

# High density of CD204-positive macrophages predicts worse clinical prognosis in patients with breast cancer

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Recent studies have indicated the clinical significance of tumor-associated macrophages (TAM) in several malignant tumors including breast cancer. Although recent studies have focused on CD68-positive or CD163-positive TAM in breast cancer, no study has investigated the significance of CD204-positive TAM in breast cancer. We found that CD204 expression on macrophages was evaluated following stimulation with the conditioned medium (CM) of breast cancer cell lines. Paraffin sections of 149 breast cancer samples which were diagnosed as invasive ductal carcinoma were immunohistochemically analyzed for CD68, CD163 and CD204 expression. The results of analyses indicated that a high number of CD204-positive TAM was associated with worse clinical prognoses, including relapse-free survival, distant relapse-free survival and breast cancer-specific survival; however, neither the numbers of CD68-positive or CD163-positive TAM were associated with clinical courses. Of the clinicopathological factors investigated, estrogen receptor, Ki-67 index, hormone subtype, and histological grade were significantly related to the increased number of CD163-positive and CD204-positive TAM. These data indicate the clinical significance of CD204-positive TAM in breast cancer progression and CD204 is a marker for predicting clinical prognosis in breast cancer.

Invasive breast cancer is the most common cancer in women and accounts for 23% of all cancers in women.<sup>(1,2)</sup> Primary breast cancer exhibits many differences in morphology and in the expression of clinical biomarkers, such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth receptor 2 (HER2).<sup>(3,4)</sup> Such heterogeneity affects the complexity of pathological diagnosis and treatment protocols.

Tumor tissues, including breast cancer, consist not only of cancer cells but also of host-derived normal cells, such as lymphocytes, fibroblasts and macrophages.<sup>(5)</sup> Macrophages that infiltrate in tumor tissues are called tumor-associated macrophages (TAM), and activated TAM are known to secrete several kinds of pro-tumor molecules and EGFR ligands, including epidermal growth factor, heparin-binding EGF-like growth factor and oncostatin M.<sup>(6,7)</sup> A higher density of infiltrating CD68<sup>+</sup> macrophages was shown to correlate well with an increased number of vessels and a poor clinical course in patients with breast cancer.<sup>(8)</sup> Conversely, depletion of macrophages abrogated neovascularization in a murine model of breast cancer.<sup>(9)</sup> Since the study of Pollard *et al.*, which demonstrated that breast cancer metastasis was significantly abrogated in macrophage-deficient *Csf1op/Csf1op* (*op/op*) mice,<sup>(10)</sup> macrophage activation has been found to contribute to breast cancer progression and metastasis by means of many studies using gene engineered mice.<sup>(11,12)</sup>

The receptor CD204 (scavenger receptor class A [SR-A]) is specifically expressed on macrophages, and recent studies have demonstrated that high expression of CD204 is observed on M2-like pro-tumor macrophages.<sup>(13–15)</sup> M2-like macrophages have pro-tumor characteristics that involve the production of angiogenic factors and immunosuppressive molecules.<sup>(11–13)</sup> The progression and metastasis of ovarian cancer and pancreatic cancer were significantly inhibited in CD204-deficient mice, and CD204-deficient macrophages showed lower pro-tumor activity than that of CD204-positive macrophages.<sup>(16,17)</sup> Macrophages derived from CD204-deficient mice showed anti-tumor activity through the secretion of nitric oxide and interferon,<sup>(17)</sup> and targeting of CD204-positive macrophages abrogated ovarian cancer progression.<sup>(18)</sup> These findings indicated that CD204 is involved in the pro-tumor activation of macrophages.

In the present study, we found that CD204 expression on human monocyte-derived macrophages was significantly increased by co-culture with the conditioned medium (CM) of breast cancer cell lines, although other macrophage-specific receptors, such as CD163 and CD206, were little changed by such co-culture. This observation suggested the significance of CD204 expression on TAM in breast cancer. However, few studies have investigated CD204 in breast cancer. Therefore, we evaluated the relationship between CD204 expression on

TAM and clinicopathological factors in patients with invasive breast cancer.

## Materials and Methods

**Breast cancer cell lines and conditioned medium.** Three human breast cancer cell lines (MCF7, MDA-MB-453 and OCUB-M) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). All cells were cultured in DMEM/Ham's F-12 (Wako, Japan) with 10% FBS. The CM of all three cell lines was collected as previously described.<sup>(14)</sup>

**Macrophage culture.** Peripheral blood mononuclear cells (PBMC) were obtained from three healthy voluntary donors in accordance with protocols approved by the Kumamoto University Hospital Review Board. CD14<sup>+</sup> monocytes were isolated by using CD14-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). These monocytes were plated in 6-well plates ( $2 \times 10^5$ /well) and were cultured with 2% human serum, granulocyte macrophage-colony stimulating factor (1 ng/mL, GM-CSF, WAKO, Tokyo, Japan) and macrophage-colony stimulating factor (100 ng/mL, M-CSF, WAKO) for 7 days to induce differentiated macrophages.

