

Intratumoral and peritumoral expression of CD68 and CD206 in hepatocellular carcinoma and their prognostic value

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Abstract. The aims of the present study were to determine whether the changes in density and location of CD68-positive and CD206-positive macrophages contribute to progression of hepatocellular carcinoma (HCC) and to evaluate prognostic values of these cells in post-surgical patients. A retrospective study involving 268 HCC patients was conducted. CD68-positive and CD206-positive macrophage infiltration in HCC tissues and adjacent tissues was examined by immunohistochemistry (IHC) and the relationship between the clinicopathological features and prognosis was analyzed. Receiver operating characteristics (ROC) curve was used to calculate diagnostic accuracy. There was an increase in CD68-positive and CD206-positive macrophage infiltration in adjacent tumor tissues compared with tumor tissues. ROC curve identified their optimal diagnostic cut-off values. The survival analysis showed that increased CD68 expression in adjacent tissues conferred superior overall survival (OS) and disease-free survival (DFS), while increase of CD206 in tumor yielded inferior OS and DFS. Cox regression analysis suggested both CD68-positive macrophages in adjacent area and intratumor CD206-positive macrophages as independent prognostic biomarkers for post-surgical HCC patients. Finally,

a combination of CD68/CD206 and HBV-positive further improved prognostic stratification, especially in DFS. These results provide the first evidence for region- and subset-dependent involvement of CD68 and CD206 cells in HCC progression. A combination of CD68/CD206 density and HBV-positivity improves further predictive value for post-operative recurrence of HCC. Quantification of CD68/CD206 macrophages and their distribution can be exploited for better postsurgical management of HCC patients. These findings provide a basis for developing novel treatment strategies aimed at re-educating macrophages in tumor microenvironment.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies ranking as the fifth most common cancer worldwide (1). The incidence of advanced HCC cases who underwent multiple metastasis and dismal prognosis is increasing and usually the lack of effective treatment options will lead to serious social burdens (2). Currently, radical or partial hepatectomy represents the standard of care for patients with early diagnosis. However, distant metastases and postoperative relapse remain tremendous challenges. Therefore, identification of potential new therapeutic targets and prognostic biomarkers would be of great benefit to HCC patients.

Recent advance suggests that inflammation plays a critical role in carcinogenesis. As pivotal mediators in the inflammatory response, macrophages could generally be polarized into two principal subtypes: classically activated macrophages (M1) and alternatively activated macrophages (M2) (3). However, the two classes of macrophages play a diverse role in exhibiting pro- and anti-inflammatory activities, respectively. Tumor-associated macrophages (TAMs) are recruited to tumors by various cytokines and growth factors (4). They are widely documented as a fundamental cellular component within the tumor immune microenvironment, even having distinct phenotypes of TAMs at different stages of the tumor (5). TAMs substantially affect the fate of cancer cells and the clinical outcome by interacting in a complicated manner with neoplastic cells (6,7). Generally, TAMs

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Abbreviations: HCC, hepatocellular carcinoma; TAMs, tumor-associated macrophages; IHC, immunohistochemistry; ROC, receiver operating characteristics; OS, overall survival; DFS, disease-free survival; AUC, area under curve; M1, classically activated macrophages; M2, alternatively activated macrophages, TNM, tumor-node-metastasis

Key words: hepatocellular carcinoma, tumor-associated macrophages, CD68, CD206, prognosis

are of M2-like phenotype and have a protumor role. This is supported by the evidence that these macrophages can promote tumor progression and metastasis by orchestrating tumor cell motility, invasion, angiogenesis and antitumor immunity (8,9). In majority of solid tumors, TAMs were often associated with decreased patient survival (10-12). Nonetheless, conflicting results have been observed by several investigators in some types of cancers, for example, non-small cell lung cancer and papillary renal cell carcinoma (13,14).

CD68 is commonly considered to be a pan-macrophage marker (15,16). However, CD68 cannot distinguish M1 or M2 subtype from all the pan-macrophages (15). In addition to its existence in resident macrophages of multiple tissues, CD68 is also expressed in tumor-infiltrating macrophages. CD206 is known to be expressed on the surface of most classes of macrophages and dendritic cell subpopulations, and is routinely used to identify the M2 phenotype (17). CD206 is involved in antigen presentation, endocytosis and phagocytosis, signal transduction, innate host defense and the adaptive immune response (18). To date, both CD68 and CD206 have been the subjects of extensive structural and functional investigation in human diseases, especially in tumors. The associations of CD68-positive or CD206-positive macrophages with poor prognosis have been extensively documented in various types of malignancies, such as oral squamous cell carcinoma, breast cancer and HCC (19-24). However, the clinical relevance of macrophages infiltration to distinct histologic locations to HCC prognosis remained unclear. A better understanding of the roles of CD68 and CD206 macrophages in HCC may provide valuable information for prevention and treatment of HCC. Herein, we initiated a comparative follow-up study to explore their distribution and expression, as well as prognostic value in primary HCC.

Materials and methods

Patients and specimens. A total of 268 anonymized patients with primary HCC were collected and pathologically confirmed by Pathology Department of the First Affiliated Hospital of Anhui Medical University were enrolled in the present study. All the patients aged >18 years underwent surgical curative resection with follow-up from October 2011 to December 2016. Concurrence of other malignant diseases, autoimmune disease, syphilis and human immunodeficiency virus were excluded from the study. None of the cases received chemotherapy and/or radiotherapy previous to surgery. Total of 266 primary tumor tissues were grouped into case subjects and 243 tumor adjacent tissues were evaluated as the control group. Paired, formalin-fixed and paraffin-embedded (FFPE) primary tumor tissues and tumor adjacent tissues specimens were obtained from the whole patient population. This study was approved by the Human Research Ethics Committee of Anhui Medical University. Informed consents were obtained from all the participating patients.

In the present study, the hematoxylin and eosin slides were independently examined, and the histological diagnoses were re-evaluated systematically by two experienced pathologists who were blinded to the clinical outcome of the samples based on WHO classifications of HCC. Clinical stages of tumor progression were determined according to

the tumor-node-metastasis (TNM) classification system of the International Union against Cancer/American Joint Committee on Cancer (IUAC/AJCC) (Edition 7). The following clinical data were obtained in a well-designed questionnaire, including TNM stage, demographics (sex and age), family history, alcohol consumption, laboratory data including alpha fetoprotein (AFP), serum albumin and total bilirubin (TBIL).

