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Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma

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Tumor associated macrophages (TAMs) are the most abundant cancer stromal cells educated by tumor microenvironment to acquire trophic functions facilitating angiogenesis, matrix breakdown and cancer cell motility. Tumor associated macrophages have anti-inflammatory properties or "alternatively" activated (M2) phenotype expressing CD204 and/or CD163. To know the role of TAMs in the growth and progression of esophageal squamous cell carcinomas (ESCCs), we calculated intratumoral CD204, CD163 or CD68 expressing macrophage count (MϕC) and CD34-positive microvessel density (MVD) by immunohistochemistry in 70 cases of surgically resected ESCCs and compared them with the clinicopathological factors and prognosis of patients. MϕC had positive linear association with MVD. High CD204$^+$ MϕC were significantly correlated with more malignant phenotypes including depth of tumor invasion, lymph and blood vessel invasion, lymph node metastasis as well as clinical stages. On the other hand, CD163$^+$ MϕC did not associate with these clinicopathological factors with the exception of depth of tumor invasion and blood vessel invasion. Patients with high CD204$^+$ MϕC ESCCs showed poor disease-free survival ($P = 0.021$). Conditioned media of five ESCC cell lines (TE-8, -9, -10, -11 and -15) induced mRNA as well as protein expression of CD204 but not of CD163 with upregulation of *vascular endothelial growth factor-A* mRNA in TPA treated human acute monocytic leukemia cell line THP-1. These results overall indicate that CD204 is a useful marker for TAMs contributing to the angiogenesis, progression and prognosis of ESCCs whose specific tumor microenvironment may educate macrophages to be CD204$^+$ M2 TAMs. (*Cancer Sci* 2013; 104: 1112–1119)

Esophageal cancer is the sixth leading cause of cancer death worldwide.[1] In Japan, more than 18 000 people were diagnosed with and over 10 000 patients died of this malignancy in 2006.[2] This high mortality rate is attributable to the anatomical location of the esophagus in deep mediastinum preventing early diagnosis. Moreover, even in superficial esophageal cancers invading no further than the submucosa, half of the cases have regional lymph node metastasis.[3] The incidence of adenocarcinoma related to the Barrett's esophagus are increasing up to 60% in the United States, esophageal squamous cell carcinoma (ESCC) is still the main histological type in Asian countries including Japan. Epidemiological studies have revealed that tobacco and alcohol are externally increasing the risk of ESCC, with genetic polymorphisms including *ALDH2* and *TP53* internally increasing the risk of ESCC.[1,4–6] Despite the accumulation of this epidemiological evidence for carcinogenesis, pathological mechanisms underlying the highly aggressive behavior of ESCC remain obscure.

Tumor tissue is composed of variable numbers of cancer cells and stromal cells. The interaction between them may produce a cancer specific microenvironment for the tumor progression. Macrophages are the most abundant cancer stromal cells involved in the host immune system, and in several kinds of cancer, tumor-associated macrophage (TAM) infiltration has been found to correlate with a poor prognosis.[7] In the viewpoint of oncology, macrophages have two different functions, a tumor-suppressive (M1) and a tumor-supportive (M2) function.[8–10] M1 macrophages are characterized by high expression of pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6, IL-12 and tumor necrosis factor, whereas M2 macrophages, which are characterized by IL-4^{high}, IL-10^{high} and IL-12^{low}, play important roles in tumorigenesis, angiogenesis, matrix remodeling and metastasis.[11]

Tumor-associated macrophages have been mainly assessed by CD68 immunostaining. However, CD68 is a broad macrophage marker that cannot discriminate the M2 phenotype from others. Recent studies have reported strong expression of CD204 or CD163, macrophage scavenger receptor A or a membrane protein belonging to the scavenger receptor cysteine-rich domain family, respectively, by the M2 macrophage.[11–13] CD204 or CD163 positive TAMs have been reported to correlate with tumor progression and poor patient outcome in glioma, ovarian epithelial tumors, biliary, lung, pancreatic, endometrial, renal, breast and oral cancers.[13–21] However, the role of M2 polarized TAMs in ESCC has not been reported yet.

Materials and Methods

Tissue samples. A total of 70 sporadic human ESCCs surgically removed at Kobe University Hospital (Kobe, Japan) were used. The patients consisted of 55 men and 15 women with an age range of 54–88 years and mean age of 65.7 years. None of them received adjuvant chemotherapy or radiotherapy before surgery. Informed consent was obtained from all patients, and the study was approved by the Kobe University Institutional Review Board. All resected specimens were fixed in 10% formalin and embedded in paraffin. Histological and clinicopathological evaluation was performed according to the Japanese Classification of Esophageal Cancer proposed by the Japan Esophageal Society[22] along with the TNM classification of the Union for International Cancer Control (UICC).[23]

Cell cultures. Five ESCC cell lines (TE-8, TE-9, TE-10, TE- 11 and TE-15) were obtained from RIKEN BioResource Center (Tsukuba, Japan).[24] The individuality of the TE series ESCC cell lines was confirmed by short tandem repeat analysis at RIKEN and at the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. THP-1 human acute monocytic leukemia

