ANTICANCER RESEARCH *41*: 4857-4865 (2021)
doi:10.21873/anticanres.15299

Infiltration of CD204-overexpressing Macrophages Contributes to the Progression of Stage II and III Colorectal Cancer

YOICHIRO TADA[1], YOSHIAKI MATSUMI[2], KAZUSHI HARA[1], WATARU MIYAUCHI[1], KEN SUGESAWA[1], CHIHIRO UEJIMA[1], AKIMITSU TANIO[1], KYOICHI KIHARA[1], MANABU YAMAMOTO[1], SHUICHI TAKANO[1], TERUHISA SAKAMOTO[1], YUJI NAKAYAMA[3], TOSHIMICHI HASEGAWA[1] and YOSHIYUKI FUJIWARA[1]

[1]Division of Gastrointestinal and Pediatric Surgery, Department of Surgery, School of Medicine, Faculty of Medicine, Tottori University, Yonago, Japan;
[2]Division of Chemical Biology, Technical Department, Tottori University, Yonago, Japan;
[3]Division of Radioisotope Science, Research Initiative Center, Organization for Research Initiative and Promotion, Tottori University, Yonago, Japan

Abstract. *Background/Aim: M1 macrophages have antitumour effects, while M2 macrophages promote tumour proliferation and invasion. The clinical significance of the M2-specific marker CD204 has not been elucidated in colorectal cancer (CRC). We investigated the prognostic significance of CD204- and CD68-positivity in specimens from patients with CRC and examined the effects of M2 polarized-macrophages on the proliferative and invasive potentials of CRC cell lines in vitro. Materials and Methods: Surgical tumour specimens from 206 patients with Stage II and III CRC were examined by immunohistochemistry. Proliferation and invasion assays and flow cytometry were used to investigate CD204 expression in macrophages co-cultured with three CRC cell lines. Results: Infiltration of CD204-positive cells was significantly associated with shorter overall survival and relapse-free survival; no association was observed for CD68. M2-polarized macrophages significantly promoted proliferation and invasion of CRC cells. Conclusion: Higher infiltration of CD204-positive macrophages into the tumour-microenvironment might be prognostically important in CRC.*

Colorectal cancer (CRC) is the third most common malignancy in the world, with an increasing burden (1). The prognosis of CRC has been improved by developments in operative techniques and discoveries of effective antitumour drugs; however, it is still unsatisfactory. Recent research on the tumour microenvironment (TME) has increased our understanding of tumour immunity and led to the discovery of new therapeutic targets (2). The TME consists of lymphocytes, fibroblasts, endothelial cells and macrophages; of these, it has become clear that macrophages play important roles in tumour development. The tumour-associated macrophages (TAMs) (3) specifically can be classified into two phenotypes: classically activated M1 macrophages and alternatively activated M2 macrophages. M1 macrophages have functions that include killing tumour cells, promoting inflammation and inhibiting tumour growth, whereas M2 macrophages function in remodelling tissues, suppressing adaptive immunity, and promoting angiogenesis and tumour growth (4, 5).

High numbers of M2 macrophages have been correlated with poor prognosis of multiple cancer types (6-13). A variety of macrophage surface markers have been applied in different evaluations; for example, CD163, CD206 and CD204 are commonly used to identify M2 macrophages. Reports have shown correlations between high expression of CD163 or CD206 with poor prognosis of CRC (14, 15); however, none have found a similar correlation for CD204. By contrast, high expression of CD68, a pan-macrophage marker, has been correlated with good prognosis of CRC (16, 17). Research on the role of CD68 expression in both M1 and M2 macrophages, which have antagonistic functions, has yielded inconclusive results.

In the present study, we investigated associations between the expression of the macrophage markers CD204 and CD68 and prognosis using immunohistochemistry (IHC) of curatively resected CRC specimens. We then further assessed whether polarized M2 macrophages contributed to cell growth and invasive activities of CRC cells using an *in vitro* co-culture system.

Correspondence to: Yoichiro Tada, Division of Gastrointestinal and Pediatric Surgery, Faculty of Medicine, Tottori University, 36-1 Nishi-cho, Yonago 683-8504, Japan. Tel: +81 859386567, Fax: +81 859386569, e-mail: yoichirotada@yahoo.co.jp

Key Words: CD204, CD68, M2 macrophage, colorectal cancer.