**Western blot analysis.** The macrophages were stimulated with IL-4 (20 ng/mL, WAKO), IL-10 (20 ng/mL, WAKO) or CM of breast cancer cell lines (concentration: 50%) for 48 h. Then the macrophages were collected, and cellular proteins were solubilized in Tris buffer containing 2% SDS and 10% glycerol. The amount of protein was quantified using the bicinchoninic acid assay. Equal amounts of protein were then separated on SDS-PAGE, and were subsequently transferred to a polyvinylidene fluoride membrane. The following mouse antibodies were used for western blotting: anti-CD204 (SRA-E5; TransGenic, Kumamoto, Japan), anti-CD163 (PM-2K; TransGenic) and anti-CD206 (5C11; Acris Antibodies, San Diego, CA, USA). For immunoblotting of CD204, cell lysates were first pretreated with N-glycosidase (Roche, Basel, Switzerland). The membranes were re-blotted with an anti- $\beta$ -actin antibody as an internal control.

**Tissue samples.** Paraffin-embedded tumor samples from 149 patients who were diagnosed with invasive ductal carcinoma from 2001 to 2012 in Kumamoto University Hospital were examined. Cases were classified as Luminal A/B-like, Her 2-positive and triple negative groups by means of immunostaining, as described previously,<sup>(19)</sup> and cases of Luminal A-like and Luminal B-like were classified as Luminal-like group. All samples were obtained with informed consent from patients in accordance with protocols approved by the Kumamoto University Hospital Review Board. Tissue samples were fixed in 10% neutral buffered formalin and were embedded in paraffin using routine methods, and tissue sections of the largest cross-sectional area (one slide for each case) and no tissue microarray samples were used for this study.

**Immunohistochemistry.** Sections (3- $\mu$ m thick) obtained from paraffin-embedded tumor samples were immunohistochemically stained. The following mouse monoclonal antibodies were used: anti-CD204 (SRA-E5; TransGenic), anti-CD163 (10D6; Novocastra, Newcastle, UK), anti-CD68 (PG-M1; DAKO, Glostrup, Denmark) and anti-Ki-67 (MIB-1; DAKO, Glostrup, Denmark).<sup>(20)</sup> After the samples were reacted with these first antibodies, the samples were incubated with HRP-labeled secondary anti-mouse antibody (Nichirei, Tokyo, Japan). The reaction was visualized using the Diaminobenzidine (DAB) system (Nichirei). Normal mouse

immunoglobulin (DAKO) was used as a negative control and no signal was observed in these sections. CD68-, CD163- and CD204-positive cells were counted in four randomly selected areas of a high power field ( $\times 200$ ) of a microscope by two pathologists (S.T. and M.Y.) who were blinded to information about the patients' backgrounds or their prognosis. The data of the Ki-67 labeling index was previously counted by our research group.<sup>(21,22)</sup> Double immunostaining of CD204, CD68, CD163 and Ki-67 was performed as previously described.<sup>(14)</sup> In brief, the sections were reacted with anti-Ki-67, CD163 or CD204 antibodies and visualized with DAB. After the sections were washed with citrate buffer (pH 2.2), they were reacted with anti-CD68 or CD204 antibodies and visualized with HistoGreen (Linaris, Heiderberg, Germany).

