

***Peripheral TEM frequency is superior to AFP and PIVKA-II levels as a diagnostic marker for***

*HCC*

In order to evaluate the feasibility of TEM frequency as a diagnostic marker of HCC, we examined whether or not the frequency of TEMs is correlated with various clinical parameters of HCC patients. No correlation was found between TEM frequency and other HCC-specific markers such as α-fetoprotein (AFP) or protein induced by the absence of vitamin K or antagonist II (PIVKA-II) (Fig. 5A). In addition, peripheral TEM frequency was not correlated with any of the levels of several angiogenic factors, such as VEGF, ANG-2, sVEGFR-1 and MIF (Supplementary figure 3). As for the diagnostic value of TEM frequency for differentiating HCC from chronic liver disease (CLD; chronic hepatitis and liver cirrhosis patients) or liver cirrhosis, its sensitivity and specificity were 86.1 and 71% or 81.3 and 90%, respectively (Table 3). ROC analyses revealed that TEM frequency was superior to AFP, PIVKA-II and ANG-2 levels as a diagnostic marker for HCC (Fig. 5B and Table 3).

## Discussion

In this study, we defined TEMs as $CD14^+CD16^+TIE2^+$ and examined their frequency, localization and correlation with micro-vessel densities in the liver. We demonstrated that 1) the frequency of TEMs is significantly increased both in PBMC and in the liver of HCC patients and positively correlates with the degree of micro-vessels in the HCC tissue; 2) its frequency dramatically changes in parallel with complete ablation or recurrence of HCC; 3) the peripheral frequency of TEMs can serve as a better diagnostic marker of HCC than AFP, PIVKA-II and ANG-2 levels. These results show that certain HCC-derived factors are responsible for the generation of TEMs, the degree of which is correlated with the intensity of vascular formation.

According to the patterns of CD16 and CD14, it has been reported that monocytes can be categorized into distinct subsets, such as classical ($CD14^+CD16^-$) and non-classical ones ($CD14^+CD16^+$) (20). Such populations are regarded as functionally distinct, since the percentage of $CD14^+CD16^+$ cells predominantly increases under inflammatory conditions such as chronic hepatitis and inflammatory bowel disease (21, 22). We identified TEMs in $CD14^+CD16^+$ monocytes but not in $CD16^-$ monocytes, suggesting that TIE2 is predominantly induced in $CD16^+$ monocytes. However, the precise mechanisms of TIE2 induction on monocytes have been largely unknown. Furthermore,

it is yet to be clarified whether $CD16^+$ monocytes are differentiated from $CD16^-$ monocytes or not. Multiple factors are reported to enhance CD16 expression on monocytes, such as macrophage-colony-stimulating factor (M-CSF), IL-10 and transforming growth factor (TGF)-β1 (23). In addition, some studies disclosed that hypoxia and MIF contributed to TIE2 expression on monocytes in vitro (14, 24). Cumulative data have been published showing that cancer cells, including HCC, are dichotomously capable of releasing various inflammatory (TNF-α, IL-1β) and anti-inflammatory (TGF-β and IL-10) cytokines, as well as hematopoietic factors (M-CSF and MIF) (7, 25-29). However, in this study, no correlation was found between the frequency of TEMs and any of serum angiogenesis factors (Supplementary figure 3). It is thus plausible that, not a sole but a mixture of such HCC-derived factors contributes to the generation of TEMs from $CD16^+$ monocytes with the aid of a hypoxic microenvironment. In our preliminary study, a combination of cytokines and growth factors were able to induce TIE2 on $CD16^+$ monocytes (manuscript in preparation).

Although the presence of TEMs has been reported in cancer tissues from patients with colorectal, pancreatic or renal cancer (15), the actual roles of TEMs in the angiogenesis process of HCC have yet to be elucidated. In this study, we showed that the peripheral and intrahepatic frequency of TEMs is positively correlated with the density of micro-vessels in the liver. Additionally, we found that TEMs accumulate in the liver and are located in the perivascular area of HCC tissues. In support for our observations, Venneri et al. reported that TEMs preferentially localize in the vicinity of tumor

blood vessels and avascular viable areas in human cancer specimens but are not found in non-neoplastic tissues adjacent to tumors (15). These results suggest that TEMs may take some part in HCC-related vascular formation. Further investigation is still needed to disclose the molecular mechanisms of TEMs accumulating in the liver. In analogy of $CD14^{+}CD16^{+}$ monocytes, some interactions between chemokine and its receptors, such as CCR5 or CX3CR1, may be involved in such process (22), all of which are shown to be expressed in TEMs as well (Fig. 1D).