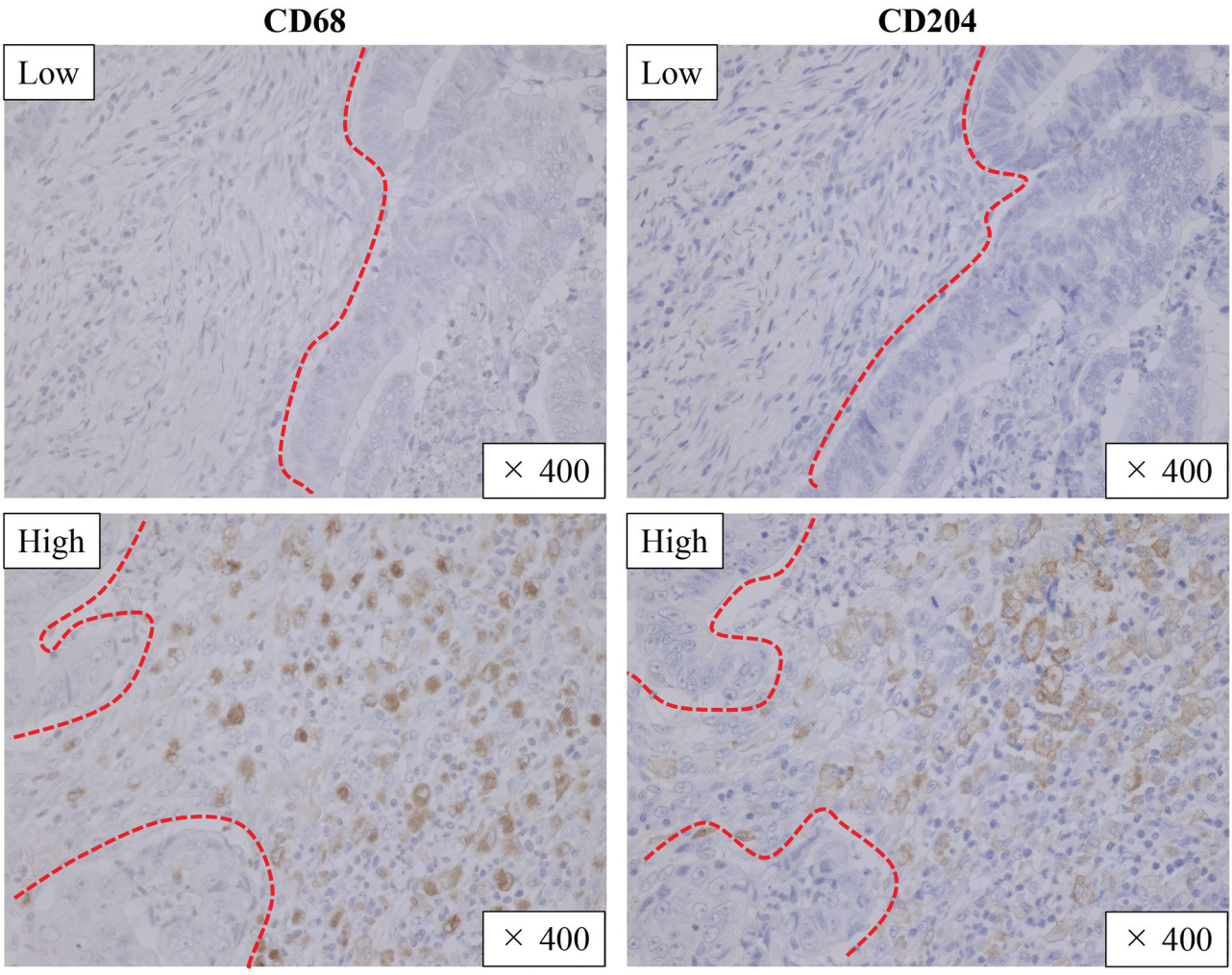

Figure 1. *Immunohistochemistry of CD68 and CD204 in colorectal cancer. Representative specimens of the 206 subjects, which were classified into low- and high-expression groups, are shown. Red dotted lines indicate the tumour invasive front. 400× magnification.*

Materials and Methods

Patients and tumour specimens. Specimens were obtained from 239 consecutive patients who underwent curative resection of CRC at the Tottori University Hospital between January 2007 and December 2012. After excluding 10 patients who had undergone emergency surgery, 6 who were lost to follow-up and 17 who were classified as Stage I or IV according to the 8th Edition of the Union for International Cancer Control-TNM classification, 206 CRC patients were finally enrolled. Patients were followed every 3 months until death or December 2017 (5 years after the last operation). Overall survival (OS) and relapse-free survival (RFS) were defined as the time from the date of surgery to the date of death and the date of relapse, respectively. We obtained informed consent from all patients and the study was approved by the institutional review board of Tottori University (19A052).

IHC staining. Sections (4-μm thickness) were examined using IHC. The sections were deparaffinized, and antigens retrieved by microwave oven heating in citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked by immersing the slides in 3% hydrogen peroxide in methanol for 30 min and 10% Block-Ace (DS Pharma Biomedical, Osaka, Japan) for 30 min, followed by incubation with primary antibodies against CD204 (1:500; SRA-E5; TransGenic, Kumamoto, Japan) or CD68 (1:100; PG-M1; DAKO, Tokyo, Japan) overnight at 4˚C. The incubated sections were subsequently exposed to the second antibody, EnVision™ +Dual Link System-HRP (DAKO), visualized with ImmPACT DAB (Vector Laboratories, Burlingame, CA, USA), and then counterstained with haematoxylin. Two independent investigators, who were given no information about the patients, evaluated the expression grades of CD204 and CD68 at the invasive tumour front. The levels of expression were initially classified as very low, low, high and very high, and ultimately divided into two groups: lower (very low and low expression) and higher (high and very high expression) (Figure 1).

Cell lines. The human CRC cell lines DLD-1 and SW620 were purchased from the American Type Culture Collection (Rockville,

MD, USA) and LoVo was purchased from Riken BNL Research Center (Tsukuba, Japan). All cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Cosmo Bio Co., Ltd, Tokyo, Japan), 100 U/ml penicillin and 100 U/ml streptomycin (PS) (FUJIFILM Wako Pure Chemical Co., Japan), at 37˚C in a humidified atmosphere with 5% CO_2.

M2 macrophage culture. Human monocytes were isolated from whole blood of healthy volunteers using the EasySep Direct Human Monocyte Isolation Kit (STEMCELL Technologies Inc., Vancouver, BC, Canada). Macrophages were obtained using ImmunoCult-SF Macrophage medium (STEMCELL Technologies Inc), according to the manufacturer's instructions. Briefly, on day 0, isolated monocytes were plated at 4×10^5 cells/ml in ImmunoCult-SF Macrophage medium supplemented with 50 ng/ml macrophage colony stimulating factor. On day 4, half of the original medium volume was added. On day 6, 10 ng/ml interleukin-4 was added only to cultures used to obtain activated macrophages, which were harvested after 2 additional days of culture. All cultures were grown in an incubator at 37˚C in a humidified atmosphere with 5% CO_2. All other chemicals were purchased from STEMCELL, unless otherwise specified.