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cell line was purchased from the American Type Culture Collection (Mannasas, VA, USA).[25] We routinely maintained ESCC cell lines in RPMI-1640 (Wako, Osaka, Japan) and THP-1 cells in DMEM (Wako) with 10% FBS (Sigma-Aldrich, Tokyo, Japan) and 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA). Conditioned media of TE series ESCC cell lines (TECM) were prepared by plating 5×10^6 tumor cells in 10 mL complete medium in 100-mm dishes for 24 h, thereafter changing the medium to complete DMEM supplemented with 10% human AB serum (Lonza, Walkersville, MD, USA) instead of fetal bovine serum. After 2–3 days, the supernatants were harvested, centrifuged and stored in aliquots at −80°C.[26] To induce macrophage-like differentiation, 5×10^6 THP-1 cells were treated with 200 nM TPA (Cell Signaling, Danvers, MA, USA) for 2 days.[27] Then, TPA-treated THP-1 cells were exposed to 50% TECM for 2 days.

Immunohistochemistry and immunofluorescence. We used a modified version of the immunoglobulin enzyme bridge technique with a Linked Streptavidin-Biotin kit (DakoCytomation, Glostrup, Denmark) as described elsewhere.[28] Specific mouse monoclonal antibodies against CD68 (Kp-1, 1:100; DAKO), CD163 (10D6, 1:100; Novocastra, Newcastle upon Tyne, UK), CD204 (SRA-E5, 1:50; Trans Genic, Kobe, Japan) and CD34 (NU-4A1, 1:50, Nichirei, Tokyo, Japan) were used for the primary reaction. After gentle rinsing with 0.05 M of Tris-HCl, the sections were incubated with biotinylated goat anti-mouse IgG and streptavidin conjugated to HRP. Chromogenic fixation was carried out by immersing the sections in a solution of 3,3′-diaminobenzidine. Sections were counterstained with Mayer's hematoxylin. For immunofluorescence, 4×10^5 THP-1 cells were grown and treated on glass coverslips and then fixed with pre-cooled methanol. Cells were stained with antibodies against CD204 (Trans Genic) and CD163 (Novocastra). Cy3 conjugated anti-mouse IgG (GE Healthcare, Little Chalfont, UK) were used as the secondary antibody. The nuclei were stained with DAPI (Wako). CD163- and CD204-positive cells were counted under fluorescein microscope.

RT–PCR and quantitative RT-PCR. Reverse transcription–polymerase chain reactions were carried out as described previously.[29] Total mRNA was extracted from THP-1 cells using an RNA extraction kit (RNeasy Kit; Qiagen, Hilden, Germany). Reverse transcription-PCR amplifications of *CD204*, *CD163* and control gene *GAPDH* were done. The PCR products were then subjected to electrophoresis in 1.5% agarose gel. Real-time RT-PCR amplifications of *vascular endothelial growth factor -A* (*VEGF-A*) and control gene *GAPDH* were performed using ABI StepOne Realtime PCR system (Applied Biosystems, Foster City, CA, USA). The threshold cycle (Ct) values were determined by plotting the observed fluorescence against the cycle number. Ct values of *VEGF-A* were analyzed using the comparative threshold cycle method and normalized to those of *GAPDH*. The relative gene expression levels were estimated using the following formula: relative expression = $2^{-(\mathrm{Ct}[target\ \ gene]-\mathrm{Ct}[GAPDH])}$. Primers (*CD204*, 5′-CCA GGG ACA TGG GAA TGC AA -3′ [forward] and 5′-CCA CTG GGA CCT CGA TCT CC -3′ [reverse]; *CD163*, 5′-CGA GTT AAC GCC AGT AAG G -3′ [forward] and 5′-GAA CAT GTC ACG CCA GC -3′ [reverse]; *VEGF-A*, 5′-TGC TCT ACC TCC ACC ATG CCA AGT-3′ [forward] and 5′-GCG CAG AGT CTC CTC TTC CTT CAT-3′ [reverse]; *GAPDH*, 5′-ACC ACA GTC CAT GCC ATC AC-3′ [forward] and 5′-TCC ACC ACC CTG TTG CTG TA-3′ [reverse]) were designed according to a previous report.[15]

Macrophage count and microvessel density. In the microvessel count (MϕC) using anti-CD34 antibody, positive staining was assessed by light microscopy in areas of the tumor containing the highest numbers of capillaries and small venules with lumens. The highly vascular areas were identified by scanning the tumor sections at low power. The vessel count was performed on a 200× field. The mean number of microvessels per 0.7386 mm^2 was calculated as microvessel density (MVD).[30] Counting of macrophages performed in three independent high-power microscopic fields (400×; 0.0625 μm^2) of cancer nest within the areas of microvessel count. CD68-, CD163- and CD204-positive round cells were counted as macrophages. The mean number of macrophages per 0.7386 mm^2 was calculated as MϕC. Three pathologists (M.N., S.S. and H.Y.), blinded to the patients' clinical data, performed the counting.