Reagents. The following primary antibodies were used: mouse anti-CD68 antibody (KP1) (cat. no. ab845), mouse anti-CD206 antibody (cat. no. ab117644) and they were obtained from Abcam (Cambridge, MA, USA). Anti-CD68 antibody was prediluted. The anti-CD206 antibody was applied at a dilution of 1:200. Both immunohistochemical MaxVision kit and diaminobenzidine (DAB) chromogenic reagent kit were purchased from Fuzhou Maixin Biotech, Co., Ltd. (Fuzhou, China).

Immunohistochemistry. We detected the expression of CD206 and CD68 in two different areas with IHC staining and further calculated their distribution densities. FFPE tissue sections (4 μ m-thick) were processed for IHC staining with mouse anti-human CD68 antibody and mouse anti-human CD206 antibody as described. IHC assay was performed with MaxVision IHC kit and DAB chromogenic reagent kit. Briefly, the protocol was as described below. First, the tissue sections were deparaffinized and hydrated, immersed in ethylene diamine tetraacetic acid buffer (PH 8.0) and heated for 15 min in a microwave oven for antigen retrieval. Next, the primary antibody against CD68 or CD206 was added to the specimen sections at 4°C and incubated overnight, which were then developed using the immunohistochemical MaxVision kit. The primary antibody was removed, and the secondary antibody was incubated with the samples (1:1,000) at 37°C for 30 min. The kit solution was incubated with tissue samples at room temperature for 20 min, and thereafter, DAB chromogenic reagent was added. Finally, hematoxylin staining was carried out. The primary antibody was replaced with phosphate buffered saline, which was used as the negative control. IHC staining of positive samples was repeated twice.

Counting. The tan or brown granules in the cytoplasm of cancer or adjacent cancer macrophages observed in the IHC assay were considered positive staining. Quantitative analysis of CD68 and CD206 in the stained sections was independently performed by two pathologists blinded to the clinical data. The mean score of five high-power fields (HPF) (\times 400) was counted for each slide by an optical microscope of Leica (DM2500; Leica Microsystems, Wetzlar, Germany).

Follow up study. Follow-up evaluations were performed every 3 months for the first year and annually thereafter for locally advanced tumor stages. Among all the 268 patients, 199 patients who completed the follow-up were further enrolled in survival analysis (69 cases were dropped). The scores of CD68-positive and CD206-positive were compared with demographical, clinical and histopathological characteristics. All these variables were tested as predictors of patient's OS and DFS. OS was defined as the interval between the dates

of surgery and death or the last follow-up. DFS was defined as the interval between the dates of surgery and the first recurrence or the latest date of follow-up.

Statistical analysis. Statistical Package for Social Sciences version 16.0 (SPSS 16.0; SPSS Inc., Chicago, IL, USA), MedCalc version 11.4.2.0 (MedCalc Software, Mariakerke, Belgium) and Graphpad Prism version 5.0 (Graphpad Software, Inc., La Jolla, CA, USA) were used in the present study. Quantitative variables following a normal distribution were presented as mean \pm standard deviation (SD), and the difference between/among groups were tested using t-test or one-way ANOVA. Quantitative variables with a skewed distribution were presented as medians with interquartile ranges (IQRs), and the differences between/among groups were tested using Mann-Whitney U test and Kruskal-Wallis test. Correlations of CD68/CD206-positive macrophages between the intratumor and the tumor adjacent tissue or between the two types of infiltrated macrophages were tested using the non-parametric Spearman's rank analysis. Then, the receiver operating characteristics (ROC) curve analysis was plotted to calculate the diagnostic accuracy of CD68 and CD206. The optimum cut-off value for diagnosis was determined by maximizing the sum of sensitivity and specificity. In addition, Kaplan-Meier survival curves and log-rank test were used to analyze the correlations between the CD68-positive and the CD206-positive macrophage numbers and survival of postsurgical HCC patients. Finally, Cox proportional hazards regression was employed for univariate and multivariate analysis of OS and DFS. Differences were considered statistically significant at two-sided $P < 0.05$.

Results

The clinicopathological characteristics of HCC patients. This study comprised 268 patients with HCC and their clinicopathological parameters are given in Table I. The majority of cases were male (229, 85.4%) and nearly 50% of cases were aged under 54 years. Among the cases, near 80% (209, 78%) had positive HBV and nearly half (112, 41.8%) HCC cases presented liver cirrhosis. Of these patients, 264 (98.51%) were considered early stage HCC: Child-Pugh-A (n=238, 88.8%), Child-Pugh-B (n=26, 9.7%) and Child-Pugh-C (n=4, 1.5%).

Distribution of CD68 and CD206 in HCC tissues and adjacent area. Liver histology results for CD68-positive and CD206-positive macrophages are depicted in Fig. 1. Among all specimens in our studies, CD68 expression was specific to macrophages and was shown in cell membrane and cytoplasm in brown color. Furthermore, the cells positively stained for CD206 often displayed typical macrophage morphology. CD68-positive and CD206-positive macrophages were infiltrated in both primary tumor tissues (Fig. 1A and C) and tumor adjacent tissues (Fig. 1B and D). However, the adjacent tissue was infiltrated with higher density of both CD68-positive and CD206-positive macrophages compared with intratumor compartments ($P < 0.001$ and $P < 0.001$, respectively). The average levels of CD68 and CD206 staining are shown in Table IV. Additionally, in tumor adjacent tissues, the density of infiltrated CD68-positive macrophages was much higher than

Table I. Clinicopathological characteristics of HCC patients.

Clinical variables	n (%)
Sex	
Male	229 (85.4)
Female	39 (14.6)
Age (years)	
<54	133 (49.6)
≥ 54	135 (50.4)
Alcohol intake	
Yes	95 (35.4)
No	173 (64.6)
HBV	
Positive	209 (78.0)
Negative	59 (22.0)
Liver cirrhosis	
Present	112 (41.8)
Absent	156 (58.2)
PHT	
Present	19 (7.1)
Absent	249 (92.9)
TBIL ($\mu\text{mol/l}$)	
<34	257 (95.9)
≥ 34	8 (3.0)
NG	3 (1.1)
AFP (ng/ml)	
<400	161 (60.1)
≥ 400	107 (39.9)
Child-Pugh class	
A	238 (88.8)
B	26 (9.7)
C	4 (1.5)
Tumor size (cm)	
<5	130 (48.5)
≥ 5	138 (51.5)
Lymph node metastasis	
Present	4 (1.5)
Absent	253 (94.4)
NG	11 (4.1)
TNM stage	
I + II	203 (75.8)
III + IV	65 (23.2)
Degree of histologic differentiation	
High	28 (10.5)
Moderate	111 (41.4)
Low	104 (38.8)
NG	25 (9.3)

HBV, hepatitis B virus; PHT, portal hypertension; TBIL, total bilirubin; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis; NG, not get.