Invasion assay. Cell invasion was assessed using a 96-well invasion assay (Cell Biolabs, Inc., San Diego, CA, USA). Medium only (RPMI containing 10% FBS and 1% PS), inactivated macrophages (1.0×10^4 cells/well) or activated macrophages (2.0×10^4 cells/well) were added to the feeder tray, followed by seeding with DLD-1, SW620, or LoVo cells suspended in serum-free medium into the membrane chamber. After a 24-h incubation, the invasive cells were dissociated from the underside of the membrane using a cell detachment buffer, and the dye+lysis buffer solution (Cell Biolabs) was added. The fluorescence intensity was measured with an Infinite F500 plate reader (TECAN, Kawasaki, Japan) at 485 nm/535 nm, and the cell invasive potential was assessed.

Proliferation assay. Cell proliferation assay was carried out using insert-integrated 96-well plates (Corning, NY, USA) and assessed using the Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan). DLD-1, SW620 and LoVo cells were each seeded at 1.0×10^4 cells/well into the feeder tray, followed by the addition of medium only (RPMI with 10% FBS and 1% PS), or a suspension of inactivated macrophages or activated macrophages seeded at 2.0×10^4 cells/well, into the membrane chamber. After 3 days of incubation, the CCK-8 diluted to 10% in RPMI was added to all feeder wells and incubated for 1 h. Absorbance was measured using an Infinite F50 microplate reader (TECAN) at 450 nm/620 nm, and the cell proliferative capacity was assessed.

Flow cytometry. Staining was carried out in the presence of Fc-receptor blocking solution (Biolegend, San Diego, CA, USA). We used the following antibodies and isotype controls: APC anti-human CD14 antibody (M5E2) (Biolegend), V500 mouse anti-human CD45 antibody (HI30) (BD PharMingen, San Jose, CA, USA), PE mouse anti-human MSR1 (CD204) (U23-56) (BD PharMingen), APC mouse IgG2a, κ isotype control antibody (MOPC-173) (Biolegend), V500 mouse IgG1, κ isotype control antibody (X40) (BD PharMingen), and PE mouse IgG2a, κ isotype control antibody (MOPC-21) (BD PharMingen). Cells (5.0×10^5) were suspended in 500 μl phosphate-buffered saline containing DAPI (DOJINDO). The expression of CD204 was examined to confirm that monocytes were differentiated into M2 macrophages. Flow cytometry was performed by a BD LSRFortessa– X-20 (BD Bioscience, San Jose, CA, USA) and data were analysed using FlowJo (Tree Star, Oregon City, OR, USA).

Statistical methods. All statistical analyses were carried out by EZR version 3.6.0 [Saitama Medical Center, Jichi Medical University, Saitama, Japan; (18)] and Excel 2021 (Microsoft) software. Two-sided p-values <0.05 were considered statistically significant. Student's t-tests, or χ^2 or Fisher's exact tests, were used for the comparison of means. The Kaplan-Meier method was used to evaluate OS and RFS, and the differences were determined using the Cox proportional hazards model. Significant variables in the univariate analysis ($p<0.1$) were also analysed using the multivariate statistical method.

Results

Expression of CD204 and CD68 and clinicopathological variables. Of the 206 patients, 95 (46.1%) showed high expression of CD204 and 109 (52.9%) showed high expression of CD68. Table I shows associations between the expression of these markers and the following clinicopathological characteristics at presentation: age, sex, tumour location and size, depth of invasion, lymph node metastasis, lymph vessel invasion, vascular invasion, chemoradiation, carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) levels. Clinically, CD204 positivity was significantly associated with tumour location ($p<0.0225$) and tumour size ($p<0.0331$), whereas CD68 positivity was significantly associated with tumour size ($p<0.0228$) and lymph node metastases ($p<0.008$).

Expression of CD204 and CD68 and survival. Next, we investigated the correlations between CD204 and CD68 expression and patient prognosis. At the end of follow-up, 74 patients had relapsed and 71 were dead. Kaplan-Meier analysis showed that the patients with high CD204 expression had significantly shorter rates of OS (log lank $p=0.00648$) and RFS ($p=0.00673$) compared with those with low CD204 expression (Figure 2A and C). The difference in OS did not appear until the third year following surgery, while the difference in RFS apparent from the beginning. There were no significant relationships between CD68 expression and OS ($p=0.843$) or RFS ($p=0.297$) (Figure 2B and D).

