Stromal Macrophage Expressing CD204 is Associated with Tumor Aggressiveness in Lung Adenocarcinoma

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Background: Tumor tissue is composed of variable numbers of cancer cells and stromal cells, and tumor-associated macrophages are recruited into cancer-induced stroma and produce a specific microenvironment. Alternatively, activated macrophages (M2 phenotype) are known to be related to tumor progression and outcome, and CD204 has been reported to be expressed in M2 macrophages in some tumors.

Methods: To investigate whether CD204-positive macrophages reflect tumor aggressiveness in adenocarcinoma of the lung, we investigated the relationships between the numbers of CD204-positive stromal macrophages and both clinicopathological features and outcome in 170 consecutive resected cases. We also examined the relationships between the numbers of CD204-positive macrophages and the expression levels of cytokines involved in the migration and differentiation of M2 macrophages.

Results: The numbers of CD204-positive macrophages were significantly correlated with several prognostic factors. The log-rank test showed a significant association between the numbers of CD204-positive macrophages and a poor outcome ($p = 0.0073$), whereas the numbers of macrophages expressing CD68, a pan-macrophage/monocyte marker, were of marginal prognostic significance ($p = 0.0789$). We evaluated associations between the levels of expression of the cytokines IL-6, IL-10, IL-12a, IL-12b, M-colony-stimulating factor, IFN-gamma-., and monocyte chemoattractant protein-1 in cancer tissue and the numbers of CD204-positive macrophages. The expression levels of IL-10 and monocyte chemoattractant protein-1, which are involved in differentiation, accumulation, and migration of M2 macrophages, were significantly correlated with the numbers of CD204-positive macrophages ($p = 0.031$ and $p = 0.031$, respectively).

Conclusion: These findings demonstrated that CD204-positive macrophages clearly reflect the tumor-promoting phenotype of tumor-associated macrophages in lung adenocarcinoma.

Key Words: Macrophage, Tumor-associated macrophages (TAMs), Lung cancer, Stroma, CD204.

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Tumor tissue is composed of variable numbers of cancer cells and stromal cells, and the cancer cells interact with the surrounding stromal cells and produce a specific microenvironment that is capable of influencing tumor progression.[1] Macrophages are the most abundant cancer stromal cells involved in the host immune system,[2] and in several kinds of cancer, tumor-associated macrophage (TAM) infiltration has been found to be correlated with a poor outcome.[3–7] Macrophages have two different functions, a tumor-suppressive (M1) function and a tumor-supportive (M2) function.[8,9] M1 macrophages are characterized by high expression of proinflammatory cytokines, including IL-1, IL-6, IL-12, and tumor necrosis factor (TNF), whereas tumor supportive macrophages (M2), which are characterized by IL-4^{high}, IL-10^{high}, and IL-12^{low}, play important roles in tumorigenesis, angiogenesis, matrix remodeling, and metastasis.[10] Recent studies have revealed high expression of CD204 in M2 macrophages.[11–13]

Several factors related to TAM recruitment and differentiation have been reported. IL-4 and IL-10 are known to polarize macrophages toward the M2 phenotype, and macrophage colony-stimulating factor (M-CSF, also known as CSF-1) and monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) have been implicated in M2 macrophage polarization, differentiation, and migration. On the other hand, classically activated M1 macrophages, which are generally considered to be potent effector cells that defend the body against pathogens and tumor cells, are induced by IFN-gamma-. alone or in concert with microbial stimuli or cytokines such as tumor necrosis factor and granulocyte macrophage-CSF.[10]

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Lung cancer is the most common cause of cancer deaths worldwide,[14] and adenocarcinoma accounts for more than half of all non-small cell lung carcinomas.[15] Although surgery is the only means of radical cure, because the 5-year disease-free survival rate for adenocarcinoma of the lung after curative resection is less than 70%,[15] the development of new effective therapeutic modalities targeting the tumor microenvironment is needed. Because investigators, including ourselves, have reported finding a correlation between the grade of macrophage infiltration and the malignancy of adenocarcinoma of the lung,[7,16,17] targeting M2 macrophages would be a novel approach to the treatment of lung adenocarcinoma.

Thus far, TAMs have been mainly assessed by CD68 immunostaining, but CD68 is a broad TAM marker that cannot discriminate the M2 phenotype from other TAM phenotypes. Recent studies have reported strong expression of CD204 by M2 macrophages, and using CD204 (scavenger receptor class A) as a marker has revealed that invasion by CD204-positive macrophages is related to tumor progression and outcome in glioma, ovarian epithelial tumors, and pancreatic cancer.[11–13,18] CD204 is the prototypic member of an expanding family of membrane receptors collectively termed scavenger receptors and a multifunctional receptor that recognizes many different types of negatively charged macromolecules.[19] The aim of this study was to examine whether CD204 can be used to detect the tumor-promoting subtype of macrophages in lung adenocarcinoma.