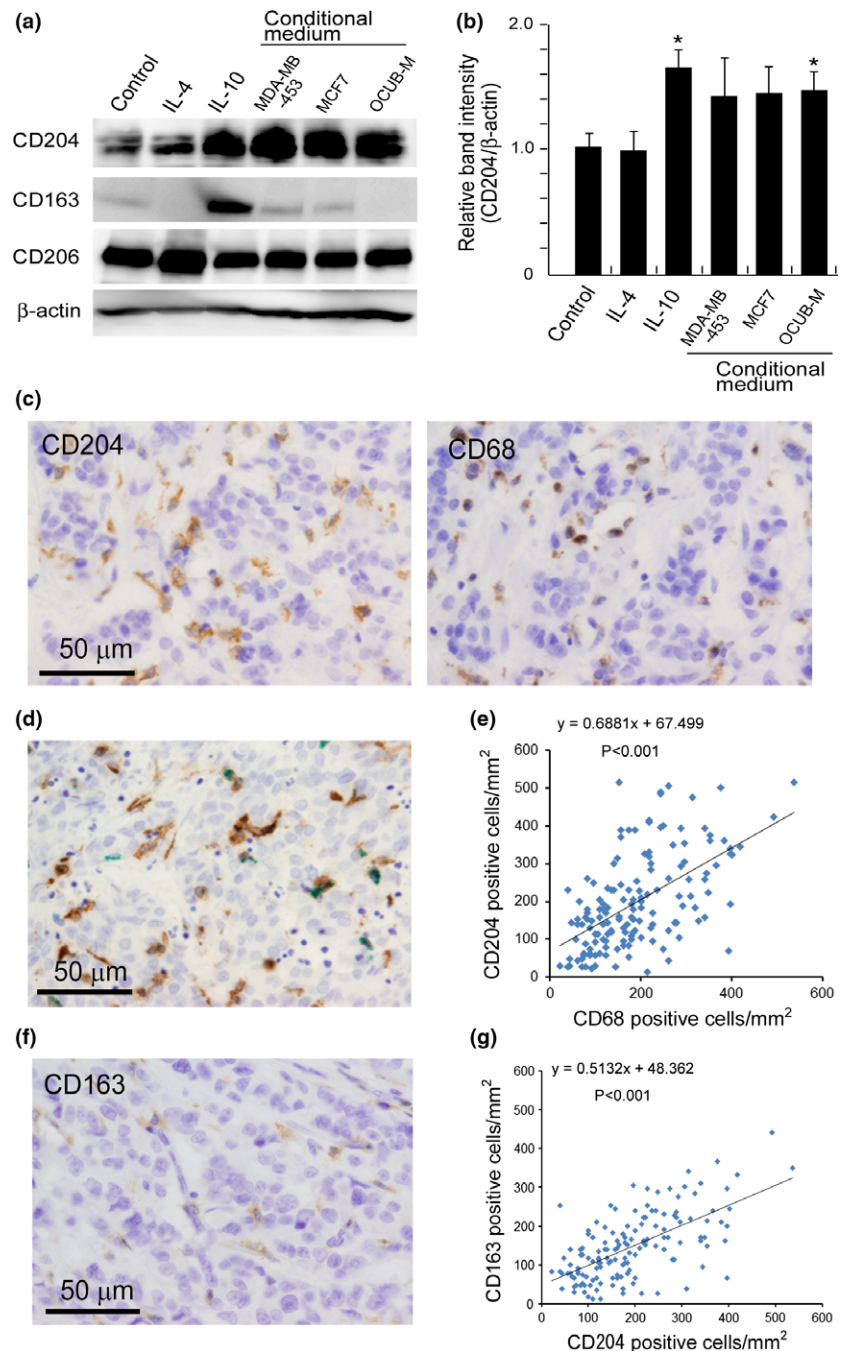
**Statistical analysis.** Statistical analysis was carried out by using JMP10 (SAS Institute, Chicago, IL, USA). A value of  $P < 0.05$  was considered statistically significant.

## Results

**Conditioned medium of breast cancer cell lines upregulated CD204 expression on macrophages.** We first tested whether incubation of macrophages with the CM of breast cancer cell lines could influence the expression of M2 macrophage markers such as CD163, CD204 and CD206. Western blot analysis indicated that CD204 expression was upregulated by the CM of all three breast cancer cell lines. The most significant change was induced by the CM of OCUB-M cells ( $P = 0.014$ ; Fig. 1a,b,  $n = 3$ ). Neither CD163 nor CD206 expression was influenced by these CM, although IL-4 and IL-10 did induce CD206 and CD163, respectively (Fig. 1a).

**Density of CD204-positive macrophages was higher than that of CD163-positive macrophages in breast cancer tissues.** CD68 is a well-established pan-macrophage marker, and some studies have shown that a high density of CD68-positive macrophages correlates well with poor clinical prognosis.<sup>(8)</sup> Therefore, we next investigated the relationship between CD68-positive and CD204-positive macrophages using invasive breast cancer cases. Immunostaining of CD68 and CD204 was performed on serial sections of breast cancer tissues and the density of CD68-positive and CD204-positive macrophages were counted in the same area (Fig. 1c). Double-immunostaining showed that some of the CD204-positive macrophages expressed CD68, whereas others express low levels of macrophages (Fig. 1d). The densities of CD68-positive and CD204-positive macrophages were well correlated; however, there seemed to be a higher density of CD204-positive macrophages (mean: 205 cells/mm<sup>2</sup>) than of CD68-positive macrophages (mean: 191 cells/mm<sup>2</sup>) (Fig. 1e). No increase in CD163 was observed on macrophages following incubation with breast cancer cell CM (Fig. 1a); however, CD163 expression on macrophages that have infiltrated breast cancer tissues has been reported.<sup>(21)</sup> We next investigated the correlation between CD204 expression and CD163 expression in all cases. In immunostaining, the signal intensity of CD163 expression was weak compared with that of CD204 expression (Fig. 1f), and the density of CD163-positive macrophages (mean: 146 cells/mm<sup>2</sup>) was lower than that of CD204-positive macrophages, as shown in Figure 1g.