To evaluate the prognostic significance of CD204 in CRC, univariate and multivariate analysis were performed using a Cox proportional hazard model (Table II). Univariate analysis revealed that CEA [hazard ratio (HR)=1.933, 95% confidence interval (CI)=1.160-3.222, $p=0.011$] and CD204 (HR=2.291, 95%CI=1.374-3.820, $p=0.001$) were prognostic factors for OS, and that lymph vessel invasion (HR=3.19, 95%CI=1.231-8.266, $p=0.017$), CEA (HR=1.546, 95%CI=0.930-2.569, $p=0.093$) and CD204 (HR=2.347, 95%CI=1.431-3.848, $p=0.001$) were prognostic factors for RFS. Finally,

Table I. *Associations of CD68 and CD204 expression with clinicopathological variables in 206 patients with stage II and III CRC.*

Clinicopathological features	Total	CD68		*p*-Value	CD204		*p*-Value
	N	Low	High		Low	High	
All cases	206	97	109		111	95	
Age (years)				0.762			0.45
<65	62	28	34		36	26	
≥65	144	69	75		75	69	
Gender				0.675			0.162
Male	96	47	49		57	39	
Female	110	50	60		54	56	
Tumour location				0.292			0.0225
Colon	143	71	72		85	58	
Rectum	63	26	37		26	37	
Tumour size (cm)				0.0228			0.0331
<4	84	48	36		53	31	
≥4	122	49	73		58	64	
Depth of invasion				0.624			1.000
T1	4	1	3		2	2	
T2-4	202	96	106		109	93	
Lymph node metastases				0.008			0.262
Absent	112	43	69		56	56	
Present	94	54	40		55	39	
Lymph vessel invasion				1.000			0.735
Not severe	197	93	104		107	90	
Severe	9	4	5		4	5	
Vascular invasion				0.223			0.352
Not severe	195	94	101		107	88	
Severe	11	3	8		4	7	
Chemoradiation				0.709			0.706
Absent	197	93	104		108	91	
Present	9	4	5		3	4	
CEA (ng/ml)				0.105			0.767
<5	138	63	75		73	65	
≥5	68	34	34		38	30	
CA19-9 (U/ml)				0.656			0.45
<37	178	88	90		98	80	
≥37	28	9	19		13	15	

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9.

multivariate analysis revealed that CEA (HR=1.997, 95%CI=1.250-3.190, p=0.004) and CD204 (HR=2.090, 95%CI=1.296-3.368, p=0.002) were prognostic factors for OS, and that lymph vessel invasion (HR=3.429, 95%CI=1.473-7.980, p=0.004), CEA (HR=1.678, 95%CI=1.050-2.682, p=0.031) and CD204 (HR=1.997, 95%CI=1.251-3.187, p=0.004) were prognostic factors for RFS.

Influence of M2 macrophages on CRC cell proliferation and invasion. To investigate how M2 macrophages contribute to worsening the prognosis of CRC patients, we co-cultured SW620, DLD-1 or LoVo cells with activated macrophages, inactivated macrophages, or medium only. Co-culturing with activated macrophages promoted both the proliferative and invasive abilities of all three CRC cell lines that were significantly greater than those of cells cocultured with inactivated macrophages or medium only (Figure 3). Flow cytometry showed that the expression of CD204 tended to be increased in activated macrophages compared with inactivated macrophages (Figure 4). These results suggested that the presence of M2 macrophages in the TME enhanced the proliferative and invasive abilities of CRC cells.

Discussion

In this study, we investigated whether high expression of CD204, a marker of M2 macrophages, and CD68, a pan-macrophage marker, were correlated with the prognosis of Stage II and III CRC and confirmed the effects of macrophages on CRC cell lines with *in vitro assays*. IHC of pathological