Statistical analysis. Pearson's analysis and the Student's *t*-test were used to evaluate the correlation between the MϕC and MVD. Relationships between clinicopathological features and immunohistochemical results were analyzed by the χ^2 test. Survival curves were estimated by the Kaplan–Meier method and differences in survival were compared by the log-rank test. Parameters that were significantly associated with disease-free survival (DFS) or overall survival (OS) rates evaluated in univariate analysis were further analyzed with multivariate analysis using Cox proportional hazard regression model. *In vitro* induction of CD163 or CD204 in THP-1 cells by TECM was evaluated by paired *t*-test. A *P*-value <0.05 was considered statistically significant. Statistical analyses were carried out using SPSS Statistics Version 21 (IBM, Chicago, IL, USA).

Results

MϕC showed significant positive correlation with MVD in ESCC. Macrophages expressing CD68, CD163 or CD204 immunoreactivities were detected in all ESCC tissues examined. Interestingly, they distributed not only in the cancer stroma or adjacent non-neoplastic connective tissue but also within the cancer nest. As demonstrated in Figure 1a–d, we observed that the number of macrophages infiltrated was higher within the tumor area with rich density of CD34 positive microvessels. Therefore, we conducted the MϕC within the area of MVD analysis. Pearson's analysis demonstrated significant correlation between MVD and $CD204^+$ MϕC ($r = 0.4534$, $n = 70$, $P < 0.0001$), $CD163^+$ MϕC ($r = 0.4552$, $n = 70$, $P < 0.0001$) or $CD68^+$ MϕC ($r = 0.4077$, $n = 70$, $P = 0.0005$) as expected (Fig. 1e). Next, we divided the ESCC cases into high and low MϕC groups according to the median value of $CD204^+$ MϕC (53.3, range 0.6–273.2), $CD163^+$ MϕC (68.9, range 3.6–283.3) and $CD68^+$ MϕC (54.4, range 0.6–320.3), then analyzed the difference of their mean value of MVD. ESCCs with high $CD204^+$, $CD163^+$ and $CD68^+$ MϕC (CD204 high, CD163 high and CD68 high) showed significantly higher MVD than those with low MϕC (CD204 low, CD163 low and CD68 low) with the *P* values of 0.0016, 0.0010 and 0.0026, respectively.

$CD204^+$ MϕC associated closely with clinicopathological factors of ESCCs. As we observed the significant positive correlation between MϕC and MVD, we subsequently asked whether MϕC had any statistical association with clinicopathological factors of ESCCs (Table 1). High $CD204^+$ MϕC in ESCC tumor nest closely correlated with more malignant phenotypes including depth of tumor invasion ($P < 0.0001$), lymphatic vessel invasion ($P = 0.0001$), blood vessel invasion ($P = 0.0006$), lymph node metastasis ($P < 0.0001$) as well as clinical stages ($P < 0.0001$). In addition, fractions of high grade intraepithelial neoplasia in low $CD204^+$ and poorly differentiated squamous cell carcinoma in the high $CD204^+$ group were significantly frequent ($P = 0.0380$). On the other hand, $CD163^+$ MϕC did not associate with these clinicopathological factors with the exception of depth of tumor invasion and blood vessel invasion. $CD68^+$ MϕC showed statistical association with depth of tumor invasion, blood vessel invasion and lymph node metastasis with