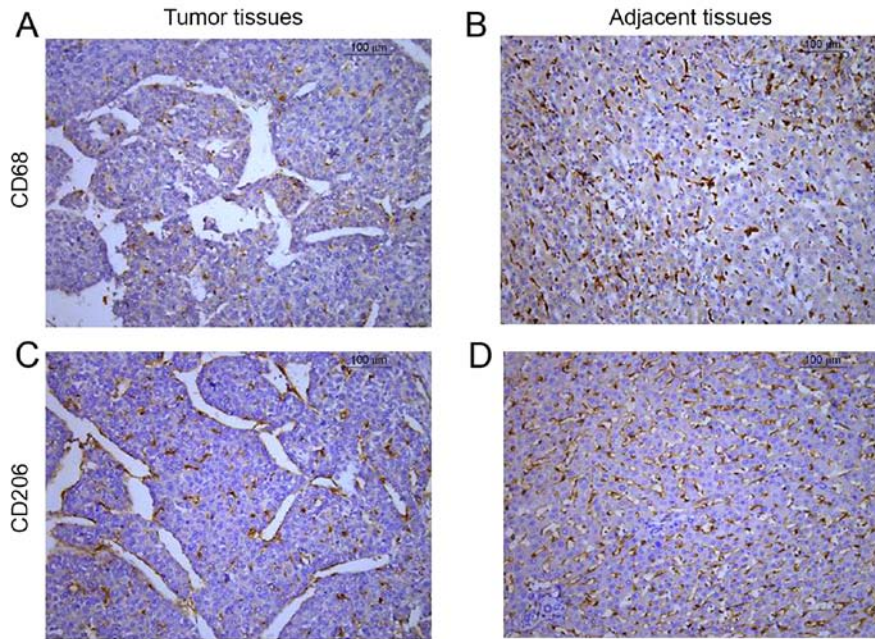


Figure 1. CD68-positive and CD206-positive macrophages in HCC tissues and adjacent tissues. Immunoreactivity to anti-CD68 (A and B) and anti-CD206 (C and D) in tumor tissues (A and C) and adjacent tissues (B and D) of HCC.

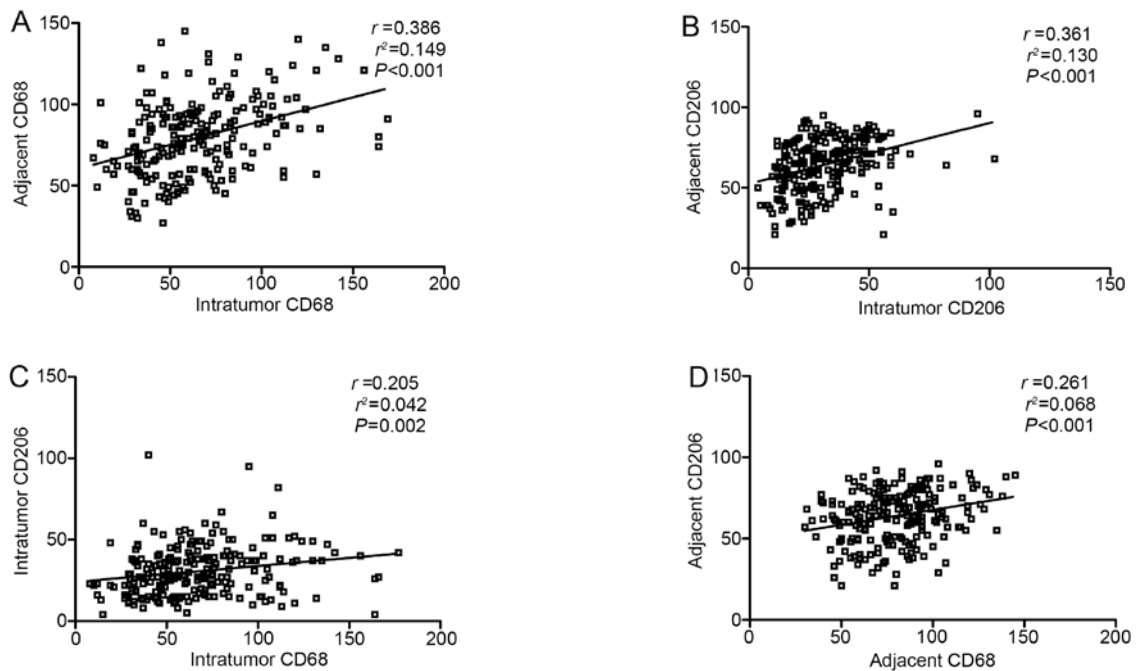


Figure 2. Correlation between infiltrated CD68-positive and CD206-positive macrophages in different areas of HCC. (A) CD68 expression between intratumor tissues and adjacent tissues ($r=0.392$; $P<0.001$). (B) CD206 expression in intratumor tissues and adjacent tissues ($r=0.360$; $P<0.001$). (C) Positive correlation between CD206 and CD68 in intratumor tissues ($r=0.237$; $P<0.001$). (D) Positive correlation between CD206 and CD68 in adjacent tissues ($r=0.261$; $P<0.001$).

that of CD206-positive macrophages ($P<0.001$) and the same pattern can be seen in the intratumor area ($P<0.001$).

Spearman's rank correlation coefficient analysis revealed a significant correlation in CD68 density between the intratumor tissues and the adjacent compartments ($r=0.392$; $P<0.001$; Fig. 2A). Similar results were obtained in the CD206-positive macrophages ($r=0.360$; $P<0.001$; Fig. 2B). This suggests a functional link between the intratumor macrophages and the adjacent macrophages. Notably, CD206

expression was positively correlated with CD68 expression in both intratumor tissues ($r=0.237$; $P<0.001$; Fig. 2C) and adjacent tissues ($r=0.261$; $P<0.001$; Fig. 2D) from HCC patients. It may imply some interaction between CD68 and CD206 cells.

Correlation of CD68/CD206 density with clinical characteristics. The relationship between the expression of CD68 and CD206 in HCC and the clinicopathological

Table II. Correlation of CD68-positive expression with clinicopathological characteristics of HCC patients.