**Higher density of CD204-positive macrophages was associated with triple-negative cancer cells and a higher Ki-67 labeling index.** We next investigated the significance of CD68-, CD163- and CD204-positive macrophages, as assessed by immunohistochemistry. After the density of macrophages was



**Fig. 1.** The expression of CD163, CD204 and CD206 on macrophages. (a) Human monocyte-derived macrophages were not stimulated (control) or were stimulated with IL-4, IL-10 or the conditioned media (CM) of three breast cancer cell lines, and the protein expression of CD204, CD163 and CD206 was evaluated using western blot analysis. β-actin was assayed as a loading control. (b) The band intensity of CD204 and β-actin in (a) was evaluated using Image J software (<http://imagej.nih.gov/ij/>). The calculated data were statistically analyzed using Student's *t*-test. \**P*-value < 0.05. (c) Representative immunohistochemical staining of CD68 and CD204 in the same area of a breast cancer sample, and (d) double-immunostaining of CD68 (green) and CD204 (brown) are shown. (e) The relationship between the density of CD68-positive and CD204-positive macrophages was evaluated using Spearman's correlation test. (f) Immunostaining of CD163 in the same area of the breast cancer sample shown in (c) is presented. (g) The relationship between the density of CD163-positive and CD204-positive macrophages was evaluated using Spearman's correlation test.

counted, the cases were divided into two groups (High and Low), as shown in Table 1. Statistical analysis showed that higher densities of CD68-, CD163- and CD204-positive macrophages were significantly associated with higher histological grade ( $P < 0.01$ ;  $P = 0.01$ ;  $P < 0.01$ , respectively) or ER-negative status (CD204;  $P = 0.03$ ; CD163;  $P = 0.04$ , respectively). Higher density of CD204-positive macrophages was observed in triple negative patient groups than in the Luminal-like group. The densities of CD68-, CD163- and CD204-positive macrophages were not correlated with age, menopausal status, tumor size or lymph node metastasis. Because a higher density of Ki-67-positive cancer cells was preferentially detected in the high CD204 group (Fig. 2a), we then tested the relationship of the densities of CD68-, CD163- and CD204-positive macrophages to the Ki-67 labeling index. Double-immunostaining of CD204

and Ki-67 showed that no Ki-67-positive macrophages were observed in cancer tissues (Fig. 2b). Higher densities of CD163-positive and CD204-positive macrophages were interestingly associated with higher Ki-67 labeling index (Fig. 2c,d and Table 1); however, there was no significant association between the number of CD68-positive macrophages and Ki-67 labeling index (Fig. 2e). The density of CD204-positive macrophages was more closely related to the Ki-67 labeling index than that of CD163-positive macrophages.

**Higher density of CD204-positive macrophages was associated with poor clinical prognosis.** Next, the correlation between the densities of CD68-, CD163- and CD204-positive macrophages and clinical prognosis was statistically evaluated. A higher density of CD204-positive macrophages was significantly associated with poor clinical prognosis, including relapse-free

**Table 1.** Correlations between the density of tumor-associated macrophages (TAM) and clinicopathological factors

Clinicopathological feature		The density of CD204-positive TAM (/mm <sup>2</sup> )				The density of CD163-positive TAM (/mm <sup>2</sup> )			The density of CD68-positive TAM (/mm <sup>2</sup> )		
		N	≤200	>200	P-value	≤145	>145	P-value	≤190	>190	P-value
Age	<50	36	21	15	0.49	17	19	0.16	21	15	0.47
	≥50	113	66	47		64	49		65	48	
Menopause	Pre-	43	24	19	0.34	22	21	0.31	24	19	0.38
	Post-	106	63	43		59	47		62	44	
Tumor size	<2 cm	62	35	27	0.33	35	27	0.39	33	29	0.13
	≥2 cm	85	51	34		46	39		53	32	
LN metastasis	Negative	95	59	36	0.13	54	41	0.25	55	40	0.47
	Positive	53	28	25		27	26		31	22	
Histological grade	1	45	34	11	<0.01†	30	15	0.01†	33	12	<0.01†
	2	65	38	27		35	30		35	30	
	3	34	10	24		11	23		13	21	
ER	Negative	34	15	19	0.03†	14	20	0.04†	17	17	0.15
	Positive	115	72	43		67	48		69	46	
PgR	Negative	59	30	29	0.06	30	29	0.24	33	26	0.34
	Positive	90	57	33		51	39		53	37	
HER2	Negative	126	73	53	0.39	69	57	0.41	72	54	0.37
	Positive	23	14	9		12	11		14	9	
Ki67	<20%	47	36	11	<0.01†	32	15	0.01†	29	18	0.23
	≥20%	98	48	50		46	52		54	44	
Subtype	Luminal	108	68	40	<0.01†‡	63	45	0.14	64	44	0.49
	HER2	23	14	9		12	11		14	9	
	TN	18	5	13		6	12		8	10	

TN, triple negative. †Statistically significant. Statistical analysis was performed by  $\chi^2$ -test and Kruskal–Wallis *H*-test. ‡Compared with triple negative group.

