European Journal of Cancer 111 (2019) 38–49

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal cancer

Taisuke Yagi [a], Yoshifumi Baba [a], Kazuo Okadome [a], Yuki Kiyozumi [a], Yukiharu Hiyoshi [a], Takatsugu Ishimoto [a], Masaaki Iwatsuki [a], Yuji Miyamoto [a], Naoya Yoshida [a], Masayuki Watanabe [b], Yoshihiro Komohara [c], Hideo Baba [a,*]

[a] *Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan*
[b] *Department of Gastroenterological Surgery, Cancer Institute Hospital, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan*
[c] *Department of Cell Pathology, Graduate School of Medical Sciences, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan*

Received 30 October 2018; received in revised form 13 January 2019; accepted 20 January 2019
Available online 26 February 2019

KEYWORDS
Oesophageal cancer;
TAM;
PD-L1;
Prognosis

Abstract ***Introduction:*** Tumour-associated macrophages (TAMs) in tumour microenvironments promote cancer cell proliferation, immunosuppression and angiogenesis, leading to tumour growth and metastasis. TAMs have become increasingly recognised as a cancer therapy target, such as in combination therapy with an immunity checkpoint inhibitor. However, the clinical and prognostic features of TAMs, and the relationship between TAMs and programmed death ligand 1 (PD-L1), remain unexplored in oesophageal cancer.
Methods: Using a non-biased database of 305 resected oesophageal cancer preparations, we evaluated the expression of two M2-like macrophage markers (CD163 and CD204) and PD-L1 on tumour cells by immunostaining. Through *in vitro* assays, we examined how TAMs influence phenotypic malignancy and PD-L1 expression.
Results: High density of CD163 (n = 160) or CD204 (n = 146) was associated with significantly worse overall survival than low expression (log rank $P = 0.0025$ and 0.018 for CD163 and CD204, respectively). The prognostic effect of TAMs was not significantly modified by any clinical factors ($P > 0.05$ for all interactions). High TAM density was significantly associated with increased PD-L1 expression. In *in vitro* assays, cell invasion and migration ability were significantly more upregulated in oesophageal cancer cell lines cocultured with

* *Corresponding author*: Fax: +81 96 371-4378.
E-mail address: hdobaba@kumamoto-u.ac.jp (H. Baba).

https://doi.org/10.1016/j.ejca.2019.01.018

activated macrophages than in control cell lines. Coculture with activated macrophages elevated the PD-L1 expression in cancer cells.
Conclusions: High TAM density in oesophageal cancer tissues was associated with shorter survival, suggesting a prognostic biomarker role for TAMs. TAMs also increase PD-L1 expression in tumour cells. Given the significant interest in cancer immunotherapies targeting TAMs and PD-L1, the current findings should have considerable clinical implications.

1. Introduction

Tumour-associated macrophages (TAMs) are a major immune component of many types of cancer [1,2]. TAMs promote tumours by secreting proangiogenic and growth factors. They also potently suppress T-cell effector functions by releasing immunosuppressive cytokines and by affecting T-cell metabolism [3–6]. Macrophages exerting these protumourigenic functions are termed M2-like macrophages, in contrast to the antitumourigenic M1-like subtype [7]. Among the several proposals for TAM-centred strategies are suppression of TAM recruitment, depletion of TAM numbers, the switching of M2-likeTAMs into the antitumour M1-like phenotype and the inhibition of TAM-associated molecules [8–10]. The presence of TAMs correlates with poor prognosis in various types of human cancers [11–19].

As demonstrated in recent clinical trials, programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) signal-blockade agents exert dramatic antitumour effects on patients with numerous types of malignancies [20–26]. Interestingly, TAMs have been shown to directly and indirectly modulate PD-1/PD-L1 expression in the tumour environment [27,28]. In fact, TAMs secrete several cytokines (e.g., interferon-γ [IFN-γ], interleukin-1β, tumour necrosis factor-α [TNF-α], transforming growth factor-β [TGF-β]) that induce PD-L1 expression [29]. In addition, TAMs reduce the effector activity of tumour-infiltrating lymphocytes [30,31]. Nonetheless, the relationship between TAM density and PD-L1 expression in oesophageal cancers has not been elucidated.

To examine the prognostic impact of TAMs, we here evaluate the expressions of two M2-like macrophage markers (CD163 and CD204) in an immunohistochemistry analysis of 305 curatively resected oesophageal cancers taken from a non-biased database. We further examine the relationship between TAMs and PD-L1 status in oesophageal cancer by double immunostaining and *in vitro* assays.

2. Material and methods

2.1. Specimens

The study subjects were 322 consecutive patients with oesophageal cancer who had undergone curative resection at Kumamoto University Hospital between January 2005 and June 2013. After excluding 17 patients who completely responded to preoperative treatment, 305 patients with oesophageal cancer were finally included in the study. Tumour staging was performed according to the American Joint Committee on Cancer Staging Manual (7th edition). In our cohort, the most frequent histological type was squamous cell carcinoma ([SCC] 279 cases, 91.4%) followed by adenocarcinoma (15 cases, 4.9%), adenosquamous carcinoma (five cases, 1.6%), basaloid SCC (five cases, 1.6%) and small cell carcinoma (one case, 0.3%). Patients were followed up as outpatients every 1–3 months after discharge until death or January 1, 2018, whichever came first. Overall survival was defined as the period from the date of surgery to the date of death. Cancer-specific survival was defined as the time from the date of surgery to the date of death attributable to oesophageal cancer. Written informed consent was obtained from each patient, and the study procedures were approved by the institutional review board of Kumamoto University.