Clinical variables	Intratumor CD68			Adjacent CD68		
	n	Mean ± SD	P-value	n	Mean ± SD	P-value
Sex			0.125			0.142
Male	190	65.42±32.11		206	77.62±23.04	
Female	35	74.71±36.18		37	83.62±21.47	
Age (years)			0.027			0.893
<54	106	61.86±27.53		117	78.45±22.66	
≥54	118	71.42±36.66		125	78.85±23.10	
Alcohol intake			0.901			0.258
Yes	79	66.49±30.13		83	80.84±24.52	
No	146	67.07±34.36		160	77.34±21.95	
HBV			0.954			0.030
Positive	171	66.80±32.99		188	76.81±21.90	
Negative	54	67.09±32.77		55	84.42±25.26	
Liver cirrhosis			0.633			0.946
Present	91	65.59±34.68		103	78.65±22.68	
Absent	134	67.73±31.68		140	78.45±23.09	
PHT			0.043			0.050
Present	19	52.26±21.37		18	68.39±18.48	
Absent	206	68.21±33.45		225	79.35±23.03	
TBIL			0.258			0.202
<34	214	67.36±32.30		232	78.81±23.01	
≥34	8	53.88±50.26		8	68.25±20.77	
AFP (ng/ml)			0.008			0.026
<400	133	62.03±30.84		146	75.88±23.18	
≥400	92	73.86±34.59		97	82.54±21.91	
Child-Pugh class			0.470			0.209
A	197	67.60±32.73		215	79.20±22.82	
B	24	63.83±36.34		24	71.21±22.82	
C	4	48.75±4.43		4	86.50±22.25	
Tumor size (cm)			0.072			0.012
<5	103	62.74±33.33		107	75.57±21.46	
≥5	115	70.66±31.21		127	83.00±23.34	
Lymph node metastasis			0.654			0.523
Present	8	72.00±26.67		8	83.63±12.31	
Absent	217	66.68±33.11		235	78.36±23.14	
TNM stage			0.143			0.491
I + II	168	64.99±33.84		185	77.97±23.37	
III + IV	57	72.39±29.40		58	80.34±21.29	
Tumor differentiation			0.767			0.283
Well	26	62.88±38.15		27	84.74±18.60	
Moderate	88	68.13±29.20		99	77.00±21.79	
Poor	91	67.21±33.43		94	79.01±24.02	

SD, standard deviation; HBV, hepatitis B virus; PHT, portal hypertension; TBIL, total bilirubin; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

features is listed in Tables II and III. We noted that a higher CD68 density was found in the older patients intratumorally

($P=0.027$). Cases with portal hypertension (PHT) appeared to have lower intratumor CD68 expression compared with

Table III. Correlation of CD206-positive expression with clinicopathological characteristics of HCC patients.

Clinical variables	Intratumor CD206			Adjacent CD206		
	n	Mean ± SD	P-value	n	Mean ± SD	P-value
Sex			0.013			0.297
Male	227	30.50±14.47		202	77.62±23.04	
Female	39	37.03±17.75		35	83.62±21.47	
Age (years)			0.828			0.185
<54	131	31.73±14.42		118	65.83±16.35	
≥54	134	31.32±15.84		118	63.10±15.18	
Alcohol intake			0.574			0.813
Yes	93	32.14±13.76		84	64.73±15.67	
No	173	31.09±15.85		153	64.22±15.93	
HBV			0.375			0.365
Positive	208	31.02±14.84		183	63.89±15.88	
Negative	58	33.02±16.21		54	66.11±15.56	
Liver cirrhosis			0.177			0.035
Present	111	29.97±13.35		98	61.82±16.22	
Absent	155	32.52±16.25		139	66.22±15.30	
PHT			0.971			0.240
Present	19	31.58±10.89		13	59.38±18.38	
Absent	247	31.45±15.43		224	64.69±15.64	
TBIL			0.349			0.007
<34	255	31.15±15.19		228	64.74±15.61	
≥34	8	36.25±13.51		6	47.00±16.86	
AFP (ng/ml)			0.486			0.338
<400	161	30.93±14.95		143	65.20±15.05	
≥400	105	32.26±15.45		94	63.18±16.89	
Child-Pugh class			0.001			0.240
A	236	31.01±14.63		213	64.96±15.63	
B	26	31.27±13.22		21	58.86±15.86	
C	4	59.00±31.52		3	63.33±26.31	
Tumor size (cm)			0.886			0.385
<5	129	31.32±15.14		113	63.46±14.92	
≥5	137	31.58±15.18		124	65.25±16.59	
Lymph node metastasis			0.734			0.859
Present	8	33.25±27.73		8	65.38±18.90	
Absent	258	31.40±14.67		229	64.36±15.73	
TNM stage			0.357			0.013
I + II	201	30.96±14.97		181	62.99±15.88	
III + IV	65	33.00±15.65		56	68.95±14.78	
Tumor differentiation			0.445			0.644
Well	28	28.07±10.65		24	66.08±13.39	
Moderate	111	31.72±13.47		99	65.07±15.34	
Poor	102	32.00±17.07		92	63.29±17.22	

SD, standard deviation; HBV, hepatitis B virus; PHT, portal hypertension; TBIL, total bilirubin; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

those without PHT ($P=0.043$), but the difference in adjacent tumor data did not reach a statistical significance ($P=0.050$). Patients whose AFP concentration was >400 ng/ml might have much higher density of CD68 TAMs in both primary

tumor tissues and tumor adjacent tissues ($P=0.008$ and $P=0.026$, respectively). Patients with high expression of CD68 in tumor adjacent tissue were prone to have a larger tumor size ($P=0.012$). As for CD206, its density in adjacent tissue

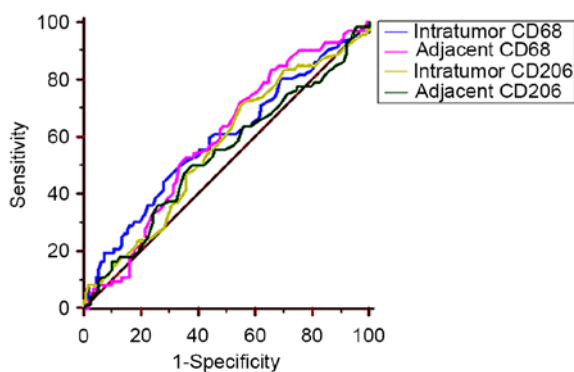


Figure 3. The optimal diagnostic cut-off for intratumor CD68 was 71/hpf and 87/hpf for adjacent tumoral CD68 in the diagnosis of HCC.

was significantly higher in patients with cirrhosis ($P=0.035$) or poor TNM stages ($P=0.013$). The HCC cases with younger age or better Child-Pugh class had lower intratumor CD206 expression ($P=0.001$). Individuals with higher level of TBIL possessed decreased expression of CD206 ($P=0.007$) in tumor adjacent tissue. However, no statistical significance was observed in the expression of CD68 and CD206 with tumor differentiation, lymph node metastasis and alcohol intake.