PATIENTS AND METHODS

Patients

Between January 1996 and March 1998, a total of 201 patients with lung adenocarcinoma underwent surgery with curative intent at the National Cancer Center Hospital East, Chiba, Japan, and after excluding the 31 cases whose surgical specimen was of poor quality, the remaining 170 cases were included in this study. All patients signed an institutional

TABLE 1. Relationship Between CD68(+)/CD204(+) Macrophage and Clinicopathological Features

	CD68			CD204		
Variables	**Low (n = 82)**	**High (n = 88)**	**p***	**Low (n = 81)**	**High (n = 89)**	**p***
Age (yr)						
<70	55	67	0.190	55	67	0.286
≥70	27	21		26	22	
Gender						
Male	31	54	0.002†	27	58	<0.001†
Female	51	34		54	31	
Smoking history						
Never	53	28	<0.001†	56	25	<0.001†
Former or current	29	60		25	64	
Differentiation						
Well	59	27	<0.001†	66	20	<0.001†
Moderately or poorly	23	61		15	69	
Pathological stage						
IA	57	38	0.001†	59	36	<0.001†
IB–IIIA	25	50		22	53	
T status						
T1	62	47	0.003†	61	48	0.004†
T2–3	20	41		20	41	
Nodal involvement						
N(−)	70	60	0.008†	76	54	<0.001†
N(+)	12	28		5	35	
Lymphatic permeation						
Ly(−)	65	43	<0.001†	63	45	<0.001†
Ly(+)	17	45		18	44	
Vessel invasion						
V(−)	62	43	<0.001†	64	41	0.001†
V(+)	20	45		17	48	
Pleural invasion						
PL(−)	71	50	<0.001†	68	53	<0.001†
PL(+)	11	38		13	36	

*Pearson χ^2 test.
†$p < 0.05$.

review board-approved informed consent. The tumors were staged according to the tumor-node-metastasis classification of the International Union Against Cancer (UICC). Mean age at the time of surgery was 62 years (range: 33–85 years), and the median follow-up period was 10.1 years. Survival time was measured from the date of surgery.

Histopathology Studies

All surgical specimens were fixed with 10% formalin and embedded in paraffin wax. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin and eosin and by the Alcian blue-periodic acid Schiff method to visualize cytoplasmic mucin production and the Verhoeff-van-Gieson method to visualize elastic fibers. Lymphatic permeation and pulmonary metastasis were evaluated in sections stained with hematoxylin and eosin. Vascular invasion and pleural invasion were evaluated by the Verhoeff-van-Gieson method. Two observers (Y.O. and G.I.) who are unaware of the clinical data independently reviewed all pathologic slides. The histologic diagnoses were based on the third revised World Health Organization histologic classification, and all the tumors are divided according the predominant subtype into four groups: a bronchioloalveolar carcinoma (BAC) group, papillary pattern (including micropapillary pattern) group, acinar pattern group, and solid adenocarcinoma with mucin production pattern group. The numbers of each predominant histologic subtype were BAC, 69; papillary, 50; acinar, 20; and solid, 31.

Tumor size was measured as the maximal diameter of the tumor on the cut sections. The pathologic stage was determined according to the classification of the UICC.

Evaluation of Clinicopathological Factors

Clinical characteristics were retrieved from the clinical records available. The following clinicopathological factors were investigated retrospectively to assess their impact on survival: age ($<$70 years versus $\geq$70 years), gender, smoking history (never smokers versus smokers), preoperative serum carcinoembryonic antigen value ($<$5.0 ng/ml versus $\geq$5.0 ng/ml), grade of differentiation (well differentiated versus moderately or poorly differentiated), pathologic stage (IA versus IB, II, and IIIA), pathologic T status (T1 versus T2, T3), pathologic nodal involvement (positive versus negative), lymphatic permeation (present versus absent), vascular invasion (present versus absent), and pleural invasion (present versus absent) (Table 1).

Antibodies and Immunohistochemistry

After reviewing the hematoxylin- and eosin-stained slides of the surgical specimens, the block containing the most extensive tumor component was selected from each specimen. The slides were deparaffinized in xylene, dehydrated in a graded ethanol series, and after washing with distilled water, the sections were placed in 0.1 M citric acid buffer. For antigen retrieval, the slides were heated twice at 95°C for 20 minutes in a microwave oven (H2800 Microwave Processor, Energy Beam Sciences Inc.) and then allowed to cool for 1 hour at room temperature. Next, the slides were washed 3 times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 minutes to inhibit endogenous peroxidase activity. After washing the slides 3 times in PBS, nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 minutes at room temperature. Individual slides were then incubated overnight at 4°C with mouse antihuman CD68 antibody (Dako, Japan) at a final dilution of 1:100 in the blocking buffer and mouse antihuman CD204 antibody (Scavenger Receptor class A-E5; Transgenic, Japan) at a final dilution of 1:100 in the blocking buffer. The slides were again washed three times with PBS and then incubated with EnVision (Dako, Denmark) for 1