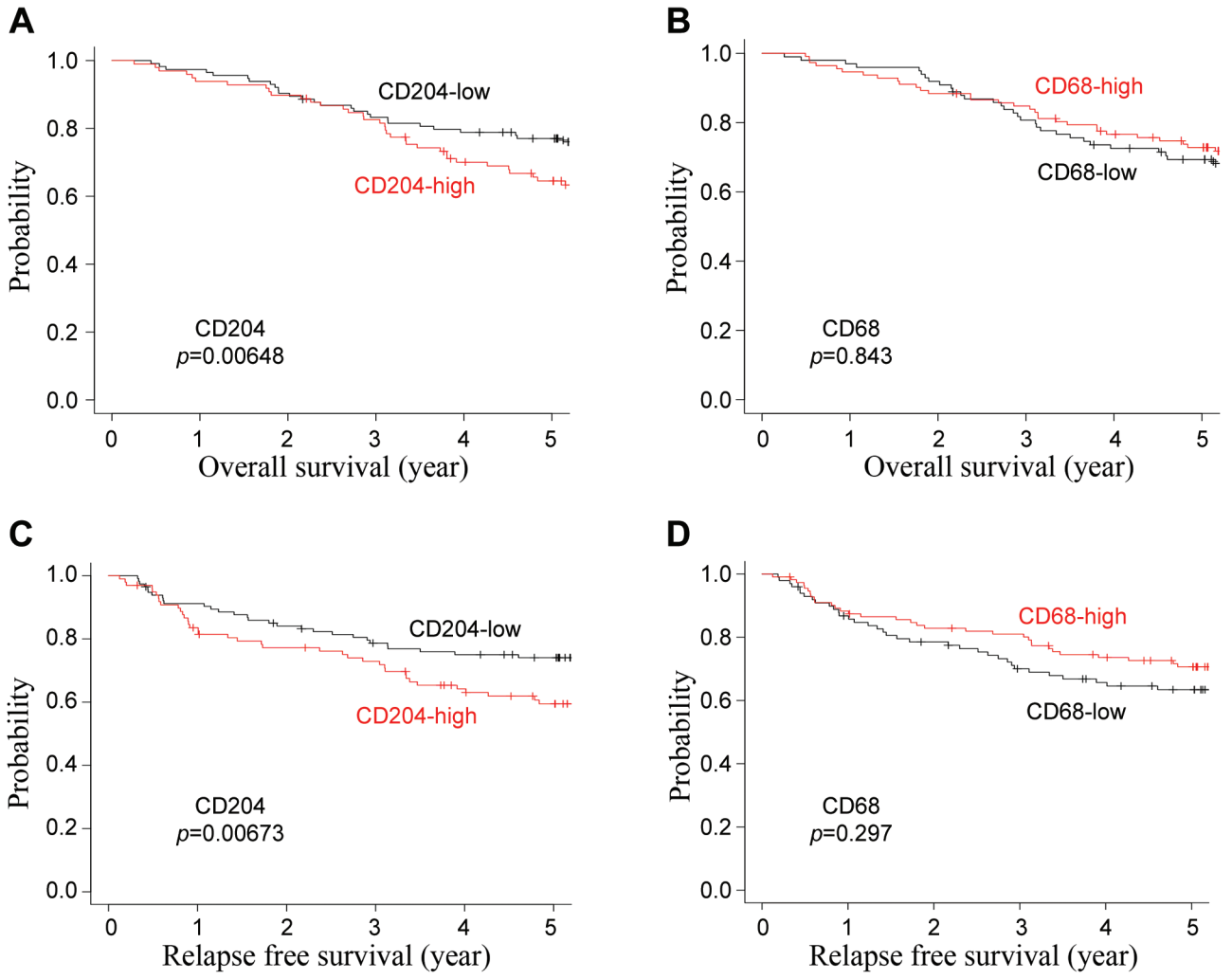

Figure 2. *Kaplan-Meier curves of overall survival (OS) and recurrence free survival (RFS) in patients with colorectal cancer (CRC). Higher expression of CD204 was significantly associated with shorter OS (A) and RFS (C). The expression levels of CD68 were not associated with OS (B) or RFS (D).*

specimens demonstrated that the rates of OS and RFS in CRC patients with high CD204 tumour expression were significantly shorter than those with low CD204 expression.

Kaplan-Meier curves for CD204 expression showed no effect on OS until the third year following surgery, but there was a significant difference in RFS from the beginning (Figure 2A and C). Though previous studies did not show this tendency (14, 15), the 2020 guidelines from the Japanese Society for Cancer of the Colon and Rectum revealed that 85% of postoperative recurrences occurred within 3 years (19). Multivariate analysis of the present study data showed that RFS was significantly correlated with lymph vessel invasion and CD204 expression. Furthermore, *in vitro* experiments demonstrated that activated macrophages with high expression of CD204 enhanced tumour invasion to a significantly greater degree than inactivated macrophages, suggesting that CD204 may be related with postoperative recurrence.

We used CD204 as a marker for M2 macrophages because of its established involvement in M2 polarization, which occurs *via* inhibition of Toll-like receptor signalling, as well as its significant correlation with poor prognosis of various cancers (20). To our knowledge, this is the first report of a correlation between CD204 expression and worse prognosis of CRC.

The previously reported correlation between high CD68 expression and improved survival (16, 17) was not found in the CRC patients in this study, either in OS or RFS. This may be explained by CD68 being a pan-macrophage marker present in both M1 and M2 macrophages. However, our *in vitro* experiments demonstrated that inactivated macrophages also promoted proliferative and invasive abilities compared with the

Table II. *Univariate and multivariate analysis of clinicopathological features associated with OS and RFS.*

Clinicopathological features	OS						RFS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age (≥65 *vs.* <65) (years)	1.528	0.849-2.749	0.157				1.378	0.791-2.402	0.257			
Gender (male *vs.* female)	1.403	0.860-2.289	0.175				1.352	0.835-2.189	0.220			
Tumour location (colon *vs.* rectum)	0.618	0.344-1.111	0.108				0.738	0.423-1.288	0.285			
Tumour size (≥4 *vs.* <4) (cm)	0.795	0.473-1.337	0.387				0.859	0.517-1.426	0.556			
Depth of invasion (T1 *vs.* T2, 3, 4)	2.633	0.335-20.710	0.358				2.591	0.334-20.120	0.363			
Lymph node metastases (absent *vs.* present)	1.155	0.685-1.948	0.588				1.286	0.777-2.129	0.329			
Lymph vessel invasion (severe *vs.* not severe)	1.760	0.648-4.779	0.268				3.19	1.231-8.266	0.017	3.429	1.473-7.980	0.004
Vascular invasion (severe *vs.* not severe)	1.149	0.432-3.053	0.781				1.163	0.402-3.370	0.781			
Chemoradiation (absent *vs.* present)	2.398	0.801-7.184	0.118				1.839	0.528-6.406	0.339			
CEA (≥5 *vs.* <5) (ng/ml)	1.933	1.160-3.222	0.011	1.997	1.250-3.190	0.004	1.546	0.930-2.569	0.093	1.678	1.050-2.682	0.031
CA19-9 (≥37 *vs.* <37) (U/ml)	1.464	0.765-2.803	0.250				1.153	0.586-2.270	0.680			
CD68 (low *vs.* high group)	0.858	0.509-1.446	0.564				0.669	0.399-1.123	0.128			
CD204 (low *vs.* high group)	2.291	1.374-3.820	0.001	2.090	1.296-3.368	0.002	2.347	1.431-3.848	0.001	1.997	1.251-3.187	0.004

OS: Overall survival; RFS: relapse-free survival; HR: hazard ratio; CI: confidence interval; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