survival (RFS), distant RFS (DRFS) and breast cancer-specific survival (BCSS) (RFS,  $P = 0.001$ ; DRFS,  $P < 0.001$ ; BCSS,  $P < 0.001$ , respectively; Fig. 3a, Tables 2–4). The ratio of CD204-positive macrophages (CD204<sup>+</sup> cells/CD68<sup>+</sup> cells) was also associated with DRFS and BCSS; however, more significant association was observed in the density of CD204-positive macrophages and clinical course. In contrast, the densities of CD68-positive and CD163-positive macrophages had little association with the clinical course (Tables 2–4). Multivariate analysis showed that the density of CD204-positive macrophages was an independent poor prognostic factor for RFS, DRFS and BCSS (RFS,  $P = 0.006$ ; DRFS,  $P = 0.001$ ; BCSS,  $P < 0.001$ , respectively; Tables 2–4). Because the cases shown in Figure 3a are comprised of three breast cancer subtypes (Luminal-like, Her2-positive and Triple negative), a similar analysis was performed for the 108 Luminal-like cases. In these cases, the density of CD204-positive macrophages was also significantly correlated with RFS and DRFS; however, there was no significant association between the density of CD204-positive macrophages and BCSS (RFS,  $P = 0.045$ ; DRFS,  $P = 0.013$ ; BCSS,  $P = 0.10$ , respectively; Fig. 3b). Although similar associations with survival rate were found in triple negative cases, there was no statistically significant association between the density of CD204-positive macrophages and clinical prognosis because of the low number of cases (data not shown).

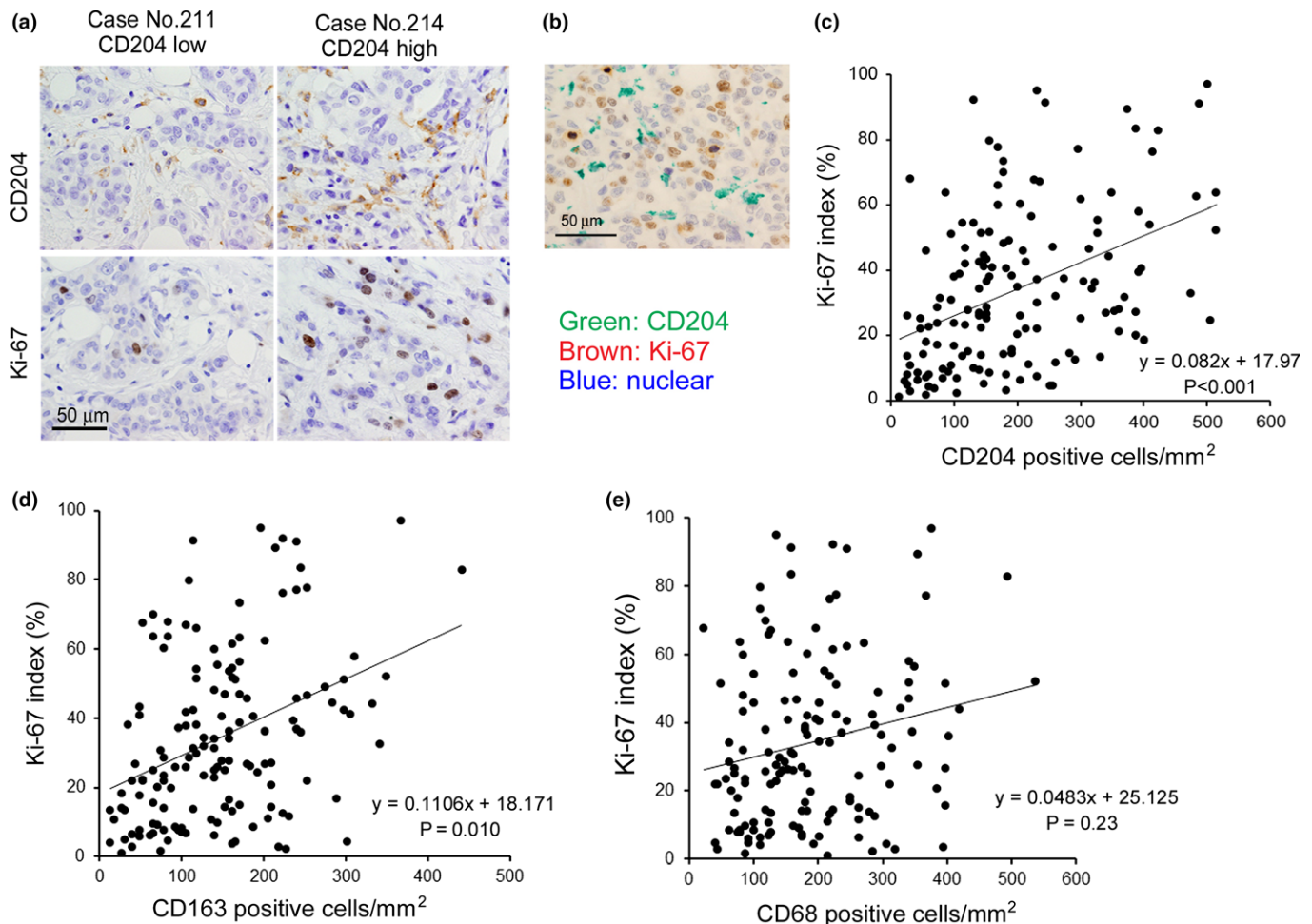
## Discussion

In the present study, we report a significant association between CD204-positive macrophages and clinicopathological factors in patients with invasive breast cancer. Since the 1990s

there have been several studies that have described a significant correlation between CD68-positive macrophages and the clinical course of patients with invasive breast cancer.<sup>(8)</sup> Although CD204 has been reported to be a marker for pro-tumor macrophages in esophageal cancer, pancreatic cancer, glioma and lung cancers,<sup>(14,15,23–25)</sup> no study of the relationship between CD204 and breast cancer samples has been published. Therefore, this study is the first report to describe the significance of CD204 in breast cancer.