Thus far, many studies have reported that the histological degree of angiogenesis in the liver is closely correlated with the prognosis or survival of patients with HCC. In agreement with these results, several serum angiogenesis factors, such as VEGF, ANG-2, sVEGFR-1 and MIF, are reported to be feasible as markers of prognosis, invasiveness or post-therapeutic recurrence in HCC patients (7-11). Multivariate analysis disclosed that the degree of angiogenesis in the liver, as assessed by MVD, is an independent factor significantly involved in the disease-free survival rate in patients with resectable HCC (17) Therefore, the positive correlation between peripheral and intra-hepatic TEM frequency and MVD in the liver observed in this study offers the possibility of TEMs being as a prognostic marker of HCC patients. In support for this, the patients with higher TEM frequency were at a more advanced Child-Pugh stage, had poorer liver function and showed higher post-therapeutic HCC recurrence in this cohort.

In order to diagnose the existence of HCC, serum AFP and PIVKA-II levels have been commonly used in clinical practice. However, the sensitivity and specificity of both markers are unsatisfactory for the detection of HCC (12). In the present study, in differentiating HCC from CLD or LC, ROC analysis revealed that the peripheral frequency of TEMs is higher than serum AFP, PIVKA-II, ANG-2 levels with respect to AUC, sensitivity and specificity. Thus, the frequency of TEMs could be better than AFP and PIVKA-II for diagnosing HCC. Evaluation of the clinical value of TEMs in the long-term prognosis of HCC patients is further needed as the observation periods in the current study were a maximum of 3.0 years. During the observation period, no significant difference was detected in such outcome between the $TEM^{high}$ and $TEM^{low}$ groups (Supplementary figure 2).

In summary, TEMs are significantly increased both in the periphery and in the liver of HCC patients and hold diagnostic value for HCC. The frequency of TEMs is well correlated with the degrees of neo-vascularization in HCC tissue. Thus, TEMs would be feasible as a diagnostic cellular marker of HCC potentially reflecting angiogenesis in the liver.

## Figure Legends

**Fig. 1: Identification and phenotypic analyses of TEMs as $CD14^+CD16^+TIE2^+$ cells in the peripheral blood.**

**A.** PBMC obtained from HCC patients were stained and analyzed by flow cytometry. $CD14^+$ monocytes were divided into two distinct subsets, $CD14^{++}CD16^-$ and $CD14^+CD16^+$ cells. These cells were examined for TIE2 expression. The numbers in the histograms depict the percentages of gated cells. Representative plots from three patients are shown.

**B.** Frequencies of TIE2 in $CD14^+$ monocytes were compared among two distinct subsets; $CD16^+$ monocytes and $CD16^-$ monocytes, See Fig.1A. The bars indicate mean ± SE of 89 patients. *: $p<0.0001$ by Mann-Whitney nonparametric U test.

**C.** Western-blot analysis of TIE2 expression in FACS-sorted $CD14^+CD16^+$ and $CD14^{++}CD16^-$ cells from HCC patients. As shown, the bands were TIE2 (140 kDa molecular weight) (the top panels) and those are β-actin (45 kDa molecular weight) (the bottom panels), respectively. The representative results of three series of experiments from 7 HCC patients are shown.

**D.** TEMs and $TIE2^-$ monocytes in the periphery were gated and analyzed for the expression of various molecules, as indicated in the histogram plots. The filled light gray line, black line and gray line depict the negative control, the expression of relevant markers in TEMs and $TIE2^-$ monocytes,

respectively. The percentage of the marker-positive cells is shown in the histograms. The upper numbers are for TEMs and the lower ones are for TIE2$^-$ monocytes. The representative plots of six series of experiments are shown.

**E.** Comparative analyses of the expression of CCR4, CCR5, CX3CR1, CD40 and CD86 between TEMs and TIE2$^-$ monocytes, assessed by FACS as described above. The bars indicate mean ± SE of six series of experiments. *: $p<0.05$, **: $p<0.005$, ***: $p<0.001$ by Mann-Whitney non-parametric U test.

**Fig. 2: Peripheral frequency of TEMs is increased in patients with HCC, with changes paralleling post therapeutic HCC recurrence.**

**A.** Frequencies of peripheral TEMs in CD14$^+$ monocytes are shown in four groups of HCV-positive patients; HS, CH, LC and HCC, see Table.1. *: $p<0.0001$ by Kruscal-Wallis test with Dunn's multiple comparison test.

**B.** Frequencies of peripheral TEMs in HCC patients at different clinical TNM stages (early stage; I+II; n=70, advanced stage; III+IV; n=19). n.s., not significant by Mann-Whitney nonparametric U test.