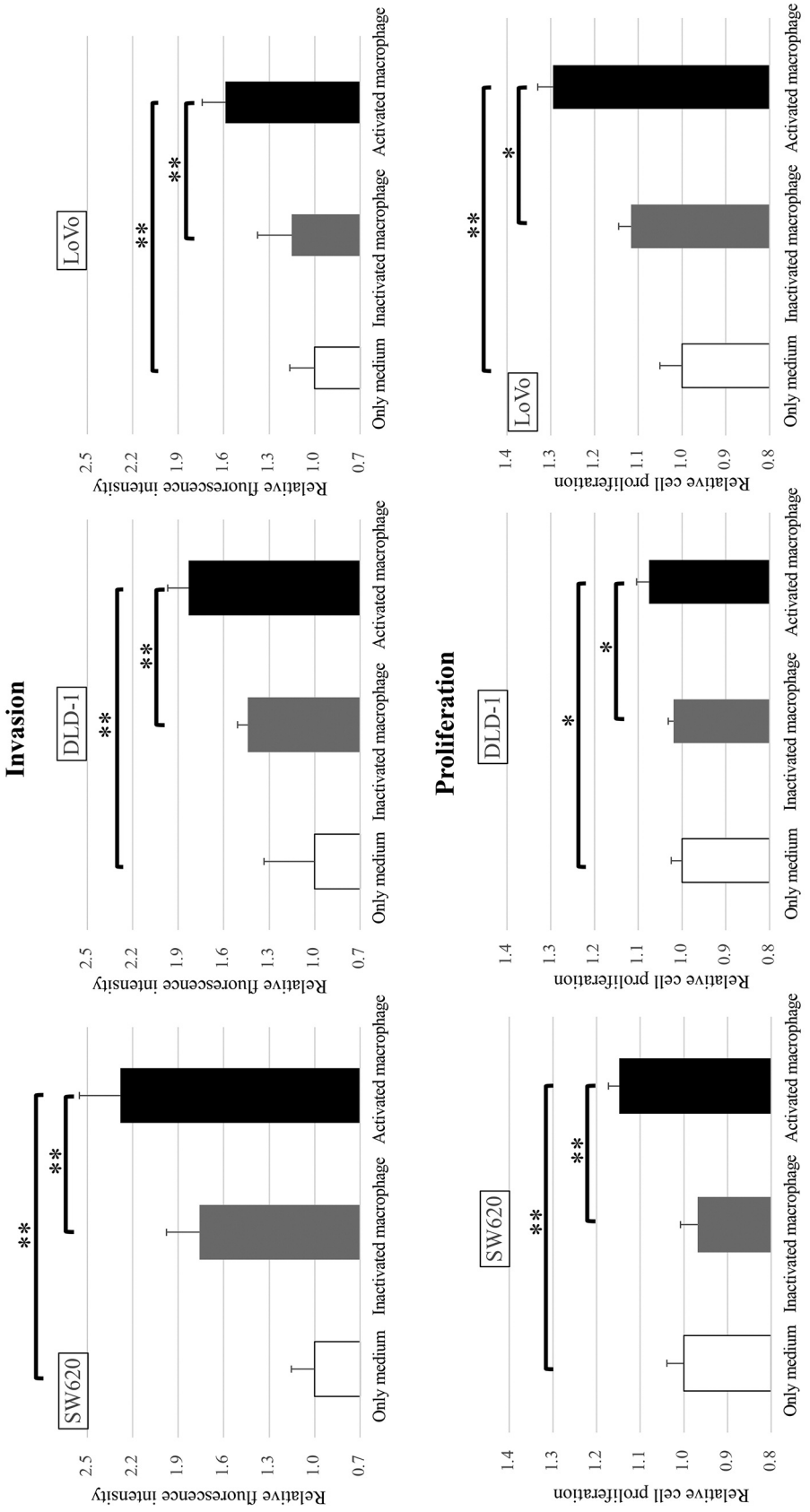

Figure 3. *Activated macrophages promoted proliferation and invasion of all colorectal cancer cell lines (SW620, DLD-1, and LoVo). Results are expressed as mean±standard deviation. *$p<0.05$; **$p<0.01$.*

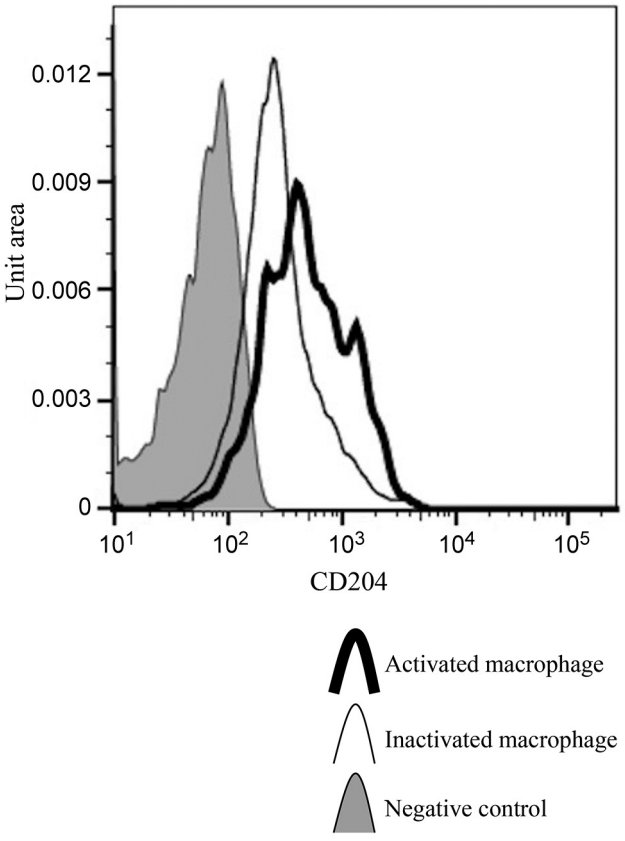

Figure 4. *Flow cytometry demonstrated that CD204 expression tended to be higher in activated macrophages than in inactivated macrophages.*

medium-only control. Generally, macrophages differentiate into M1 or M2 type depending on the microenvironment, however, they coexist and the roles of each macrophage type are poorly understood. Yang *et al*. showed that M1 macrophages did not promote proliferative or invasive abilities of CRC cells, and that a high CD163/CD68 expression ratio was correlated with poor prognosis in CRC (14). These results indicated that the ratio of M1 and M2 macrophages in the TME might have an important role in tumour malignancy.