Diagnostic prediction of CD68/CD206 staining for HCC. AFP is a valuable serological biomarker. A level >400 ng/ml was defined as cut-off value to evaluate sensitivity and specificity of CD68 and CD206. ROC curves showed that the optimal diagnostic cut-off for intratumor CD68 was 71/HPF [area under curve (AUC) 0.614, (95% CI, 0.547-0.678), sensitivity 52.2% and specificity 70.7%; $P=0.003$], and that for adjacent area was 87/HPF [AUC 0.599, (0.534-0.661), 49.5 and 70.5%, $P=0.008$] (Fig. 3). In contrast, the optimal cut-off value for intratumor CD206 was 25/HPF [AUC 0.527, (0.465-0.588), sensitivity 56.2%, specificity 52.8%; $P=0.463$] while the value for tumor-adjacent area was 83/HPF [AUC 0.534, (0.469-0.599), 44.7, 69.2%; $P=0.384$] (Fig. 3).

Prognostic significance of CD206 and CD68 expression in HCC patients after operation. The prognostic value of CD68 and CD206 expression in postsurgical HCC patients was examined and data are shown in Table V and Fig. 4. Within a median follow-up time of 44 months (1-54 months), 5-year OS and DFS rates for the 199 patients were 47 and 62%. The analyses showed that the median value for density of CD68 in intratumor was 61/HPF (CD68 density ≥ 61 /HPF was defined as CD68^{high} group, CD68 density <61 /HPF was defined as CD68^{low} group) and that in adjacent area was 78/HPF (CD68 density ≥ 78 /HPF was defined as CD68^{high} group, CD68 density <78 /HPF was defined as CD68^{low} group), respectively. Similarly, 29/HPF and 66/HPF were the medians of CD206 macrophage content for the two areas.

Survival analyses revealed that high density of CD206-positive macrophages in intratumor tissues was significantly associated with reduced OS ($P=0.013$; Fig. 4E) and DFS ($P=0.003$; Fig. 4F) in post-surgical HCC patients. On the contrary, we noted that increased CD68-positive macrophages were significantly correlated with better

Table IV. Difference in the density of CD68-positive and CD206-positive macrophages in tumor and adjacent tissue (mean \pm SD).

Staining	Density		P-value (paired t-test)
	n	Mean \pm SD	
CD68			<0.001
Intratumor	216	66.59 \pm 32.89	
Adjacent	216	79.41 \pm 23.42	
CD206			<0.001
Intratumor	236	31.55 \pm 15.03	
Adjacent	236	64.36 \pm 15.83	
Intratumor			<0.001
CD68	223	66.61 \pm 32.89	
CD206	223	30.42 \pm 15.13	
Adjacent			<0.001
CD68	220	79.89 \pm 22.64	
CD206	220	63.95 \pm 15.85	

survival only in tumor adjacent area ($P=0.020$; Fig. 4C) and patients with low CD68 expression in adjacent area exhibited a worse DFS ($P=0.018$; Fig. 4D). However, no significant correlations of CD68-positive macrophages infiltrated in intratumor ($P=0.906$ and $P=0.535$, respectively; Fig. 4A and B) and CD206-positive macrophages in adjacent compartment with prognosis ($P=0.330$ and $P=0.912$, respectively; Fig. 4G and H).

Univariate analysis of prognostic factors revealed that CD68-positive macrophages in tumor adjacent tissue and CD206-positive macrophages in intratumor had significant prognostic effects on OS in post-surgical HCC patients ($P=0.024$ and $P=0.014$, respectively) (Table V). In addition, both low CD68 density in tumor adjacent tissue ($P=0.008$) and high CD206 density in intratumor ($P=0.016$) were identified as independent predictors of poor prognosis for OS by multivariate survival analysis after adjustment of covariates (Table V). Similar results were obtained for DFS. Increased CD68-positive macrophages in tumor adjacent tissue ($P=0.016$) and enhanced intratumor CD206-positive macrophages ($P=0.003$) played significant roles by univariate analysis. Multivariate survival analysis showed both CD68 density in tumor adjacent tissue ($P=0.028$) and CD206 density in intratumor tissue ($P=0.005$) could be as independent predictors for DFS (Table V).

Combination of adjacent area CD68 density with intratumoral CD206 density analysis. Patients were classified into four groups according to their intratumoral CD206 and adjacent tumoral CD68 densities (Table V): group I ($n=65$), low CD206 and CD68 density; group II ($n=39$), high CD206 but low CD68 density; group III ($n=46$), low CD206 but high CD68 density; and group IV ($n=49$), high CD206 and CD68 density. Differences in both OS ($P=0.040$; Fig. 5A) and DFS ($P=0.015$; Fig. 5B) were significant among the four groups and group II had the worst survival.