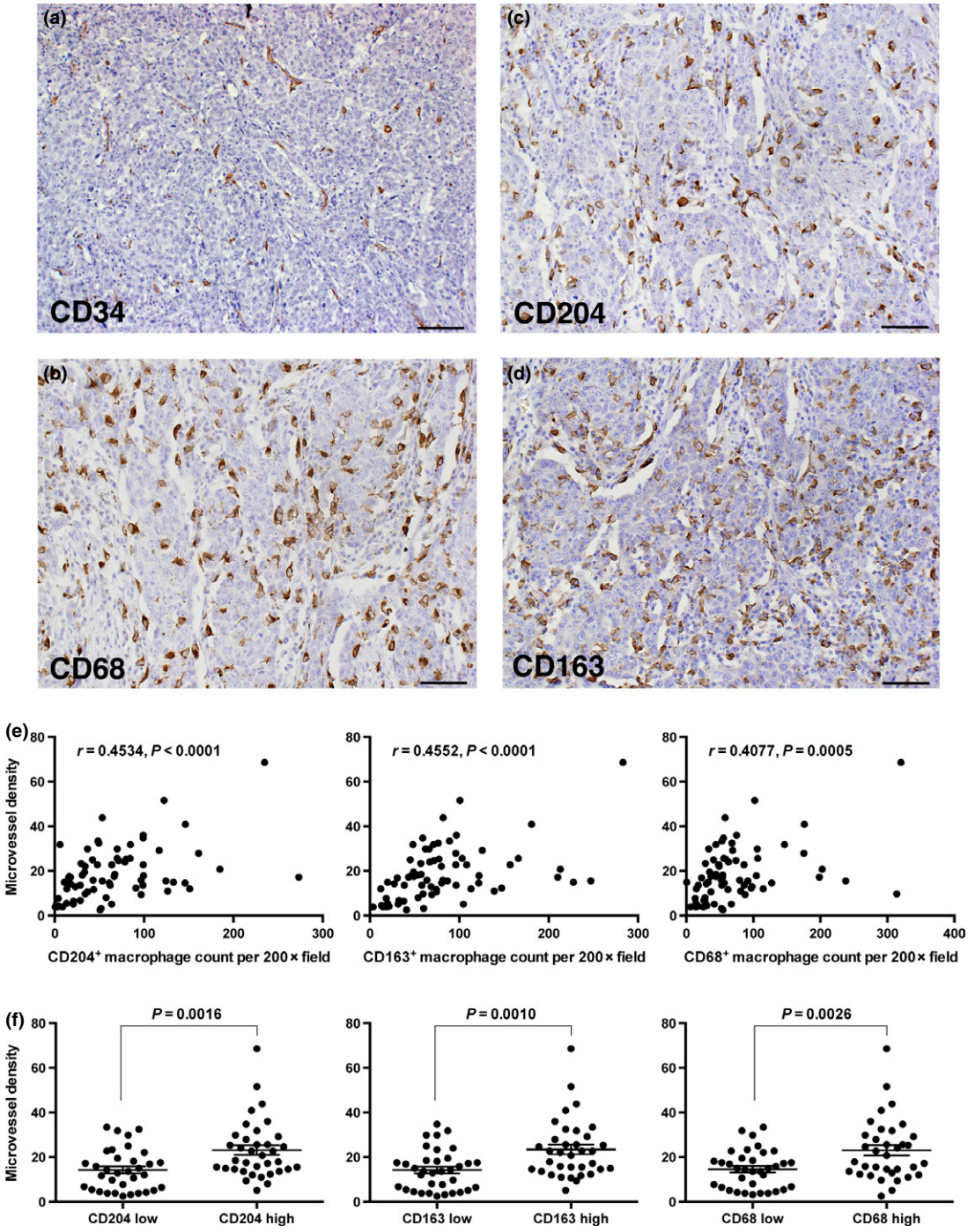

Fig. 1. Microvessel density (MVD) showed significant positive correlation with numbers of infiltrating macrophages in esophageal squamous cell carcinoma (ESCC). (a–d) Tumor areas containing the highest numbers of CD34-positive capillaries and small venules with lumens were selected and the numbers of microvessels and macrophages per 0.7386 mm^2 (200× field) were calculated as MVD and macrophage count (MϕC). Representative immunoreactivity of CD34 positive microvessels (a), $CD68^+$ macrophages (b), $CD204^+$ (c) or $CD163^+$ (d) macrophages within the same 200× field of ESCC. Bar 50 μm. Immunoperoxidase stain. (e) Significant correlation was observed between MVD and $CD204^+$ MϕC ($r = 0.4534$, $n = 70$, $P < 0.0001$), $CD163^+$ MϕC ($r = 0.4552$, $n = 70$, $P < 0.0001$) or $CD68^+$ MϕC ($r = 0.4077$, $n = 70$, $P = 0.0005$). Pearson's analysis. (f) Microvessel density increased significantly in ESCCs with high number of macrophages expressing CD204, CD163 or CD68. The median value of macrophage number in the cancer nest within the areas of microvessel count was used to divide the patients into high and low groups. Student's *t*-test. Bars, mean ± standard error of the mean (SEM).

rather higher *P*-values than those of $CD204^+$ MϕC ($P = 0.0013$, 0.0486 and 0.0486, respectively).

Correlation of MϕC levels with patient survival after surgery. Next, we performed a prognostic study in 61 out of 70 ESCC patients who received curative surgery from 2005 to 2010 and were followed up for 1–8 years (Fig. 2; Tables 2,3). Median disease-free period of patients with high $CD204^+$ MϕC ESCCs (4.75 years) was significantly short in comparison with those with low $CD204^+$ MϕC tumors (6.16 years) by univariate analysis calculated by Kaplan–Meier method ($P = 0.021$, Fig. 2a; Table 2). On the other hand, $CD163^+$ MϕC and $CD68^+$ MϕC were not associated with DFS of ESCC patients.

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Table 1. Tumor infiltrating macrophages in esophageal squamous cell carcinomas and their correlation with clinicopathological parameters

	Number of cases	$CD204^+$ cells†		*P*-value‡	$CD163^+$ cells†		*P*-value‡	$CD68^+$ cells†		*P*-value‡
		Low (n = 34)	High (n = 36)		Low (n = 35)	High (n = 35)		Low (n = 35)	High (n = 35)	
Age										
<65	37	19	18	0.6408	20	17	0.6324	18	19	1.0000
≥65	33	15	18		15	18		17	16	
Histological grade§										
HGIEN	4	4	0	0.0380*	4	0	0.1612	3	1	0.3044
WDSCC	12	6	6		5	7		7	5	
MDSCC	43	22	21		22	21		22	21	
PDSCC	11	2	9		4	7		3	8	
Depth of tumor invasion§										
T1a	19	18	1	<0.0001*	17	2	0.0001*	16	3	0.0013*
T1b	30	14	16		13	17		13	17	
T2 + T3	21	2	19		5	16		6	15	
Lymphatic vessel invasion§										
Negative	37	26	11	0.0001*	23	14	0.0999	23	14	0.0547
Positive	33	8	25		12	21		12	21	
Blood vessel invasion§										
Negative	43	28	15	0.0006*	26	17	0.0486*	26	17	0.0486*
Positive	27	6	21		9	18		9	18	
Lymph node metastasis§										
Negative	43	29	14	<0.0001*	25	18	0.0227	26	17	0.0486*
Positive	27	5	22		10	17		9	18	
Stage¶										
0 + I	38	27	11	<0.0001*	22	16	0.2301	22	16	0.2301
II + III + IV	32	7	25		13	19		13	19	