TABLE 2. RNA Primers Used

Target	Primer	Sequence(5′-3′)	Gene Size	Products (bp)	Position	Accession Number
IL-6	Forward	AAGCCAGAGCTGTGCAGATGAGTA	1131	150 (480–629)	554	NM_000600.2
	Reverse	TGTCCTGCAGCCACTGGTTC				
IL-10	Forward	GAGATGCCTTCAGCAGAGTGAAGA	1629	114 (193–306)	249	NM_000572.2
	Reverse	AGGCTTGGCAACCCAGGTAAC				
IL-12a (p35)	Forward	AGGAATGTTCCCATGCCTTCAC	1444	170 (413–582)	497	NM_000882.2
	Reverse	CCAATGGTAAACAGGCCTCCAC				
IL-12b (p40)	Forward	GGAGCGAATGGGCATCTGT	2347	112 (998–1109)	1053	NM_002187
	Reverse	TGGGTCTATTCCGTTGTGTCTTTA				
M-CSF (CSF-1)	Forward	TAGCCACATGATTGGGAGTGGA	2973	88 (530–617)	573	NM_000757.4
	Reverse	CTCAAATGTAATTTGGCACGAGGTC				
IFN-gamma-.	Forward	CTTTAAAGATGACCAGAGCATCCAA	1240	189 (372–560)	466	NM_004918
	Reverse	GGCGACAGTTCAGCCATCAC				
MCP-1 (CCL2)	Forward	GCTCATAGCAGCCACCTTCATTC	760	147 (106–252)	179	NM_002982.3
	Reverse	GGACACTTGCTGCTGGTGATTC				
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC	1310	142 (248–389)	318	NM_002046
	Reverse	ATGGTGGTGAAGACGCCAGT				

CSF, colony-stimulating factor; MCP, monocyte chemoattractant protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

hour at room temperature, and after extensive washing with PBS, the color reaction was developed in 2% 3, 3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained with Meyer hematoxylin, dehydrated, and mounted.

Immunostained cell counts were made blind to the patients' clinical data. Round cells in the stroma of the cancer tissue were counted as macrophages. Two pathologists (Y.O. and G.I) counted CD68- and CD204-positive macrophages in high-power microscopic fields (400×; 0.0625 mm^2), and reviewed all slides.

Tissue Samples, RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA were extracted from 18 patients with adenocarcinoma surgically resected at Thoracic Oncology Division, National Cancer Center Hospital East. Samples of cancer tissue and noncancer tissue in the most remote part of the same lobe of the lung as the cancer were collected and immediately homogenized in Trizol reagent (Invitrogen, CA) with multibeads shocker (Yasui Kikai) and stored at −80°C until used. Total RNA was isolated from the tissues by using a commercial RNA isolation reagent according to the manufacturer's instructions.

The RNA was reverse transcribed to synthesize cDNA by using a primerscript RT reagent kit according to the manufacturer's instructions (Takara Biochemicals, Osaka, Japan).

To quantitatively compare the mRNA level of each cytokine, we performed real-time polymerase chain reaction using SYBR Premix Ex *Taq*II (Takara, Japan) with Smart Cycler II (Takara Biochemicals, Osaka, Japan) according to the manufacturer's protocol. The sense and antisense primers used for quantitative amplification of cytokine mRNAs (IL-6, IL-10, IL-12a, IL-12b, M-CSF, IFN-gamma-., and MCP-1) and for amplification of glyceraldehyde-3-phosphate dehydrogenase as an internal control are shown in Table 2.

The amount of template cDNA was expressed by a threshold cycles (G) that was determined from the amplification curve (exponential curve) and a threshold level of polymerase chain reaction product detection. One G was equal to a twofold difference in initial template. The quantification data were analyzed with Smart Cycler System Software version 2.0d (Cepheid). The level of expression of each gene is reported as the ratio of its expression to the level of glyceraldehyde-3-phosphate dehydrogenase gene expression in the same sample. The ratio between the level of cytokine expression in the cancer tissue to its level of expression in the noncancer tissue was calculated in each case, and the median number of CD204-positive macrophages was used to divide the cases into a high CD204-positive macrophage group (above the median value) and a low CD204-positive macrophage group (below the median value).

Statistical Analysis

The correlations between the grade of expression of CD68 and CD204 in cancer stromal cells and the clinicopath-