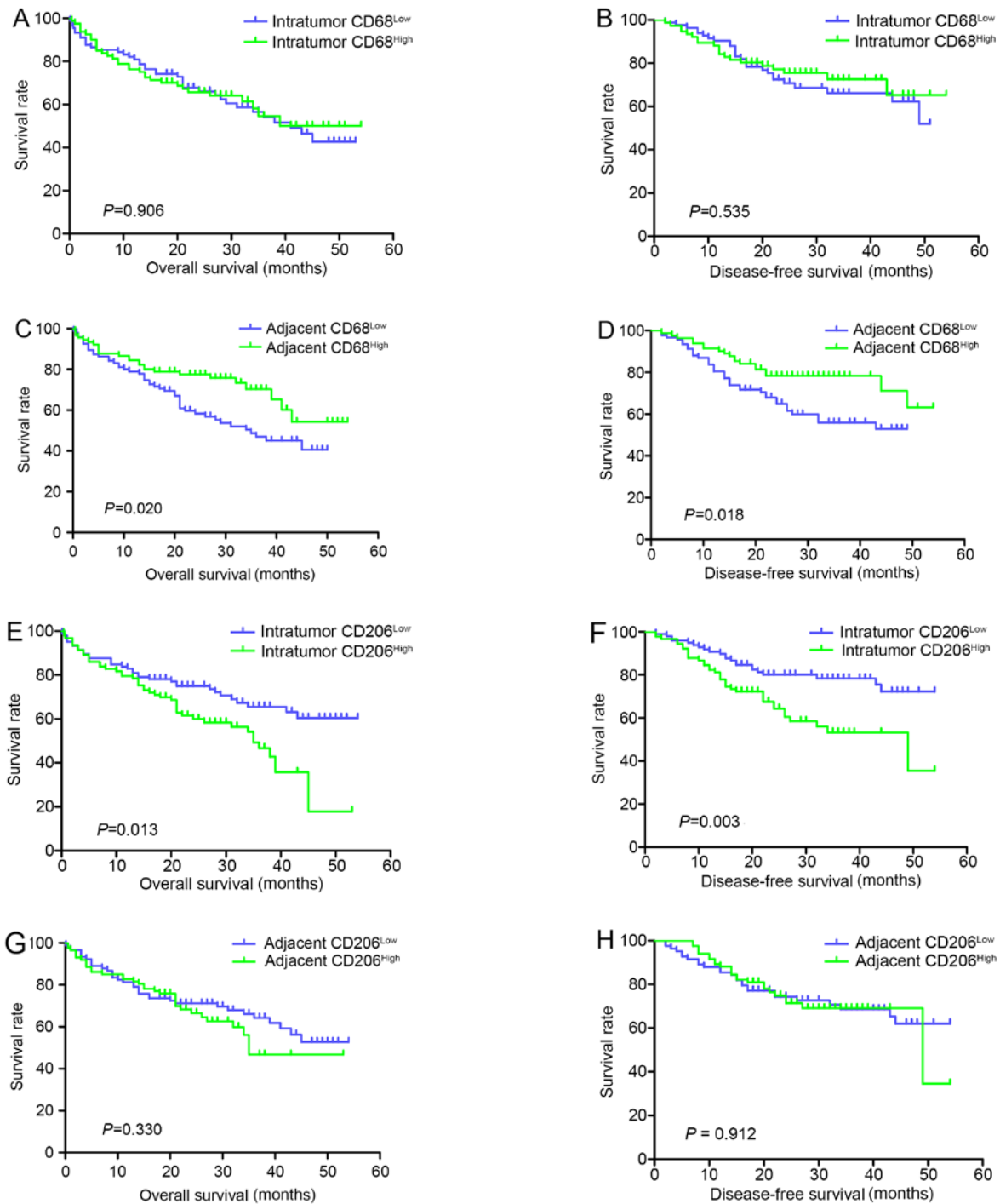


Figure 4. OS and DFS for infiltrated CD206-positive and CD68-positive macrophages in different tumor areas of post-surgical HCC patients. In HCC patients after operation, the low CD68 expression in adjacent tumor and high density of CD206-positive macrophages in intratumor tissues were significantly associated with reduced OS and DFS (C-F). However, no significant association of CD68-positive macrophages infiltrated in intratumor and CD206-positive macrophages in adjacent compartment with prognosis was found (A, B, G and H).

Combination of adjacent area CD68 density/intratumor CD206 density with HBV. To assess whether the combined use of adjacent area CD68 density and HBV infective status was better than either of these biomarkers alone, four groups were assessed as follows (Table V and Fig. 6): group I (n=69), high CD68 density and positive HBV; group II (n=27), high CD68 density but negative HBV; group III (n=80), low CD68 density but positive HBV; and group IV (n=23), low CD68 density and negative HBV. We found a significant difference in DFS (P=0.001; Fig. 6B), but not in OS (P=0.102; Fig. 6A)

among the four groups. Group III with low CD68 density in adjacent tumor but positive HBV had the shortest recurrence time.

Similarly, another grouping approach is listed in Table V and Fig. 6C and D. There were significant differences in either OS (P=0.043; Fig. 6C) or DFS (P<0.001; Fig. 6D) among the four groups. Of note, group I had the poorest DFS. However, in univariate analysis, only difference in DFS (P<0.001) is shown in Table V. In brief, the combined predictive value in DFS was much greater than that of CD68 or CD206 alone.

Table V. Univariate and multivariate analysis of different parameters with survival and recurrence in HCC patients.

Variables	OS				DFS			
	Univariate P-value	Multivariate			Univariate P-value	Multivariate		
		HR	95% CI	P-value		HR	95% CI	P-value
Alcohol intake Yes vs. no	0.257			NA	0.212			NA
Smoking Yes vs. no	0.012	1.717	1.069-2.759	0.025	0.866			NA
HBV Positive vs. negative	0.212			NA	0.003	3.898	1.400-10.854	0.009
Liver cirrhosis Present vs. absent	0.448			NA	0.961			NA
PHT Present vs. absent	0.436			NA	0.334			NA
TBIL <34 vs. ≥34	0.867			NA	0.699			NA
Albumin <35 vs. ≥35	0.026	0.360	0.205-0.632	<0.001	0.280			NA
AFP <400 vs. ≥400	0.438			NA	0.206			NA
Child-Pugh class A vs. B vs. C	0.740			NA	0.865			NA
Tumor size <5 vs. ≥5	0.311			NA	0.936			NA
Lymph node metastasis Present vs. absent	<0.001	8.238	3.126-21.710	<0.001	0.460			NA
Primary lesion	<0.001	1.531	1.295-1.811	<0.001	0.054	1.214	1.001-1.473	0.049
TNM stage: I + I vs. III + IV	<0.001	2.052	1.637-2.571	<0.001	0.326			NA
Tumor differentiation Well vs. moderate vs. poor	0.421			NA	0.206			NA
Intratumor features Low vs. high				NA				NA
CD68 density	0.907				0.538			
CD206 density	0.014	1.792	1.116-2.879	0.016	0.003	2.222	1.280-3.856	0.005
Adjacent tumor features Low vs. high								
CD68 density	0.024	0.510	0.311-0.837	0.008	0.016	0.529	0.299-0.935	0.028
CD206 density	0.334			NA	0.912			NA
Combine adjacent tumoral CD68 and intratumoral CD206	0.040			NA	0.015			NA
Combine adjacent tumoral CD68 and HBV	0.014			NA	0.326			NA
Combine intratumoral CD206 and HBV	0.070			NA	<0.001			NA

OS, overall survival; DFS, disease-free survival; NA, not adopted; HBV, hepatitis B virus; PHT, portal hypertension; TBIL, total bilirubin; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

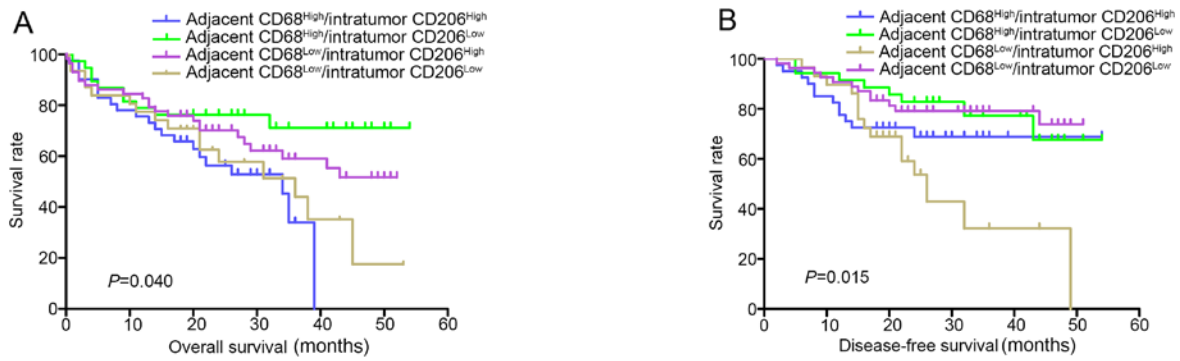


Figure 5. OS and DFS for combination of adjacent tumoral CD68 density with intratumoral CD206 density. Combined analysis of adjacent tumoral CD68 with intratumoral CD206, differences in both OS (A) and DFS (B) were significant.