†The median value of $CD204^+$, $CD163^+$ or $CD68^+$ macrophage number of cancer nest within the areas of microvessel count was used to divide the patients into high and low groups. ‡Data were analyzed by χ^2-test and $^*P < 0.05$ was considered statistically significant. §According to the Japanese Classification of Esophageal Cancer.[22] ¶According to the Tumor Node Metastasis (TNM) classification by Union for International Cancer Control.[23] HGIEN, high grade intraepithelial neoplasia; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma. T1a, tumor invades mucosa; T1b, tumor invades submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia; WDSCC, well-differentiated squamous cell carcinoma.

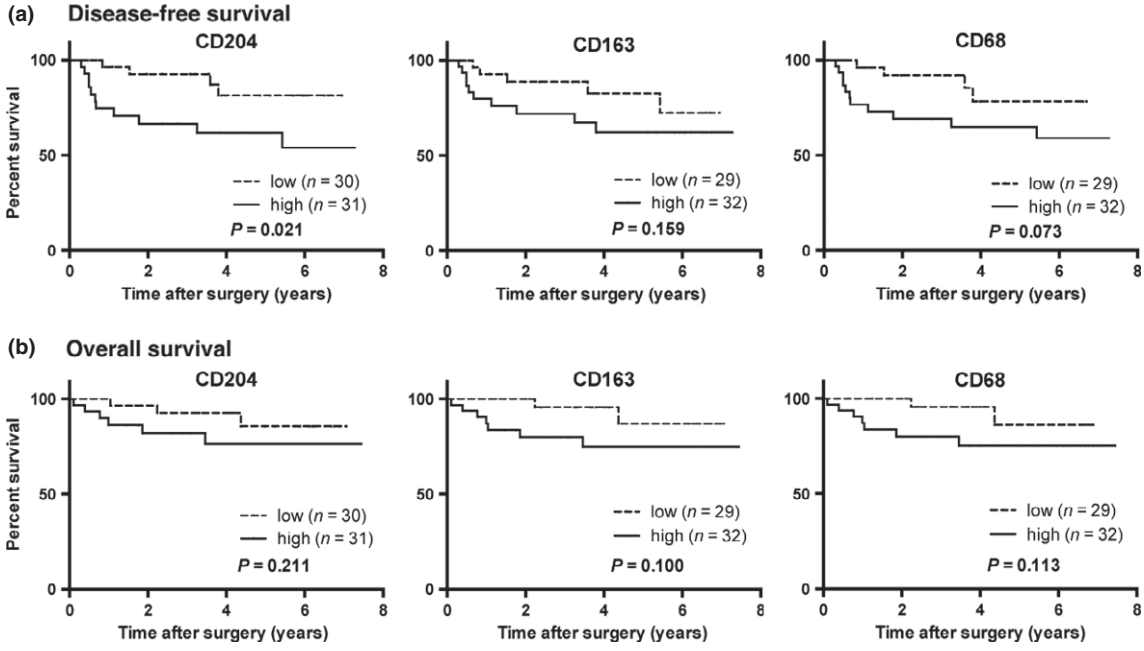

Fig. 2. Kaplan–Meier analysis of esophageal squamous cell carcinomas (ESCCs). (a) Disease-free survival (DFS) after curative surgery of ESCCs showed significant difference between cases with (__) high (n = 31) and (…) low (n = 30) $CD204^+$ MϕC (P = 0.021), while $CD163^+$ or $CD68^+$ MϕC did not have significant impact on DFS. (b) Overall survival was not significantly affected by any MϕC.

Table 2. Relationship between clinicopathological features of human esophageal squamous cell carcinoma and disease-free survival

Variable	Univariate analysis			Multivariate analysis		
	Number	Median survival (years)	*P*-value†	HR	95% CI	*P*-value†
Age						
<65	29	4.769	0.246			
≥65	32	6.028				
Gender						
Male	49	5.073	0.380			
Female	12	6.175				
Depth of invasion‡						
T1	42	6.494	<0.001*	9.587	2.031–45.251	0.004*
T2 + T3	19	3.007				
Histological grade‡						
HGIEN + WDSCC	12	5.604	0.713			
	49	5.542				
MDSCC + PDSCC						
Lymphatic vessel invasion‡						
Negative	32	6.370	0.002*			
Positive	29	4.398				
Blood vessel invasion‡						
Negative	38	5.767	0.159			
Positive	23	4.871				
Lymph node metastasis‡						
Negative	38	6.440	<0.001*	3.034	0.610–15.079	0.175
Positive	23	3.882				
Stage§						
0 + I	35	6.613	<0.001*			
II + III + IV	26	3.854				
$CD204^+$ cells¶						
Low	30	6.155	0.021*	0.627	0.148–2.657	0.526
High	31	4.750				
$CD163^+$ cells¶						
Low	29	5.915	0.159			
High	32	5.074				
$CD68^+$ cells¶						
Low	29	5.852	0.073			
High	32	4.978				