In the present study, we first showed that CM of breast cancer cell lines significantly increased CD204 expression on macrophages; however, we have never identified the cancer-cell derived molecules associated with upregulation of CD204. Breast cancer cells are known to secrete colony stimulating factor 1/2 and monocyte chemoattractant protein-1, which is closely associated with macrophage activation.<sup>(26)</sup> Cyr61 is known to be secreted from breast cancer cells,<sup>(27)</sup> and cancer cell-derived Cyr61 increased CD204 expression on macrophages via activation of MEK/ERK pathway.<sup>(28)</sup> These molecules are suggested to be involved in CD204 overexpression in TAM.

CD68 is a widely-used marker for pan-macrophages, as described above. Our study demonstrated that the densities of CD68-positive and CD204-positive macrophages were very similar but the density of CD204-positive macrophages was slightly higher than that of CD68-positive macrophages. A similar observation was seen in our previous research related to human glioma.<sup>(14,29)</sup> Because CD68 is a lysosomal-associated membrane protein, and not a cell membrane protein, this molecule seemed to be downregulated in some macrophages, which were possibly with less phagocytic features. It is also interesting to note that the Ki-67 index of tumor cells



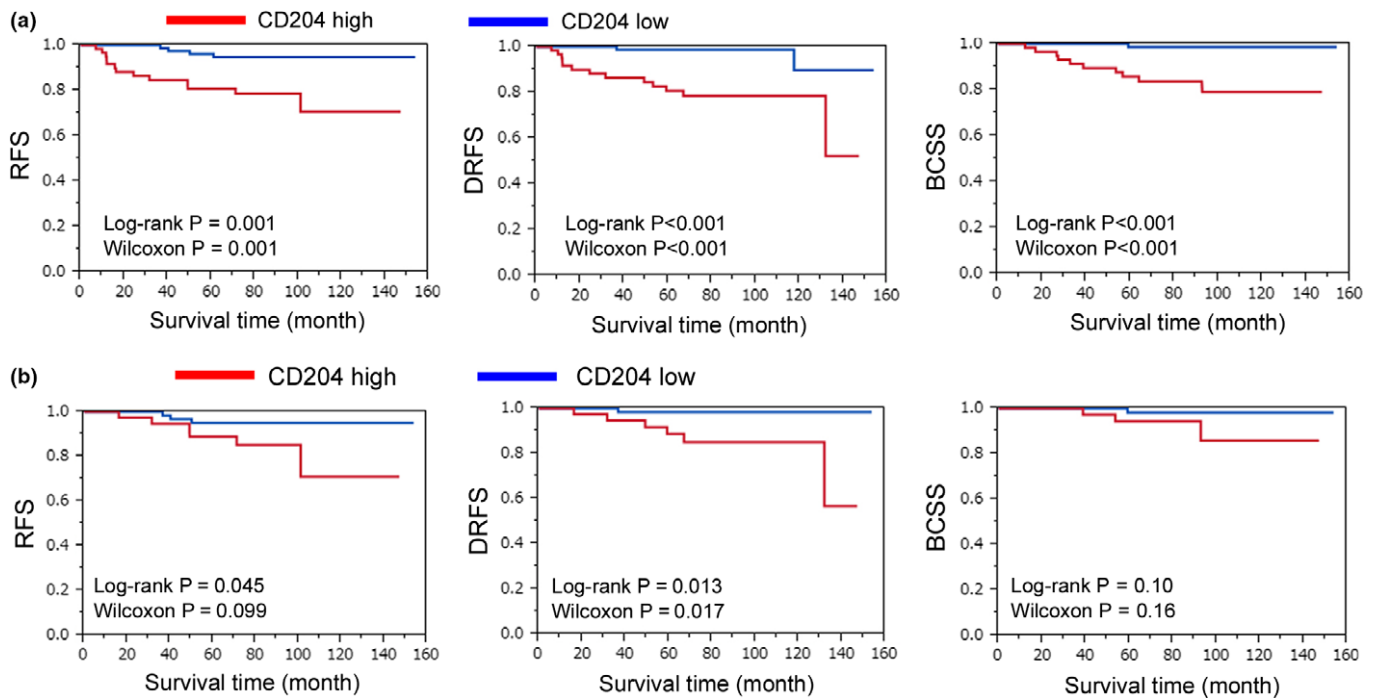
**Fig. 2.** Correlation between the density of CD204-positive macrophages and tumor cell proliferation. (a) The immunostaining data of two cases (a high CD204 case and a low CD204 case) are shown. Serial sections of cancer tissues were stained with the anti-CD204 antibody and the anti-Ki-67 antibody, and pictures of the same area are shown. (b) Double immunostaining of CD204 and Ki-67 in a representative breast cancer sample was performed in a high CD204 case. (c) The correlation between the density of CD204-positive macrophages and the Ki-67 index was evaluated using Spearman's correlation test. The correlations between the density of CD163-positive macrophages and the Ki-67 index (d) and between the density of CD68-positive macrophages and the Ki-67 index (e) were tested using Spearman's correlation test.

was significantly associated with the density of CD204-positive macrophages, but not with the density of CD68-positive macrophages.