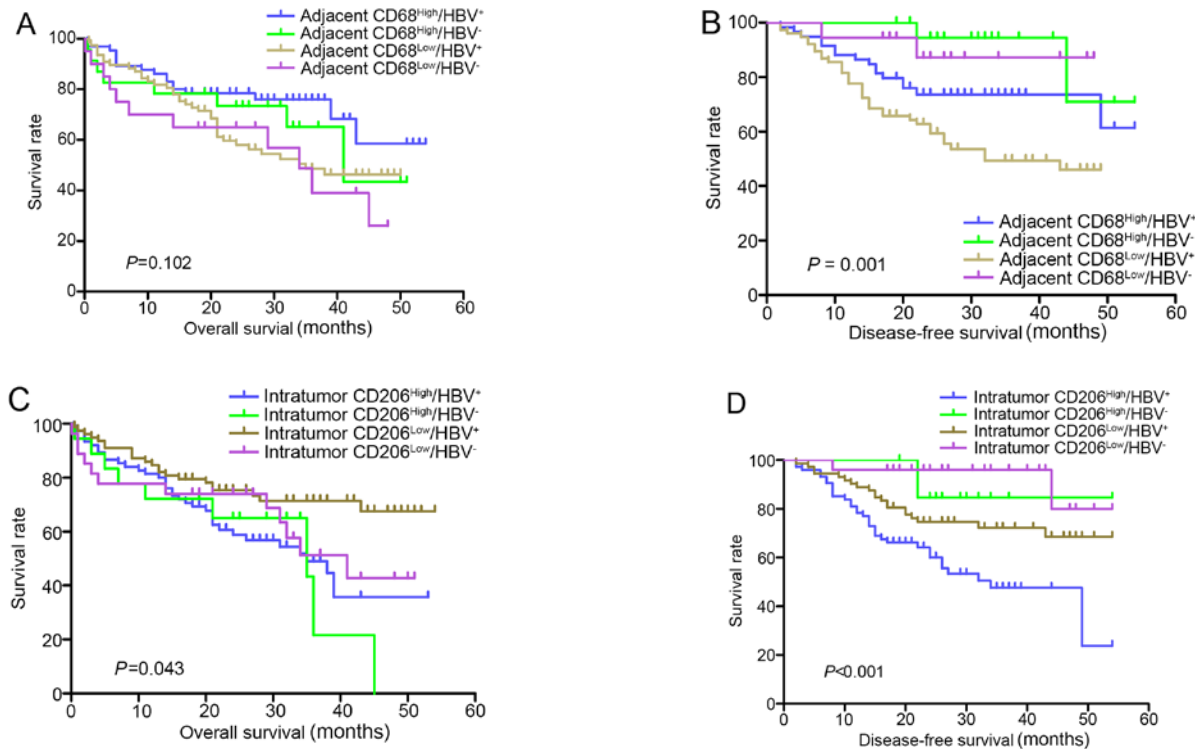


Figure 6. OS and DFS for combination of adjacent tumoral CD68 density/intratumoral CD206 density with HBV. Combination of either adjacent CD68 or intratumoral CD206 with HBV, was significantly different in DFS (B and D). There was significant difference in OS for the combination of intratumoral CD206 with HBV (C), but not for combination of either adjacent CD68 or HBV (A).

Discussion

Results from the present study demonstrate that changes to CD68-positive and CD206-positive macrophage subpopulations in tumor site and adjacent region in HCC are associated with OS and DFS. To the best of our knowledge, this is the first study that shows location-specific and subtype-specific macrophage expression and their prognostic values in HCC.

In the present study, the infiltrating CD68- and CD206-positive subpopulations were observed in cancer tissues and in adjacent tissues of HCC, with predominant presence in the adjacent tumor tissues. CD68-positive macrophages were much more abundant than CD206-positive macrophages both in the intratumor and adjacent areas. This is in accordance with the fact that the total number of CD68-positive macrophages

is greater than that of CD206-positive M2 macrophages. According to the infiltrating macrophage count, there was a notable increase of CD68 expression in non-tumor tissues in line with the increased macrophage expression and inflammatory response in HCC demonstrated previously by others (25,26). This result is in contrast to several investigations which showed overexpression of CD68 in tumor tissue (27). This suggests the expression profile of CD68 in tumor and adjacent tumor-free region is specific to HCC. The increased number of CD68-positive macrophages in HCC adjacent samples in our analysis may be explained by heterogeneity of macrophages in response to different microenvironment signals. Thus, the subtype of CD68-positive macrophages in adjacent tissue is probably different from that in intratumor tissue and they may represent resident macrophages (CD68-positive Kupffer