†Disease-free survival was estimated by Kaplan–Meier method compared by log-rank test and a value of $*P < 0.05$ was considered statistically significant. ‡According to the Japanese Classification of Esophageal Cancer.[22] §According to the Tumor Node Metastasis (TNM) classification by Union for International Cancer Control.[23] ¶The median value of $CD204^+$, $CD163^+$ or $CD68^+$ macrophage number of cancer nest within the areas of microvessel count was used to divide the patients into high and low groups. CI, confidence interval; HGIEN, high grade intraepithelial neoplasia; HR, hazard ratio; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma. T1, tumor invades mucosa or submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia; WDSCC, well-differentiated squamous cell carcinoma.

Overall survival of the ESCC patients was not affected by any MϕC status statistically in the present study (Fig. 2b; Table 3).

Conditioned media of ESCC cell lines induced CD204 and *VEGF-A* expressions in differentiated THP-1 human monocytic leukemia cells. Finally, to know the character of the macrophages in esophageal cancer tissues, we evaluated the expression of M2 markers in THP-1 human monocytic leukemia cells stimulated with 200 nM TPA or with 200 nM TPA followed by 50% TECMs (Fig. 3a). 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment induced floating THP-1 cells (Fig. 3b) to be attached to the surface of culture dish with flat amoeboid morphology and various cytoplasmic projections (Fig. 3c). They continued to adhere to the plate substrate with extension of their cytoplasmic projections by the subsequent treatment with any TECM tested (Fig. 3d). Then we checked the expression of CD163 and CD204, cytological markers for M2 macrophages used in immunohistochemical analyses of ESCCs, in THP-1 cells at each point of the present *in vivo* differentiation. Reverse transcription-PCR analysis demonstrated that THP-1 cells express *CD163* mRNA from the untreated state and the expression level did not alter significantly by treatment with TPA or TECMs (Fig. 3e, upper panel). On the other hand, although the expression level of *CD204* mRNA was extremely low in untreated as well as TPA treated THP-1, it was dramatically induced by each TECM (Fig. 3e, middle panel). Significant induction of CD204 but not CD163 by TECM exposure was also confirmed by counting the number of positive cells by immunofluorescence (Fig. 3f,g). Interestingly, exposure of TPA treated THP-1 cells with TECMs induced *VEGF-A* expression (Fig. 3h).

Discussion

Clinicopathological significances of TAMs in ESCCs have been reported using CD68 as single immunohistochemical marker and various counting philosophies. Initial studies paid attention to the infiltrating inflammatory cells including $CD68^+$ macrophages mainly in the stroma surrounding the tumor and concluded that local immunocyte accumulation around the tumor as a whole served as a favorable prognostic factor of

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Table 3. Relationship between clinicopathological features of human esophageal squamous cell carcinoma and overall survival

Variable	Univariate analysis			Multivariate analysis		
	Number	Median survival (years)	*P*-value†	HR	95% CI	*P*-value†
Age						
<65	29	6.349	0.239			
≥65	32	6.087				
Gender						
Male	49	5.781	0.459			
Female	12	6.835				
Depth of invasion‡						
T1	42	6.324	0.215			
T2 + T3	19	5.788				
Histological grade†						
HGIEN + WDSCC	12	NA	0.099			
MDSCC + PDSCC	49					
Lymphatic vessel invasion‡						
Negative	32	6.841	0.005*	5.827	0.652–52.069	0.115
Positive	29	5.475				
Blood vessel invasion‡						
Negative	38	6.738	0.003*	4.186	0.789–22.201	0.093
Positive	23	5.162				
Lymph node metastasis‡						
Negative	38	6.385	0.195			
Positive	23	5.887				
Stage§						
0 + I	35	6.351	0.266			
II + III + IV	26	5.966				
$CD204^+$ cells¶						
Low	30	6.473	0.211			
High	31	6.035				
$CD163^+$ cells¶						
Low	29	6.615	0.100			
High	32	5.931				
$CD68^+$ cells¶						
Low	29	6.443	0.113			
High	32	5.950				

†Overall survival was estimated by Kaplan–Meier method compared by log-rank test and a value of $*P < 0.05$ was considered statistically significant. ‡According to the Japanese Classification of Esophageal Cancer.[22] §According to the Tumor Node Metastasis (TNM) classification by Union for International Cancer Control.[23] ¶The median value of $CD204^+$, $CD163^+$ or $CD68^+$ macrophage number of cancer nest within the areas of microvessel count was used to divide the patients into high and low groups. CI, confidence interval; HGIEN, high grade intraepithelial neoplasia; HR, hazard ratio; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma; T1, tumor invades mucosa or submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia; WDSCC, well-differentiated squamous cell carcinoma.