**C.** In patients who underwent RFA therapy or resection of HCC, the frequencies of TEMs in CD14$^+$ monocytes were examined serially after confirmation of complete ablation or operation to remove

HCC lesions. The bold arrows depict the time point of the RFA therapy or the operation. Both panels depict the frequencies of TEMs in patients before and after the treatment. The left panel shows the results of TEMs in patients without HCC recurrence (n=12) as assessed by CT/MRI examinations, while the right panel shows the results in patients with HCC recurrence (n=5), respectively. *: $p<0.05$ by Paired t-test.

**D.** In patients with HCC who underwent RFA or the operation, the recurrence-free survival rate after the treatment was compared between those with $TEM^{high}$ (frequency of TEMs $\geq$ 2.75; n=45) and $TEM^{low}$ (frequency of TEMs < 2.75; n=44) using the Kaplan-Meier method, with the log-rank test for comparison. $TEM^{high}$ and $TEM^{low}$ , see Table 2. p = 0.047.

**Fig. 3: TEMs are observed in perivascular areas of HCC tissue, and their frequency is higher in HCC tissue than in the periphery.**

**A.** Immunofluorescence staining was performed as described in Materials and Methods. The staining for CD14 (green), CD16 (red) and TIE2 (red) identifies $CD14^+TIE2^-$, $CD14^+TIE2^+$, $CD14^+CD16^-$ cells and $CD14^+CD16^+$ monocytes in human liver tissue (blue: nuclei counterstained with Dapi). Representative results of the resected samples obtained from 12 HCC patients are shown. The panels show $CD14^+TIE2^+$ cells (A, B, D) and $CD14^+CD16^+$ cells (C) in the perivascular area of HCC tissue (magnification, 400×), Bold arrows depict $CD14^+TIE2^+$ cells, thin arrows $CD14^+CD16^+$ cells, bold

arrowheads vascular endothelial cells ($TIE2^+CD14^-$) and thin arrowheads $CD14^+TIE2^-$ cells.

B. The frequencies of TEMs in 9 patients with HCC are compared for those in the peripheral blood or in the liver. Liver-infiltrating leukocytes are divided into two distinct groups: leukocytes infiltrating non-tumor tissue (NIL) and tumor-infiltrating leukocytes (TIL). These cells were stained using anti-human CD14, CD16 and TIE2 mAbs. The analyses were performed as described in Materials and Methods. The samples were obtained from nine patients who underwent tumor resection. *: $p<0.05$, **: $p<0.0005$, by Paired t-test.

C. The correlation is analyzed between the frequencies of TEMs in PBMC and those in tumor-infiltrated lymphocytes. Analysis (n=14) was based on Pearson's correlation coefficient. P = 0.003, $R^2 = 0.53$

**Fig. 4: The degree of microvessels in HCC is correlated with the frequency of TEMs**

A. Assessment of neo-vascularization in HCC tissue was performed by staining $CD34^+$ cells in the resected samples obtained from patients with HCC. Immunohistochemical staining for CD34 was done as described in Materials and Methods. The $CD34^+$ cells were mainly confined to the cytoplasm of vascular endothelial cells as brownish yellow granules. Microvessels were represented by brownish yellow capillaries or small cell clusters. Representative results are shown. The panel shows a tumor cell area of a high grade.

**B.** The correlation is analyzed between TEM frequencies and the counts of $CD34^+$ cells (MVD values) in relevant patients. The panels show the correlation of peripheral (left: n=11) or intra-tumoral TEM frequencies (right: n=9) and MVD ($p = 0.0009$, $R^2 = 0.72$ and $p = 0.04$, $R^2 = 0.44$, respectively). Analysis was based on Pearson's correlation coefficient.

**Fig. 5: Frequency of TEMs is superior as a diagnostic for HCC to common tumor markers or angiogenesis factor.**

**A.** Correlation between TEM frequency and AFP (n=87) or PIVKA-II (n=81) was analyzed using Pearson's correlation coefficient. $P = 0.45$, $R^2 = 0.007$ or $P = 0.27$, $R^2 = 0.02$, respectively.

**B.** ROC analyses were performed in order to assess the diagnostic value of TEM frequency for differentiating HCC (n=89) from chronic liver disease (CLD, n=79) or liver cirrhosis (LC, n=30). The left panel shows the diagnostic value of TEMs, PIVKA-II, AFP and angiopoietin-2 (ANG-2) for HCC from CLD and the right panel shows those for HCC from LC, respectively.