cells). Specifically, CD68-positive macrophages in adjacent tumor are more similar to macrophages in normal or inflamed tissues that possess spontaneous antitumor activity. Therefore, these cells would be beneficial in the prognosis of HCC (28). These data suggest a possibility that as a front line defense to prevent tumor growth, the adjacent non-tumor liver tissue is endowed with abundant CD68-positive macrophages. Positive correlation of increased amount of adjacent CD68-positive macrophages with good HCC prognosis in the present study could be explained by the ability of macrophages that are not yet re-programmed by tumor cells to kill cancer cells. In other words, CD68-positive macrophages in adjacent tissue may have antitumor activity as they are uneducated into M2 phenotype by cancer, while in tumor the opposite happens. In tumor area, a genetic exchange between the primary tumor cells and the associated macrophages may lead to the expression of a more invasive phenotype of macrophages. On the other hand, immunohistochemistry with antibodies to CD206 also identified a higher CD206 density in adjacent tissue in HCC patients, which is similar to a study examining the higher proliferation of endothelial cells to IL-6/sIL-6R in HCC peritumor (29). Generally, CD206 can identify mannose and fucose glycoconjugates and has been recognized as an important regulator in the phagocytosis of intracellular pathogens. Herein we speculate that this phagocytosis in tumor may consume a large number of CD206 so that the count of CD206 would be reduced dramatically in intratumor. However, the mechanism for overexpression of CD68 and higher density of CD206 in the adjacent liver tissue remains unclear. One possible mechanism is tumor cell-micro environmental crosstalk which is complex. Another possibility is that a line of defense surrounding tumor formed by tumor microenvironment may block the entrance of macrophages into the tumor. Furthermore, the tumor itself could be a possible stimulator either stimulating chronic inflammation or causing a host hepatocyte reaction (26), resulting in elevated production of cytokines and more CD68/CD206 at the tumor-host margin. Moreover, the heterogeneity of macrophages in HCC raises a further possibility that there are different distributions and phenotypes of macrophages in different tumor parts and nearby areas. We propose that some genetic heterogeneity among different individuals may lead to the different baseline expression level of CD68/CD206. In this regard, previous evidence concerning the density of TAMs in HCC partly contrasts with our data. These discrepancies could be due to differences in the methodology, sample types, or patient characteristics. Two recent studies showed no association of CD68-positive macrophages in tumor region with clinicopathological characteristics in HCC but the tumor-adjacent region was not examined (30,31). Additionally, associations between CD68-positive and CD206-positive macrophages in both tumor and adjacent tissues were also found in this study. A similar relationship between CD68-positive and CD163-positive macrophages in HCC was also previously reported (21). This may suggest a possible interaction between macrophage subpopulations.

In the present study, low CD68 expression in tumor was associated with age and PHT, which could partly explain the protective role of CD68 in this study. On the contrary, patients with cirrhosis, poor TNM stages and Child-Pugh class tended to have high CD206 density, indicating the M2 role of

CD206 in cancers. On the whole, a lower correlation between CD68-positive/CD206-positive macrophages and clinicopathological characteristics in HCC patients may imply some other genetic heterogeneity within different individuals. Our results also suggest that CD68 expression either in intratumor or in adjacent tumor may be a potential diagnostic measure of HCC. However, the effectiveness of this emerging marker requires further assessment.

Prognostic factors are of paramount interest in the risk assessment of cancer patients. This study demonstrated that the dense CD68 infiltration in the tumor adjacent tissues, but not in primary tumor tissues, was strongly associated with favorable survival of both OS and DFS for post-operative HCC patients in univariate and multivariate analyses. This is consistent with another report on esophageal squamous cell carcinoma (32). However, CD68 has been frequently shown to correlate with an adverse prognosis in a variety of malignancies, such as oral squamous cell carcinoma and HCC (21-24). Recently, dual prognostic significance of CD68-positive TAMs has been reported in pancreatic ductal adenocarcinoma (PDAC) (33). In this study, CD68-TAMs at the tumor-stroma interface may promote PDAC distant metastasis, while in patients who have received chemotherapy, TAMs play an opposite role. Additionally, some authors suggested that CD68-positive macrophages did not affect the outcome of patients with classical Hodgkin lymphoma (34). A similar finding was reported recently by Shu *et al* (30), however, we thought that the results could not comprehensively and accurately reveal the prognostic value of CD68 as they only analyzed HCC tissues for CD68-positive macrophages rather than adjacent tissues.

Macrophages in normal or inflamed tissues possess spontaneous antitumor activity, while TAMs seem to have distinct phenotypes with diverse functional programs within different local environment (28). This might suggest that CD68-positive macrophages, exhibiting M1-like phenotype, serve as a protective phenotype and likely lessen HCC damage. Moreover, TAMs in HCC are heterogeneous and present different phenotypes as heterogeneity and plasticity are hallmarks of macrophages (22). Possibly, there was different depletion or recruitment of heterogeneous populations of protumor and antitumor macrophages in the liver. Also, plasticity of macrophages allowed them to acquire either protumor or antitumor functions (35). Thus, it was not surprising to find various functional phenotypes of macrophages in adjacent tissue versus intratumor tissue, as seen in the present study. Positive correlation of increased amount of adjacent CD68-positive macrophages with HCC prognosis in this study could be due to the macrophages that were not yet re-programmed into M2 phenotype by tumor cells and still possessed the ability of killing cancer cells.

In our HCC patient cohort, the increased CD206-positive M2 macrophages at primary tumor tissues were correlated with poor prognosis. The bulk of available information indicates that CD206-positive M2 macrophages appear to possess mostly tumor promoting property (36,37). This is in line with a poor prognosis and hence the prognostic value of intratumor CD206 macrophages in HCC patients. Although intratumoral CD206-positive M2-macrophages were reported to promote tumor cell dissemination, the adjacent CD206-positive macrophages may play a different, even opposite role. Herein,

intratumoral CD206-positive macrophages contributed to a propensity of metastases and poor prognosis for HCC. Our results strongly demonstrate that intratumoral CD206 is an independent factor of prognosis for postsurgical HCC patients. In contrast, another study reported an association of decreased CD206-positive macrophages with loss of interstitial cells of Cajal in patients with diabetic gastroparesis, indicating a cytoprotective role for CD206-positive macrophages (38). These data may prompt a therapeutic strategy that targets intratumoral CD206 by reprogramming its protumor phenotype into an effective antitumor activity. Our findings with CD206-positive macrophages are consistent with two recent reports that CD206 in tumor region of HCC was linked to poor prognosis. However, by examining CD68/CD206-positive macrophages in both tumor tissues and adjacent tissues, our models have higher predicative power.

Furthermore, we found a much greater advantage in combining capability of intratumor CD206 with adjacent CD68 in prognostic prediction of HCC. There are greater numbers of younger patients with HBV-related HCC in Asian countries including China (39), as shown in the present study (Table I). The findings revealed a better prognostic value, especially in recurrence. According to the Cox regression model that included the levels of CD68/CD206-positive macrophage content, smoking, HBV, albumin, lymph node metastasis, primary lesion and TNM, it was concluded that primary lesion, adjacent CD68 and intratumor CD206 were independent prognostic factors for both OS and DFS.

In conclusion, this study clearly demonstrates higher densities of CD68 and CD206 in the adjacent liver tissue than those in tumor tissue. The dense CD206 macrophage infiltration at the tumor is an independent predictor of poor survival in postsurgical HCC patients. High density of CD68 macrophage infiltration in adjacent tissue is capable of predicting improved survival. The findings from this study suggest the complexity of macrophages within and surrounding tumor microenvironment, a novel role for CD68/CD206-positive macrophages and a basis for targeting these cells as new therapeutic strategies for HCC patients.

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