This study has several limitations. First, patients undergoing preoperative chemotherapy, radiotherapy or chemoradiotherapy were included. Previous studies revealed that several chemotherapy drugs (cyclophosphamide, docetaxel, doxorubicin, 5-fluorouracil) increased macrophage infiltration, resulting in tumour resistance and recurrence (21). Radiotherapy is often used as part of the treatment for various cancers. Wan *et al*. showed that irradiated tumour cell-released microparticles have antitumour effects by inducing ferroptosis and polarizing macrophages from M2 to M1, enriching the Janus kinase/signal transducers and activators of transcription signaling pathways and the mitogen-activated protein kinase signaling pathway (22). In this study, chemoradiation was not correlated with CD204 expression. Further research is needed to confirm the effects of chemotherapy, radiotherapy and chemoradiotherapy on the polarization status of macrophages. Second, there are several M2-specific markers, including CD163, CD206, CD204 and Arginase 1 (ARG1). Previous studies on the association between M2 macrophages and CRC used CD163 or CD206 (14, 15), but not ARG1; marker selection can be complicated by the presence of various M2 subtypes. To clarify the impact of M2 macrophages on CRC more comprehensively, the potential association between ARG1 expression and CRC should be investigated. Third, all of the clinical specimens used in this study were obtained from Japanese patients. In a previous study, the number of M2 macrophages tended to be higher in black patients, who also had more breast cancer-related deaths compared with white patients (23). Further studies are needed to investigate the commonality of our results across racial/ethnic groups.

In conclusion, we demonstrated that high expression of the M2-specific marker CD204 was significantly correlated with the poor prognosis of Stage II and III CRC patients, whereas the pan-macrophage marker CD68 was not. Additionally, co-culturing of CRC cell lines with M2 macrophages expressing CD204 affected their proliferative capacity and invasive potential. These results indicated that CD204 might be an important prognostic marker and a new therapeutic target for CRC patients.

Conflicts of Interest

The Authors have no conflicts of interest to disclose regarding this study.

Authors' Contributions

YT designed the study, analysed the data and drafted the article. YF contributed to the final approval of the manuscript. All Authors read and approved the final manuscript.

Acknowledgements

The Authors thank Michelle Kahmeyer-Gabbe, PhD, from Edanz (https://jp.edanz.com/ac), for editing a draft of this manuscript.

References

1 Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. CA Cancer J Clin *65(1)*: 5-29, 2015. PMID: 25559415. DOI: 10.3322/caac.21254

2 Becker LM and LeBleu VS: Endoglin targeting in colorectal tumor microenvironment. Clin Cancer Res *24(24)*: 6110-6111, 2018. PMID: 30076135. DOI: 10.1158/1078-0432.CCR-18-2023

3 Erreni M, Mantovani A and Allavena P: Tumor-associated macrophages (TAM) and inflammation in colorectal cancer. Cancer Microenviron *4(2)*: 141-154, 2011. PMID: 21909876. DOI: 10.1007/s12307-010-0052-5

4 Mantovani A, Sozzani S, Locati M, Allavena P and Sica A: Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol *23(11)*: 549-555, 2002. PMID: 12401408. DOI: 10.1016/s1471-4906(02)02302-5

5 Mantovani A and Locati M: Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: lessons and open questions. Arterioscler Thromb Vasc Biol *33(7)*: 1478-1483, 2013. PMID: 23766387. DOI: 10.1161/ATVBAHA.113.300168

6 Koide N, Nishio A, Sato T, Sugiyama A and Miyagawa S: Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. Am J Gastroenterol *99(9)*: 1667-1674, 2004. PMID: 15330899. DOI: 10.1111/j.1572-0241.2004.30733.x

7 Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD, Bast MA, Rosenwald A, Muller-Hermelink HK, Rimsza LM, Campo E, Delabie J, Braziel RM, Cook JR, Tubbs RR, Jaffe ES, Lenz G, Connors JM, Staudt LM, Chan WC and Gascoyne RD: Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med *362(10)*: 875-885, 2010. PMID: 20220182. DOI: 10.1056/NEJMoa0905680

8 Komohara Y, Hasita H, Ohnishi K, Fujiwara Y, Suzu S, Eto M and Takeya M: Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. Cancer Sci *102(7)*: 1424-1431, 2011. PMID: 21453387. DOI: 10.1111/j.1349-7006.2011.01945.x

9 Shigeoka M, Urakawa N, Nakamura T, Nishio M, Watajima T, Kuroda D, Komori T, Kakeji Y, Semba S and Yokozaki H: Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. Cancer Sci *104(8)*: 1112-1119, 2013. PMID: 23648122. DOI: 10.1111/cas.12188

10 Yamaguchi T, Fushida S, Yamamoto Y, Tsukada T, Kinoshita J, Oyama K, Miyashita T, Tajima H, Ninomiya I, Munesue S, Harashima A, Harada S, Yamamoto H and Ohta T: Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. Gastric Cancer *19(4)*: 1052-1065, 2016. PMID: 26621525. DOI: 10.1007/s10120-015-0579-8