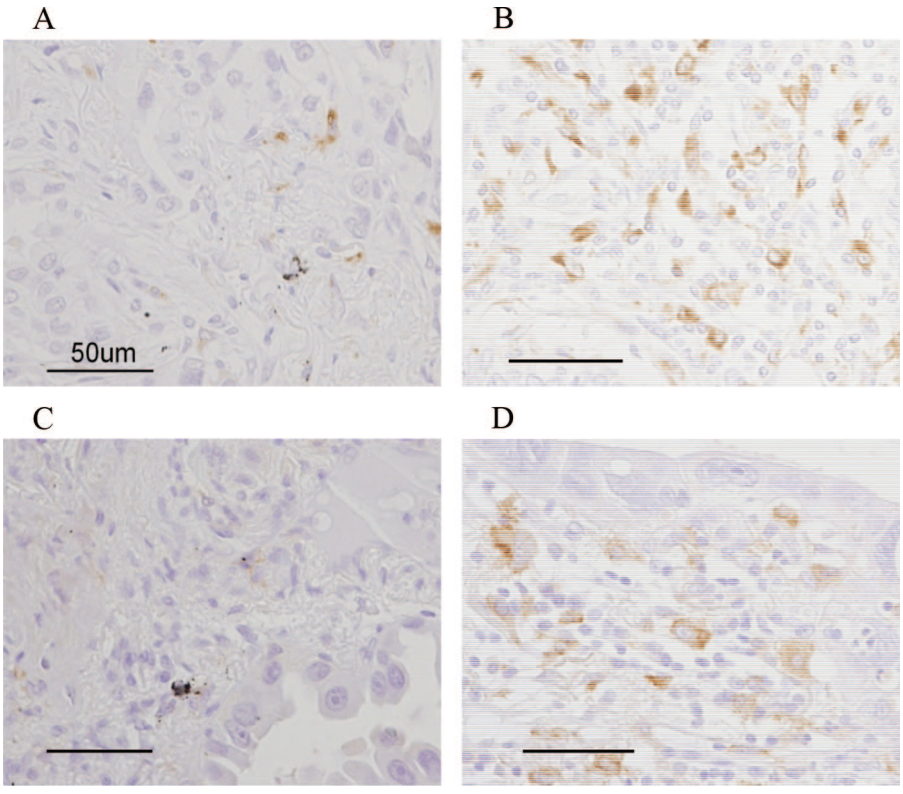

FIGURE 1. Immunostaining of stromal $CD68^+$ macrophages (*A* and *B*) and $CD204^+$ macrophages (*C* and *D*). *A*, CD68 macrophages (low number), (*B*) CD68 macrophages (high number), (*C*) CD204 macrophages (low number), and (*D*) CD204 macrophages (high number).

ological factors were evaluated by the χ^2 test. Comparison of the numbers of CD68-positive macrophages and CD204-positive macrophages in each predominant subtype group was performed by one-way layout analysis of variance. All pairs of groups were compared by using the Bonferroni test. Overall survival was measured from the date of surgery to the date of death from any cause or to the date the patient was last known to be alive. Survival curves were estimated by the Kaplan-Meier method, and differences in survival were compared by the log-rank test. Differences of the ratios of expression of each cytokine between high and low CD204-positive macrophage group were tested for statistical significance by the Mann-Whitney *U* test. The distribution of CD204-positive macrophages and CD68-positive macrophages and the distribution of CD204 expression and ratios of expression of each cytokine were tested for correlations by calculating Spearman rank correlation coefficients. A p value less than 0.05 was considered significant. Statistical analysis software (SPSS, Version 11.0) was used to perform the analyses.

RESULTS

CD68-Positive Macrophages and CD204-Positive Macrophages in Cancer Stroma

A series of 170 specimens of adenocarcinoma of the lung were examined for CD68 and CD204 expression in the cancer stroma (Figures 1*A–D*). The distribution of CD204-positive macrophages and CD68-positive macrophages in each tumor was strongly correlated ($p < 0.001$, $r^2 = 0.5910$) (Figure 2*A*). The mean number of CD68-positive macrophages and CD204-positive macrophages was 30.6 ± 1.8 (median: 25, range: 0–120) and 24.8 ± 2.0 (median: 15, range: 0–143), respectively (Figure 2*B*).

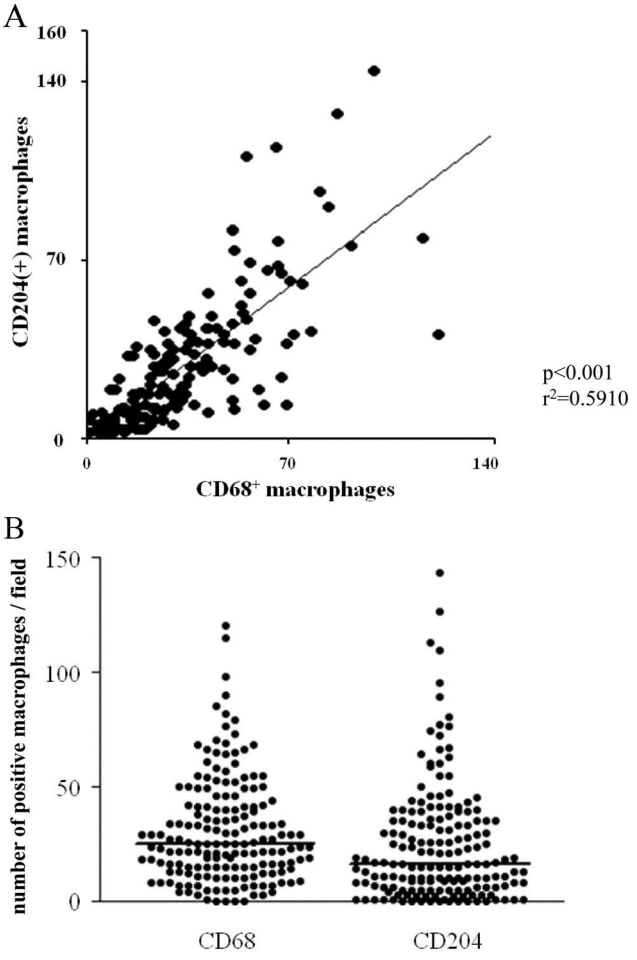

FIGURE 2. Distribution of $CD68^+$ and $CD204^+$ macrophages. *A*, Correlations between the distribution of $CD68^+$ macrophages and $CD204^+$ macrophages of each case are shown. The numbers of $CD204^+$ macrophages were significantly correlated with the numbers of $CD68^+$ macrophages ($r^2 = 0.5910$, $p < 0.001$). *B*, The mean number of CD68-positive macrophages and CD204-positive macrophages was 30.6 ± 1.8 (median: 25, range: 0–120) and 24.8 ± 2.0 (median: 15, range: 0–143), respectively.