**Supplementary figure 1: Frequency of TEMs is comparable in HCC patients regardless of etiology.**

The frequencies of peripheral TEMs are shown for HCC patients with or without HCV infection (n=89 and n=26, respectively). The group without HCV infection included patients with HBV

infection or those without HBV nor HCV (HBV-HCC and Non-B, Non-C [NBNC] HCC patients). n.s., not significant by Mann-Whitney non-parametric U test.

**Supplementary figure 2: Peripheral frequency of TEMs is not related to the overall survival in patients with HCC.**

In patients with HCC who underwent RFA or the operation, the overall survival rate after the treatment was compared between those with $TEM^{high}$ (frequency of TEMs $\geq$ 2.75; n=45) and $TEM^{low}$ (frequency of TEMs < 2.75; n=44) using the Kaplan-Meier method, with the log-rank test for comparison. $TEM^{high}$ and $TEM^{low}$, see Table 2. P=0.36

**Supplementary figure 3: Correlation between the frequency of TEMs and serum levels of angiogenesis factors**

All plots indicate the correlation between TEM frequency and VEGF, ANG-2, sVEGFR-1 or MIF. The numbers of patients examined were 32, 37, 23 and 31, respectively. Analyses were based on Pearson's correlation coefficient.

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**Table.1: The clinical backgrounds of the subjects**

| Clinicopathologic characteristics | CH | LC | HCC |
|---|---|---|---|
| Gender: Male/female | 21/28 | 13/17 | 63/26 |
| Age: mean±SD | 63.4±7.8 | 67.5±8.8 | 70.0±7.0 |
| Alanine aminotransferase (IU/L) | 56.1±43.5 | 54.0±28.6 | 46.3±29.5 |
| Prothrombin time (%) | 91.5±14.8 | 73.4±9.7 | 77.7±15.4 |
| Platelet ($\times 10^4/mm^3$) | 15.8±5.0 | 8.6±3.7 | 11.5±5.7 |
| Albumin (g/dl) | 4.0±0.3 | 3.5±0.4 | 3.5±0.5 |
| Total bilirubin (mg/dl) | 0.7±0.3 | 0.9±0.4 | 0.9±0.6 |
| Child-Pugh grade: A/B | 49/0 | 21/9 | 59/30 |
| α-fetoprotein (ng/ml) | 8.7±10.7 | 42.6±80.6 | 264.0±1281.7 |
| TNM stage: I/II/III/IV | | | 33/37/15/4 |
| BCLC stage: A/B/C/D | | | 44/13/26/6 |

TNM, Tumor, Lymph Node and Metastasis; BCLC, Barcelona-Clinic Liver Cancer; CH, chronic hepatisis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

**Table.2: The comparison of clinical parameters of HCC patients between those with higher frequency of TEMs and those with lower frequency**

| Clinicopathologic characteristics | Peripheral TEMs | | |
|---|---|---|---|
| | High (n=45) | Low (n=44) | *P* |
| Gender: Male/female | 32/13 | 31/13 | 1.000[†] |
| Age: mean±SD | 71.4±6.6 | 68.7±7.3 | 0.065[‡] |
| Child-Pugh grade: A/B | 24/20 | 34/10 | **0.043**[†] |
| MELD score | 9.1±2.3 | 8.2±1.8 | **0.010**[§] |
| α-fetoprotein (ng/ml) | 471.0±1785.2 | 52.3±101.4 | 0.101[§] |
| TNM stage: I+II/III+IV | 35/10 | 35/9 | 1.000[†] |
| BCLC stage: A+B/C+D | 25/20 | 32/12 | 0.123[†] |
| Vascular invasion: present/absent | 4/41 | 2/42 | 0.677[†] |
| Tumor size: <3/>3cm | 36/9 | 34/10 | 0.800[†] |
| Tumor number: single/multiple | 21/24 | 25/19 | 0.399[†] |
| Alanine aminotransferase (IU/L) | 51.5±34.5 | 41.0±22.6 | 0.251[§] |
| Prothrombin time (%) | 73.3±14.5 | 82.6±15.0 | **0.004**[§] |
| Platelet ($\times 10^4/mm^3$) | 10.8±5.2 | 12.2±6.2 | 0.319[§] |
| Albumin (g/dl) | 3.4±0.4 | 3.6±0.6 | **0.045**[§] |
| Total bilirubin (mg/dl) | 0.9±0.6 | 0.9±0.5 | 0.820[§] |

P value of less than 0.05 was expressed by thick one.

[‡]Student's *t* test, [†]$\chi 2$ test or Fisher exact test, [§]Mann-Whitney *U* test.