11 Miyasato Y, Shiota T, Ohnishi K, Pan C, Yano H, Horlad H, Yamamoto Y, Yamamoto-Ibusuki M, Iwase H, Takeya M and Komohara Y: High density of CD204-positive macrophages predicts worse clinical prognosis in patients with breast cancer. Cancer Sci *108(8)*: 1693-1700, 2017. PMID: 28574667. DOI: 10.1111/cas.13287

12 Kubota K, Moriyama M, Furukawa S, Rafiul HASM, Maruse Y, Jinno T, Tanaka A, Ohta M, Ishiguro N, Yamauchi M, Sakamoto M, Maehara T, Hayashida JN, Kawano S, Kiyoshima T and Nakamura S: $CD163^{+}CD204^{+}$ tumor-associated macrophages contribute to T cell regulation *via* interleukin-10 and PD-L1 production in oral squamous cell carcinoma. Sci Rep *7(1)*: 1755, 2017. PMID: 28496107. DOI: 10.1038/s41598-017-01661-z

13 Yagi T, Baba Y, Okadome K, Kiyozumi Y, Hiyoshi Y, Ishimoto T, Iwatsuki M, Miyamoto Y, Yoshida N, Watanabe M, Komohara Y and Baba H: Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal cancer. Eur J Cancer *111*: 38-49, 2019. PMID: 30822683. DOI: 10.1016/j.ejca.2019.01.018

14 Yang C, Wei C, Wang S, Shi D, Zhang C, Lin X, Dou R and Xiong B: Elevated $CD163^{+}/CD68^{+}$ ratio at tumor invasive front is closely associated with aggressive phenotype and poor prognosis in colorectal cancer. Int J Biol Sci *15(5)*: 984-998, 2019. PMID: 31182919. DOI: 10.7150/ijbs.29836

15 Feng Q, Chang W, Mao Y, He G, Zheng P, Tang W, Wei Y, Ren L, Zhu D, Ji M, Tu Y, Qin X and Xu J: Tumor-associated macrophages as prognostic and predictive biomarkers for postoperative adjuvant chemotherapy in patients with stage II colon cancer. Clin Cancer Res *25(13)*: 3896-3907, 2019. PMID: 30988081. DOI: 10.1158/1078-0432.CCR-18-2076

16 Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A and Palmqvist R: High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res *13(5)*: 1472-1479, 2007. PMID: 17332291. DOI: 10.1158/1078-0432.CCR-06-2073

17 Zhou Q, Peng RQ, Wu XJ, Xia Q, Hou JH, Ding Y, Zhou QM, Zhang X, Pang ZZ, Wan DS, Zeng YX and Zhang XS: The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. J Transl Med *8*: 13, 2010. PMID: 20141634. DOI: 10.1186/1479-5876-8-13

18 Kanda Y: Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant *48(3)*: 452-458, 2013. PMID: 23208313. DOI: 10.1038/bmt.2012.244

19 Hashiguchi Y, Muro K, Saito Y, Ito Y, Ajioka Y, Hamaguchi T, Hasegawa K, Hotta K, Ishida H, Ishiguro M, Ishihara S, Kanemitsu Y, Kinugasa Y, Murofushi K, Nakajima TE, Oka S, Tanaka T, Taniguchi H, Tsuji A, Uehara K, Ueno H, Yamanaka T, Yamazaki K, Yoshida M, Yoshino T, Itabashi M, Sakamaki K, Sano K, Shimada Y, Tanaka S, Uetake H, Yamaguchi S, Yamaguchi N, Kobayashi H, Matsuda K, Kotake K, Sugihara K and Japanese Society for Cancer of the Colon and Rectum: Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2019 for the treatment of colorectal cancer. Int J Clin Oncol *25(1)*: 1-42, 2020. PMID: 31203527. DOI: 10.1007/s10147-019-01485-z

20 Komohara Y, Takemura K, Lei XF, Sakashita N, Harada M, Suzuki H, Kodama T and Takeya M: Delayed growth of EL4 lymphoma in SR-A-deficient mice is due to upregulation of nitric oxide and interferon-gamma production by tumor-associated macrophages. Cancer Sci *100(11)*: 2160-2166, 2009. PMID: 19694752. DOI: 10.1111/j.1349-7006.2009.01296.x

21 Zhao Y, Zhang C, Gao L, Yu X, Lai J, Lu D, Bao R, Wang Y, Jia B, Wang F and Liu Z: Chemotherapy-induced macrophage infiltration into tumors enhances nanographene-based photodynamic therapy. Cancer Res *77(21)*: 6021-6032, 2017. PMID: 28916656. DOI: 10.1158/0008-5472.CAN-17-1655

22 Wan C, Sun Y, Tian Y, Lu L, Dai X, Meng J, Huang J, He Q, Wu B, Zhang Z, Jiang K, Hu D, Wu G, Lovell JF, Jin H and Yang K: Irradiated tumor cell-derived microparticles mediate tumor eradication *via* cell killing and immune reprogramming. Sci Adv *6(13)*: eaay9789, 2020. PMID: 32232155. DOI: 10.1126/sciadv.aay9789

23 Kim G, Pastoriza JM, Condeelis JS, Sparano JA, Filippou PS, Karagiannis GS and Oktay MH: The contribution of race to breast tumor microenvironment composition and disease progression. Front Oncol *10*: 1022, 2020. PMID: 32714862. DOI: 10.3389/fonc.2020.01022

Received July 30, 2021
Revised August 22, 2021
Accepted August 23, 2021