Correlations Between the Numbers of CD68-Positive Macrophages and CD204-Positive Macrophages and the Clinicopathological Factors

The median numbers of infiltrating macrophages were used to divide the cases into two groups: high; above the median value, low; below the median value. The relationship between the two groups and the individual clinicopathological features are shown in the Table 1. Both CD68 expression and CD204 expression were significantly correlated with gender, smoking history, tumor differentiation, pathologic stage, pathologic T status, pathologic nodal involvement, lymphatic permeation, vascular invasion, and pleural invasion.

Correlations Between the Numbers of CD68-Positive Macrophages and CD204-Positive Macrophages and the Predominant Histologic Subtype

The relationships between the numbers of infiltrating macrophages and predominant histologic subtypes are shown in the Figure 3. The average numbers of CD68-positive macrophages according to predominant subtype were BAC, 18; papillary, 30; acinar, 38; and solid, 55, and the numbers were significantly higher in the predominantly papillary subtype ($p = 0.004$), the predominantly acinar subtype ($p < 0.001$), and the predominantly solid subtype ($p < 0.001$) than in the BAC subtype (Figure 3*A*). The average numbers of CD204-positive macrophages according to predominant subtype were BAC, 11; papillary, 24; acinar, 32; and solid, 51, and the numbers were significantly higher in the predominantly papillary subtype ($p = 0.008$), the predominantly acinar subtype ($p = 0.001$), and the predominantly solid subtype ($p < 0.001$) than in the BAC subtype (Figure 3*B*). The numbers of CD68-positive macrophages and CD204-positive macrophages were both significantly higher in the solid subtype than in the papillary subtype ($p < 0.001$ and $p = 0.010$, respectively) and acinar subtype ($p = 0.001$ and $p = 0.018$, respectively).

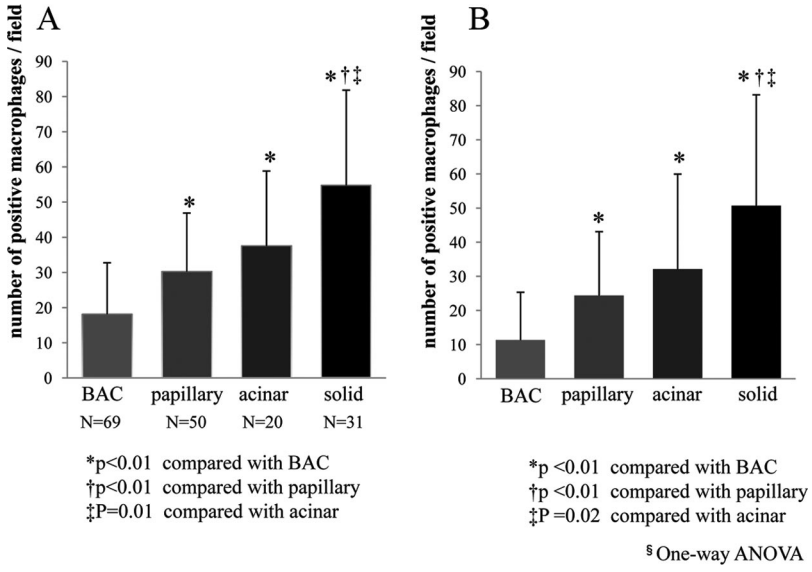

FIGURE 3. Numbers of $CD68^+$ macrophages (*A*) and $CD204^+$ macrophages (*B*) in each histologically predominant subtype (bronchioloalveolar carcinoma [BAC], papillary, acinar, and solid).

Relationship Between the Numbers of CD68-Positive Macrophages and CD204-Positive Macrophages and Overall Survival

Univariate analyses by the log-rank test were performed to compare survival according to numbers of infiltrating macrophages (Table 3), and overall survival curves obtained by the Kaplan-Meier method, with statistical significance assessed using the log-rank test, are shown in Figure 4. Although Survival time was significantly shorter in the high CD204-positive macrophage group ($CD204^{high}$ group) ($p = 0.0073$), $CD68^{high}$ did not significantly show prognostic impact compared with $CD68^{low}$ ($p = 0.0789$). Age ($p = 0.048$), gender ($p = 0.0019$), smoking history ($p = 0.0002$), CEA ($p = 0.0150$), differentiation ($p < 0.001$), pathologic stage ($p < 0.001$), pathologic T status ($p < 0.001$), nodal involvement ($p < 0.001$), lymphatic permeation ($p < 0.001$), vessel invasion ($p < 0.001$), and pleural invasion ($p < 0.001$) were also correlated with shorter survival time.