CD163 has also recently been found to be a marker for pro-tumor macrophages in several kinds of malignant tumors,<sup>(13,30)</sup> and some studies have demonstrated the significance of CD163-positive macrophages in invasive breast cancer.<sup>(31–33)</sup> Macrophage expression of CD163 was previously shown to be induced by the CM of MDA-MB231 cells, but not by the CM of MCF-7 or T47D cells.<sup>(31)</sup> MDA-MB231 cells have been shown to have more stem-cell like properties or mesenchymal differentiation than MCF-7 cells.<sup>(34)</sup> Cancer cells that display high stem-cell properties were shown to significantly induce immunosuppressive M2 macrophages via soluble factors, including M-CSF, TGF- $\beta$  and MIC-1.<sup>(35)</sup> The fact that no CD163 upregulation was observed in our study of macrophage culture with the CM of MCF7, MDA-MB-453 and OCUB-M cells may indicate the low stem-cell properties of these cell lines. In the present study, we showed that the number of CD163-positive macrophages in breast cancer samples was lower than that of CD204-positive macrophages in the data shown in Figure 1. These observations are similar to those of

our previous study.<sup>(36)</sup> These findings might indicate that CD163 is expressed on more specialized macrophages than CD204 in breast cancer.

The Ki-67 index is a well-known marker for the prediction of clinical prognosis in patients with a malignant tumor, including breast cancer.<sup>(37,38)</sup> In the present study, we found a positive correlation between the density of CD163-positive and CD204-positive macrophages and the Ki-67 index. A similar correlation was previously seen in glioma in which Stat3 activation was significantly involved in the cell–cell interaction between glioma cells and macrophages.<sup>(39)</sup> Cancer cell-derived soluble molecules, including annexin A1, heat shock proteins, fibronectin and galectin-1, were suggested to bind and stimulate CD204.<sup>(16)</sup> Therefore, we tried to investigate the detailed mechanisms of the cell–cell interaction between breast cancer cells and macrophages using cultured cell lines and human macrophages. However, no significant cell–cell interactions between macrophages and breast cancer cell lines were observed (unpublished data). Further studies are necessary to uncover the detailed functions of CD204-positive macrophages in the breast cancer microenvironment.



**Fig. 3.** Correlation between the density of CD204-positive macrophages and clinical prognosis. (a,b) Kaplan–Meier analysis of relapse-free survival (RFS), distant relapse-free survival (DRFS) and breast cancer-specific survival (BCSS) of the CD204 low density (CD204 low) and high density (CD204 high) subgroups of patients with CD204-positive macrophages. A total of 149 patients with total invasive ductal carcinoma (a) and 108 patients with Luminal-like type (b) were analyzed.

**Table 2.** Univariate analysis and multivariate analysis in relapse free survival

Variables	Reference	Univariate analysis					Multivariate analysis			
		P-value	HR	95% CI		P-value	HR	95% CI		
				Lower	Upper			Lower	Upper	
CD204	Low vs high	Low	0.001†	5.35	1.89	19.01	0.006†	4.61	1.51	17.29
CD163	Low vs high	Low	0.892	1.07	0.40	2.80				
CD68	Low vs high	Low	0.599	1.29	0.48	3.38				
CD204/CD68	Low vs high	Low	0.054	2.56	0.98	7.05				
CD163/CD68	Low vs high	Low	0.342	0.61	0.19	1.65				
Ki67	>20% vs ≤20%	≤20%	0.147	2.34	0.76	10.15				
Age	<50, ≥50	≤50	0.629	0.62	0.23	1.83				
Menopause	Pre vs post	Pre	0.365	0.63	0.24	1.75				
Tumor size	≤2 cm vs >2 cm	≤2 cm	0.318	1.69	0.61	5.36				
LN metastasis	Positive vs negative	Negative	0.247	1.79	0.65	4.87				
HG	1,2 vs 3	1 + 2	0.035†	2.89	1.08	7.58	0.978	1.02	0.29	3.36
ER	Negative vs positive	Negative	0.011†	0.27	0.10	0.74	0.049†	0.31	0.10	0.99
PgR	Negative vs positive	Negative	0.476	0.70	0.26	1.88				
Her2	Negative vs positive	Negative	0.883	1.1	0.25	3.37				

†Statistically significant.