ESCCs.[31,32] On the other hand, Ohta *et al.*[33] focused on the potential roles of TAMs in tumor angiogenesis in ESCC tissues. They found a significant correlation of densities of $CD68^+$ TAMs in tumor and intratumoral stroma with MVD, tumor expression of VEGF and clinicopathological factors favoring biological malignancy. Subsequent reports have confirmed the association of high density of $CD68^+$ TAMs with prognosis of ESCCs.[34,35] Two main points may be indicated to overcome discrepancies of the previous reports for the generalization of the understanding of precise roles of TAMs in ESCCs.

The first point is on the criteria for the counting macrophages in cancer tissue specimens. Guo *et al.*[35] counted $CD68^+$ TAMs and stratified as those localized to cancer stroma ($M\phi I_{stroma}$) and those in contact with cancer cells or penetrating into a cancer nest ($M\phi I_{cancer}$) and reported that total number of TAMs ($M\phi I_{stroma} + M\phi I_{cancer}$) was an independent prognostic factor for survival in ESCC patients. In the present study, as we generally observed significant numbers of TAMs in the cancer nest as well as in the stroma within the cancer area in ESCC tissues, we selected counting total number of TAMs at three hot spots within the area of MVD measurement to see the precise cancer-macrophage interaction and significant correlation with angiogenesis, clinicopathological factors and DFS especially in $CD204^+$ MϕC. Our present *in vitro* results demonstrating the induction of *VEGF-A* along with CD204 in TPA treated THP-1 cells by TECMs may suggest the role of $CD204^+$ TAMs in the angiogenesis of ESCC. The association of inflammatory cell infiltration including $CD68^+$ MϕC in the stroma surrounding tumor and favorable prognosis may reflect the local host defense reaction in ESCCs. Contrarily, it has been emphasized that TAMs in tumor stroma were significantly correlated with clinicopathological parameters and tumor angiogenesis in breast cancers or adenocarcinomas of the lung.[18,20] As TAMs in the cancer nest have been shown to be fewer in comparison with those in the cancer stroma in adenocarcinomas including invasive ductal breast cancers generally in comparison with ESCCs,[15–17] densities of TAMs in the stroma may reflect the overall biological effect on adenocarcinomas.

Second is concerning the marker for the detection of TAMs. Although CD68 is convenient for immunohistochemical visualization of macrophages in tissue sections, it can not