Although we stratified the patient with age (<70 years or ≥70 years), we could observe the significant survival difference between $CD204^{high}$ group and $CD204^{low}$ group only in the group of more than 70 years ($p < 0.01$). In the group of less than 70 years, we could not observe significant difference ($p = 0.05$). Correspondingly, when stratified with pathologic stage (IA or IB–IIIA), significant differences between $CD204^{high}$ group and $CD204^{low}$ group could not be observed: IA, $p = 0.25$; IB–IIIA, $p = 0.41$, respectively (data not shown).

Correlations Between the Numbers of CD204-Positive Macrophages and Cytokine Expression in Cancer Tissues

To identify factors associated with the migration and differentiation of M2 macrophages, we examined the expression of IL-6, IL-10, IL-12a, IL-12b, M-CSF, granulocyte macrophage-CSF, IFN-gamma-., and MCP-1 and the number of CD204-positive macrophages in specimens of adenocarcinoma of the lung. The results showed that the ratios of expression of IL-10 and MCP-1 in cancer tissue to their level of expression in noncancer tissue ($n = 18$) were significantly higher in the $CD204^{high}$ group ($p = 0.031$ and $p = 0.031$, respectively) (Figure 5*A*). The differences between the two groups in expression of the other soluble factors, i.e., IL-6, IL-12a, IL-12b, M-CSF, and IFN-gamma-. were not significant. Although the IL-4 RNA levels were measured, the amount of RNA expressed was too small to be studied (data not shown).

Correlation analysis of the numbers of CD204-positive macrophages and levels of IL-10 and MCP-1 expression revealed that the numbers of CD204-positive macrophages were significantly correlated with the increases in level of IL-10 and MCP-1 (Figure 5*B*) (MCP-1: $r^2 = 0.641$; IL-10: $r^2 = 0.580$).

DISCUSSION

In this study, we first showed that the numbers of CD204-positive macrophages were correlated with several clinicopathological factors in lung adenocarcinoma, i.e., gender, smoking history, tumor differentiation, pathologic stage, T status, nodal involvement, lymphatic permeation, vessel invasion, and pleural invasion. The grade of CD204 expression was significantly correlated with cumulative survival, and the levels of expression of IL-10 and MCP-1, which are involved in polarization of M2 macrophages, were significantly correlated with the numbers of CD204-positive macrophages. These results suggest that CD204-positive macrophages could be the tumor-promoting macrophages (M2 macrophages) involved in tumor progression in lung adenocarcinoma.

Macrophage number has been shown to have prognostic significance in lung adenocarcinoma, but the immunohistochemical identification in those studies involved the use of antibodies against CD68.[7,16,17,20] In our study, we also used an antibody against CD204, and the numbers of CD204-

TABLE 3. Prognostic Significance for Overall Survival (Univariate Analysis)

Characteristics	Patient ($N = 170$) No.	Percentage	5-yr Survival (%)	p*
Age				
Median, yr (range)	63 (33–85)			0.0048†
<70 yr	122	71.8	79.3	
≥70 yr	48	28.2	62.5	
Gender				
Male	85	50.0	67.9	0.0019†
Female	85	50.0	81.1	
Smoking history				
Never	81	47.6	85.2	0.0002†
Former or current	89	52.4	64.8	
CEA				
<5.0	104	61.5	83.5	0.0150†
≥5.0	65	38.5	60.0	
Differentiation				
Well	86	50.6	89.4	<0.0001†
Moderately or poorly	84	49.4	59.5	
Pathological stage				
IA	98	57.6	86.6	<0.0001†
IB–IIIA	72	42.4	58.4	
T status				
T1	109	64.1	81.4	0.0001†
T2–3	61	35.9	62.3	
Nodal involvement				
N(−)	130	76.5	85.4	<0.0001†
N(+)	40	23.5	38.5	
Lymphatic permeation				
Ly(−)	108	63.5	87.0	<0.0001†
Ly(+)	62	36.5	50.9	
Vessel invasion				
V(−)	105	61.8	89.5	<0.0001†
V(+)	65	38.2	50.0	
Pleural invasion				
PL(−)	121	71.2	82.5	<0.0001†
PL(+)	49	28.8	55.1	
CD68-positive macrophage				
Below median	82	48.2	81.6	0.0789
Above median	88	51.8	67.8	
CD204-positive macrophage				
Below median	81	47.6	88.9	0.0073†
Above median	89	52.4	61.4	

*Log-rank test.
†$p < 0.05$.

positive macrophages allowed for better prognostic distinction than the grade of CD68-positive macrophages. This finding suggests that CD204 could outline the macrophages that promote tumor progression and affect the prognosis more clearly, thereby allowing for a more precise assessment of the density of the aggressive subpopulation of M2 macrophages. Although CD68 is known as a pan-monocyte/macrophage marker,[21] not all TAMs may be stained by CD68. The difference in staining patterns may be due to the phenomenon of monocyte/macrophage heterogeneity,[22] because different subgroups of macrophages were identified by each antibody.