In this study, we demonstrated that a high density of CD204-positive macrophages was associated with worse clinical course, including DRFS, RFS and BCSS, in patients with invasive breast cancer. A high Ki-67 index was also correlated with worse DRFS, but not with RFS and BCSS in this study. Notably, in multivariate analysis a high density of CD204-positive macrophages was the only independent factor associated with clinical course, including DRFS, RFS and BCSS. This result indicated that the density of CD204-positive

macrophages is a useful marker for prediction of clinical prognosis and might be a better marker than the Ki-67 index. In preliminary data, we tested whether macrophage-derived factors influenced the cancer cell proliferation and invasion; however, no significant involvement was observed (unpublished data).

The present study revealed that higher expression of CD204 is related to worse clinical prognosis in breast cancer. Although the detailed mechanism of CD204 upregulation

**Table 3. Univariate analysis and multivariate analysis in distant relapse free survival**

Variables	Reference	Univariate analysis				Multivariate analysis				
		P-value	HR	95% CI		P-value	HR	95% CI		
				Lower	Upper			Lower	Upper	
CD204	Low vs high	Low	<0.001†	10.24	2.82	65.54	0.001†	8.57	2.12	59.13
CD163	Low vs high	Low	0.667	1.25	0.44	3.59				
CD68	Low vs high	Low	0.728	1.20	0.42	3.34				
CD204/CD68	Low vs high	Low	0.004†	4.65	1.58	16.87				
CD163/CD68	Low vs high	Low	0.504	0.69	0.21	1.97				
Ki67	>20% vs ≤20%	≤20%	0.021†	6.31	1.25	114.65	0.567	1.83	0.28	35.69
Age	<50, ≥50	<50	0.944	1.04	0.35	3.80				
Menopause	Pre vs post	Pre	0.907	0.93	0.33	3.03				
Tumor size	≤2 cm vs >2 cm	≤2 cm	0.158	2.40	0.72	10.76				
LN metastasis	Positive vs negative	Negative	0.141	2.22	0.76	6.80				
HG	1,2 vs 3	1 + 2	0.007†	4.25	1.50	12.8	0.946	0.96	0.27	3.58
ER	Negative vs positive	Negative	0.001†	0.18	0.06	0.52	0.014†	0.21	0.05	0.73
PgR	Negative vs positive	Negative	0.051	0.35	0.11	1.01				
Her2	Negative vs positive	Negative	0.898	1.09	0.24	3.49				

†Statistically significant.

**Table 4. Univariate analysis and multivariate analysis in breast cancer specific survival**

Variables	Reference	Univariate analysis				Multivariate analysis				
		P-value	HR	95% CI		P-value	HR	95% CI		
				Lower	Upper			Lower	Upper	
CD204	Low vs high	Low	<0.001†	16.00	3.06	293.66	<0.001†	15.87	2.74	302.04
CD163	Low vs high	Low	0.989	1.01	0.29	3.35				
CD68	Low vs high	Low	0.750	0.82	0.21	2.72				
CD204/CD68	Low vs high	Low	0.002†	8.04	2.07	52.76				
CD163/CD68	Low vs high	Low	0.366	0.55	0.12	1.92				
Ki67	>20% vs ≤20%	≤20%	0.060	4.94	0.94	90.56				
Age	<50, ≥50	<50	0.551	1.56	0.40	10.24				
Menopause	Pre vs post	Pre	0.777	1.21	0.34	5.52				
Tumor size	≤2 cm vs >2 cm	≤2 cm	0.085	3.33	0.85	21.88				
LN metastasis	Positive vs negative	Negative	0.261	1.97	0.54	6.85				
HG	1,2 vs 3	1 + 2	0.031†	3.75	1.13	13.04	0.449	0.57	0.14	2.53
ER	Negative vs positive	Negative	<0.001†	0.09	0.02	0.31	0.001†	0.09	0.02	0.39
PgR	Negative vs positive	Negative	0.105	0.37	0.09	1.23				
Her2	Negative vs positive	Negative	0.964	1.04	0.15	4.03				

†Statistically significant.

has never been clarified, it is reported that hyaluronan (HA) is one of the breast cancer-derived factors that upregulates M2 marker expression, such as CD204, CD206, IL-10 and TGF- $\beta$ , via STAT3 activation.<sup>(40)</sup> In a murine ovarian cancer model, immunotherapy with anti-CD204 immunotoxin inhibited tumor progression.<sup>(7)</sup> In murine lymphoma, CD204<sup>-/-</sup> macrophages inhibited tumor growth by means of producing nitric oxide and interferon- $\gamma$ , which are M1 markers.<sup>(17)</sup> Thus, CD204 might be a target for anti-breast cancer therapy.

In conclusion, we have shown that the density of CD204-positive macrophages is significantly related to the Ki-67 index and to worse clinical prognosis in patients with breast cancer. Thus, in addition to Ki-67, CD204 is also considered to be useful for the prediction of clinical prognosis. The fact that

CD204 expression was upregulated by the conditioned medium of breast cancer cell lines further indicated the significance of CD204 in macrophage activation. However, further studies are necessary to uncover the detailed mechanisms of CD204-related macrophage activation.

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#### Disclosure statement

The authors have no conflicts of interest to declare.

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