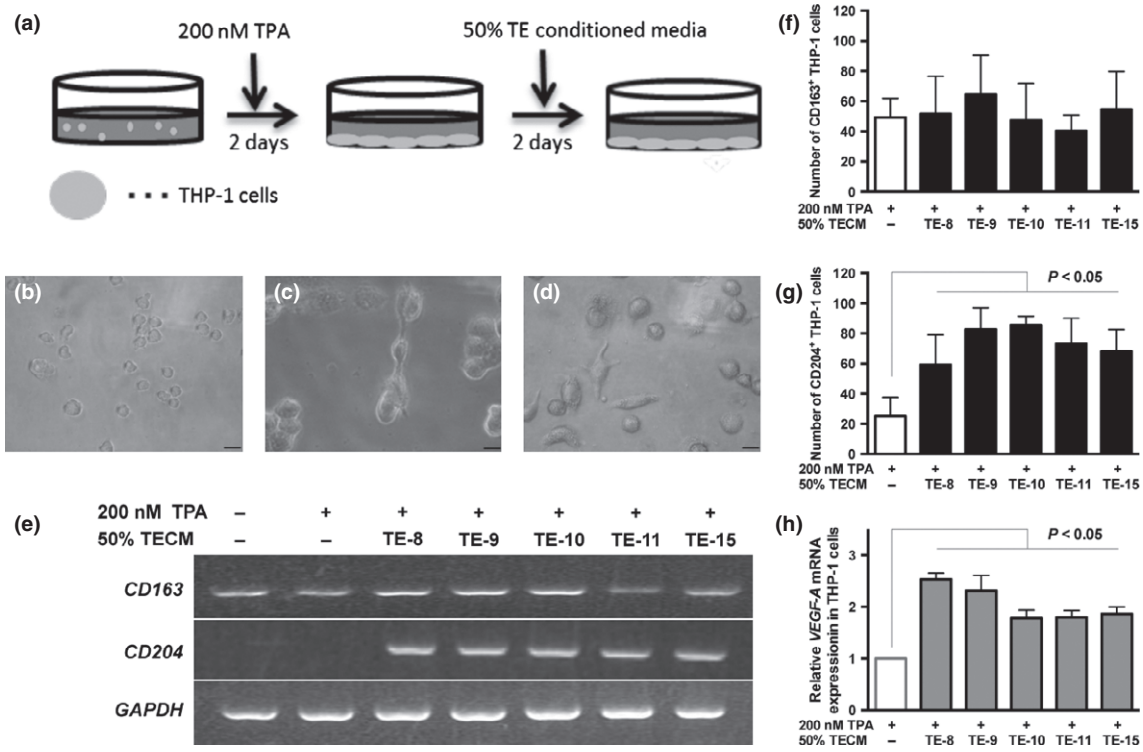

Fig. 3. Induction of CD204 and *VEGF-A* expression in 12-O-tetradecanoylphorbol-l3-acetate (TPA)-treated THP-1 human monocytic cell line by conditioned media of human esophageal cancer cell lines. (a) THP-1 cells were treated with 200 nM TPA for 2 days to induce macrophage-like differentiation, then exposed to 50% conditioned media of TE series esophageal cancer cell lines (TECMs) for 2 days. (b–d) Morphological changes of THP-1 by TPA and TECM treatment. Before treatment, THP-1 cells were round with diameter around 15 μm and did not attach to the surface of culture dish (b) After treatment with TPA, they adhered to the plate substrate with various cytoplasmic projections (c) They kept attaching with extended cytoplasmic projections with the consecutive exposure to the CM of TE-8 (d) Bar 20 μm. (e) *CD163* mRNA expression was observed in untreated, TPA-treated and TECM treated THP-1, while *CD204* expression was induced by TECM exposure with TPA-pretreatment. Reverse transcription-polymerase chain reaction analysis. *Glyceraldehyde 3-phosphate dehydrogenase* was used as a control. (f, g) Quantification of $CD163^+$ (f) and $CD204^+$ (g) THP-1 cells in culture by immunofluorescence exposed with TECMs after TPA-treatment. Mean numbers of $CD163^+$ or $CD204^+$ cells were compared with those of TPA-treated THP-1 cells. Significant induction of numbers of $CD204^+$ ($P < 0.05$) but not of $CD163^+$ THP-1 cells was observed after TECM exposure. Paired *t*-test. Bars, mean ± standard error of the mean (SEM). (h) Relative expression levels of *VEGF-A* mRNA determined by real time qRT-PCR in THP-1 cells exposed with TECMs after TPA-treatment. Each TECM demonstrated significant induction of *VEGF-A* expression ($P < 0.05$). Paired *t*-test. Bars mean ± SEM.

distinguish monocytes and subpopulations of macrophages or histiocytes.[36] CD204 and CD163 have been confirmed to be markers for the anti-inflammatory M2 macrophage phenotype useful for distinguishing the M2 skewed macrophage from the pro-inflammatory M1 macrophage.[13] Significance of $CD204^+$ and $CD163^+$ TAMs was simultaneously evaluated in limited human malignancies. In gliomas, both $CD204^+$ and $CD163^+$ TAMs was significantly correlated with the histological malignancy.[13] The numbers of $CD163^+$ and $CD204^+$ TAMs in borderline and malignant ovarian epithelial tumors were significantly higher than those in benign tumors.[14] High incidence of lymph node metastasis with increased lymphatic vessel density and a poor prognosis after surgery were detected in cases with high number of $CD163^+$ or $CD204^+$ TAMs in pancreatic cancers.[17] While, $CD163^+$, but not $CD204^+$, TAMs were correlated with nuclear grade, T classification and a poor prognosis of clear cell renal cell carcinomas (ccRCCs).[19] Results of our present study indicated that CD204 was a better marker for the TAM populations associating with tumor progression of ESCCs than CD163. Our findings may be supported by following reports on TAMs in SCCs of other organs. A high number of $CD204^+$ TAMs in lung SCCs was significantly correlated with MVD, advanced clinicopathological parameters and poor prognosis.[37] On the other hand, a significant difference was not observed in clinicopathological factors between high and low numbers of $CD163^+$ TAMs with the exception of patients age and survival in oral SCCs.[21]

This accumulating information may suggest that macrophages are heterogeneously activated into TAMs depending on the tumor type and organ specific microenvironment. Conditioned media (CM) of T98G human glioma cell line significantly upregulated expression of CD163 as well as CD204 along with induction of IL-10 and supression of tumor necrosis factor-α (TNF-α) and IL-12 in macrophages derived from peripheral blood monocytes *in vitro*.[13] In contrast, CM of ccRCC derived cell lines induced CD163 expression in cultured human macrophages.[19] Our present *in vitro* results demonstrated that CM of five different ESCC cell lines equally induced CD204 but not CD163 in TPA-treated THP-1 human monocytic leukemia cell line. These *in vitro* data correlated well with the significance of $CD163^+$ and/or $CD204^+$ TAMs in clinical gliomas, ccRCCs and ESCCs. According to the normal expression and induction signals, CD163 is exclusively expressed in moncytes and macrophages and its expression is inducible by glucocorticoid and IL-4, less so by IL-13 and IL-10.[38] On the other hand, CD204 is expressed in macrophages.[39] In THP-1 cells used in the present study, CD163 was only induced by combined treatment of phorbol-ester and glucocorticoid.[38] Therefore, it is speculated that humoral factor(s) from the cancer cells may induce tumor promoting $CD204^+$ TAMs making up a ESCC specific tumor microenvironment that contributed to the significant association

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of clinicopathological factors for biological malignancy and poor DFS.

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Disclosure Statement

The authors have no conflict of interest.

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