We also demonstrated that the quantities of MCP-1 and IL-10 were significantly correlated with the numbers of infiltrating CD204-positive macrophages within the cancer-induced stroma and that other cytokines, i.e., IL-4, IL-6, IL-12, M-CSF, and IFN-gamma-., were not significantly correlated with the numbers of CD204-positive macrophages. *MCP-1* is known to polarize, differentiate, and migrate M2 macrophages,[23,24] and IL-10 is known to function to polarize monocytes to M2 macrophages.[11] Both MCP-1 and IL-10 are derived from M2 macrophages themselves and from tumor cells.[25,26] We have previously shown that stromal MCP-1 rather than tumoral MCP-1 contributes to macrophage recruitment in breast cancer suggesting that stromal MCP-1 may recruit additional macrophages through tumor cell-macrophage interactions.[27] Zeni et al.[28] immunohistochemically demonstrated that IL-10 is expressed by macrophages and not by tumor cells in patients with non-small cell lung cancer, and IL-10 expression correlated with disease progression and the patients' outcome. These findings led us to surmise that MCP-1 and IL-10 derived from tumor cells induce differentiation, accumulation, and migration of M2 macrophages, which contribute to recruiting more CD204-positive macrophages in lung cancer tissue. IL-4 has been found to polarize macrophages, but too little IL-4 was expressed to be detected in this study.

The results of this study demonstrated that CD204-positive macrophages are engaged in histologic predominant subtype. Macrophage infiltration in the solid subtype was correspondingly much greater than in the BAC subtype. Actually, adenocarcinoma with a predominant solid subtype has been found to be correlated with certain gene profiles and a poorer outcome,[29] implying that this type of adenocarcinoma belongs to another biologic spectrum that is different from the predominantly BAC subtype. Based on all the above findings taken together, the profiles of factors involved in the polarization of M2 macrophages, including MCP-1 and IL-10, may be different in the predominant BAC subtype and solid subtype.

Because CD204-positive macrophage infiltration was also related to several factors involved in local invasion, including lymphatic permeation, vessel invasion, and pleural invasion, we speculate that CD204-positive macrophages contribute to local invasion by cancer cells. Practically, TAMs are important producers of proteases, including matrix metalloproteinases, and of a wide variety of growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) receptor family ligands that can stimulate the growth and motility of tumor cells.[9] TAMs have been reported to be the most significant source of EGF in tumors,[30] and they are associated with EGF receptor expression and poor outcome in breast cancer.[31] Pollard et al.[9,32] showed that tumor cells responded to macrophage-produced EGF ligands

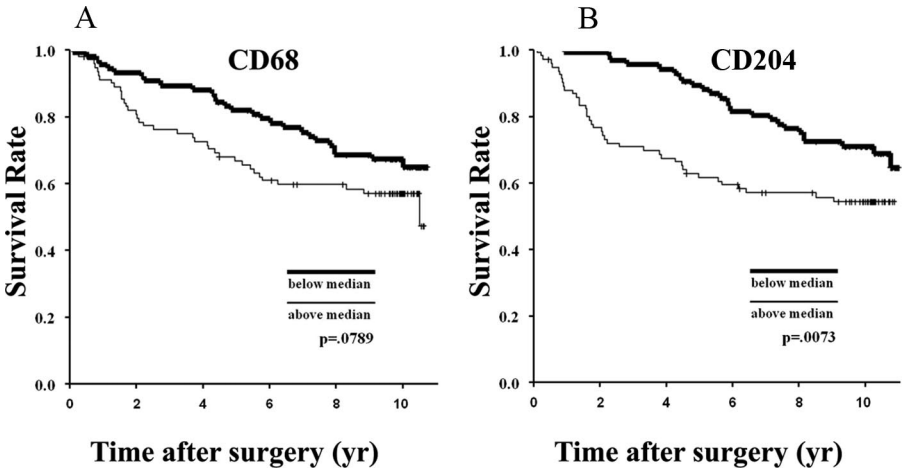

FIGURE 4. Kaplan-Meier analysis of overall survival. Kaplan-Meier analysis of overall survival according to the level of infiltration by $CD68^{+}$ macrophages (*A*) and by $CD204^{+}$ macrophages (*B*). Data were dichotomized at the median value for each parameter.

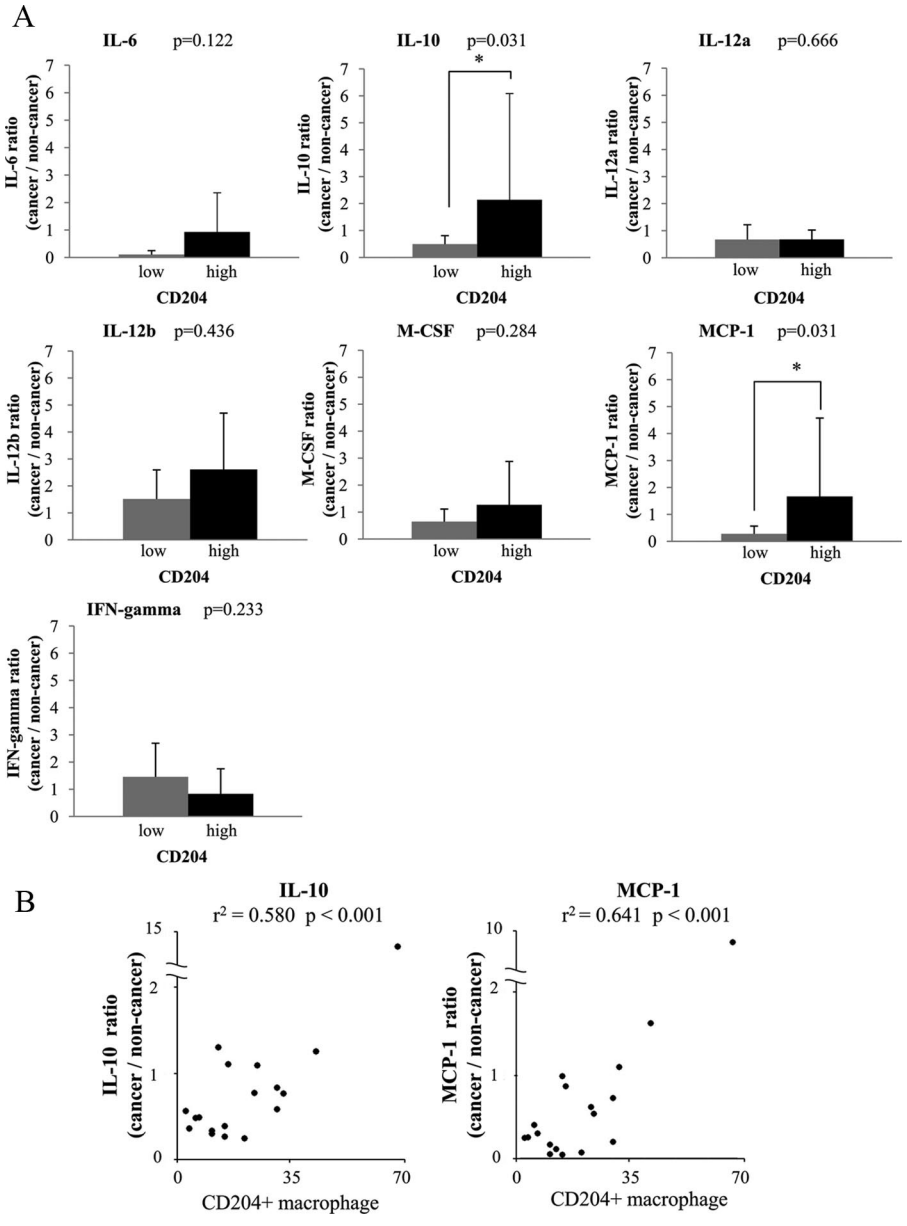

FIGURE 5. Correlations between the levels of expression of factors and numbers of CD204+ macrophages. *A*, The levels of expression shown are ratios of expression in cancer tissue to expression in noncancerous tissue. The ratios are expressed as means ± standard deviation. Expression of IL-10 ($p = 0.0031$) and MCP-1 ($p = 0.0031$) was significantly higher in the $CD204^{high}$ group ($n = 9$) than in the $CD204^{low}$ group ($n = 9$). The differences in expression of IL-6 ($p = 0.122$), IL-12a ($p = 0.666$), IL-12b ($p = 0.436$), M-CSF ($p = 0.284$), and IFN-gamma-. ($p = 0.233$) were not significant. *B*, Correlations between levels of IL-10 and MCP-1 expression (ratios of expression in cancer tissue to expression in noncancerous tissue) and the distribution of $CD204^{+}$ macrophages of each case are shown. The numbers of CD204-positive macrophages were significantly correlated with the increases in the ratios of IL-10 expression ($r^2 = 0.580$, $p < 0.001$) and MCP-1 expression ($r^2 = 0.641$, $p < 0.001$).

in vivo by chemotaxis and invasion and that the macrophages were often associated with vessels. These findings suggest that CD204-positive macrophages provide chemotactic signals that recruit tumor cells to blood vessels and enhance their egress into the vasculature.

In conclusion, the results of this study demonstrated that CD204-positive macrophages in cancer stroma would be the marker of tumor-promoting macrophages. The tumor microenvironment may also be important in terms of the treatment of lung cancer. Reducing the number of TAMs in the tumor stroma in an animal model of breast cancer has been found to effectively alter the tumor microenvironment involved in tumor angiogenesis and progression and to markedly suppressed tumor growth and metastasis.[33] Bak et al.[34] demonstrated that anti-CD204 immunotoxin reduced the peritoneal tumor burden and accumulation of ascitic fluid in a murine model of ovarian cancer. These findings support the feasibility of possible new immunotherapy for adenocarcinoma of the lung that targets CD204-positive macrophages. Thus, more accurate insight into the role of CD204-positive macrophages and consideration of the local microenvironment in regulating the functions of this type of macrophages is needed and has important implications.

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