2.2. CD163 and CD204 immunohistochemical staining

After deparaffinizing the tissue blocks, antigens were retrieved in antigen retrieval solution (pH 9, Histofine; Nichirei Biosciences) in a steamer autoclave at 121 °C for 15 min. Slides were incubated with primary antibody against CD163 (1:200; clone 10D6; Leica Biosystems) or CD204 (1:500; clone SRA-E5; TransGenic) overnight at 4 °C. The incubated slides were exposed to secondary antibody, anti-mouse EnVisionTM+/HRP (Dako Japan), and counterstained with haematoxylin. Appropriate positive and negative controls were included in each immunohistochemistry run. TAMs rarely infiltrated the normal epithelium (Fig. 1A). The densities of CD163 and CD204 expressions in the tumour were reviewed by a pathologist (Y.T.), who was unaware of other data. The CD163 and CD204 expressions in the tumour were classified as absent, mild, moderate or strong. High CD163 or CD204 expression was assigned a strong or moderate score (Fig. 1B and C).

2.3. Double immunostaining

PD-L1 and CD163 in the oesophageal cancer specimens were doubly immunostained as described previously [32]. In brief, the slides were exposed to rabbit monoclonal

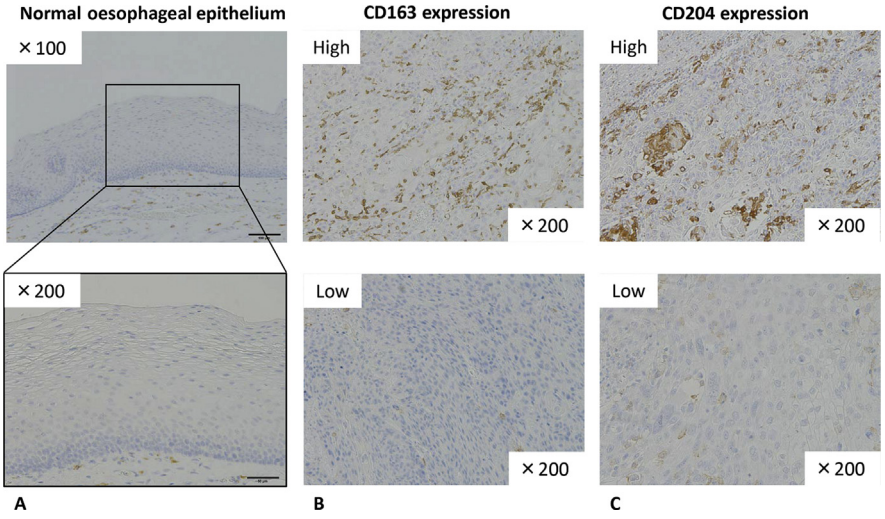

Fig. 1. CD163 and CD204 immunohistochemistry for oesophageal cancer. (A) CD163 expression in normal epithelium (upper panel) and tumour (lower panel). (B) CD163 expression in tumours. (C) CD204 expression in tumours. In all panels, the original magnification was × 200.

antibody against PD-L1 (clone E1L3N; Cell Signaling Technology) as described in a previous article [33] and then, incubated with horseradish peroxidase (HRP)-labelled goat anti-rabbit secondary antibodies (Nichirei Biosciences). Before application, the antibodies were diluted with Can Get Signal solution (Toyobo) to enhance the immunoreaction of PD-L1. Positive signals were visualised with DAB (brown) and HistoGreen (green) substrates (Fig. 5A).

2.4. Isotype control

We performed an isotype control using a FLEX Negative Control Mouse Cocktail of mouse IgG1, IgG2a, IgG2b and IgG3, IgM Ready-to-Use (IS750, Dako) for CD163 and CD204 and Universal Negative Control—Rabbit (N1699, Dako) for PD-L1 (Supplemental Figure 1).

2.5. Cell lines

The oesophageal cancer cell line TE-8 was purchased from the Japanese Cell Resource Center for Biomedical Research (Sendai), and KYSE-30 was purchased from HPA Culture Collections. TE-8 cells were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS), and KYSE-30 cells were grown in Dulbecco's modified eagle medium (D-MEM) medium (Sigma-Aldrich) supplemented with 10% FBS. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.6. Macrophage culture and coculture assay

Peripheral blood mononuclear cells were donated by healthy male volunteers. CD14+ monocytes were isolated on CD14 microbeads (Miltenyi Biotec). The monocytes were plated in 6-well plates (1×10^5 cells/well) and cultured with macrophage-colony-stimulating factor (M-CSF) granulocytes (2 ng/ml) (Wako) for 5 days to induce immature macrophages. The macrophages were either cultivated normally or activated by stimulating them with lipopolysaccharide (100 ng/ml) (PeproTech). The media of the normal and activated macrophage cultures were collected and transferred to 6-well plates containing TE-8 and KYSE-30 cells (2×10^5 cells/well). After 24 h of coculture, the TE-8 and KYSE-30 cells were washed and used in subsequent experiments.

2.7. Quantitative real-time reverse transcription-polymerase chain reaction and selection of cell lines

RNA was isolated from the cultured cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The mRNA expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan probes (Roche) and were normalised to those of β-actin. To design the qRT-PCR primers, we accessed the Universal Probe Library (Genenet), following the manufacturer's recommendations. Real-time PCR (RT-PCR) was performed with the following primer sequences and probes: PD-L1 (PD-L1_#25), 5′-GGCATCCAAGATACAAACTCAA-3′, 5′-CAGAAGTTCCAATGCTGGATTA-3′ and β-actin (ACTB_#11), 5′-ATTGGCAATGAGCGGTTC-3′, 5′-CGTGGATGCCACAGGACT-3'. All qRT-PCR reactions were performed in the LightCycler 480 System II (Roche). All data for real-time RT-PCR were obtained from triplicate experiments and are presented as the mean ± standard error.

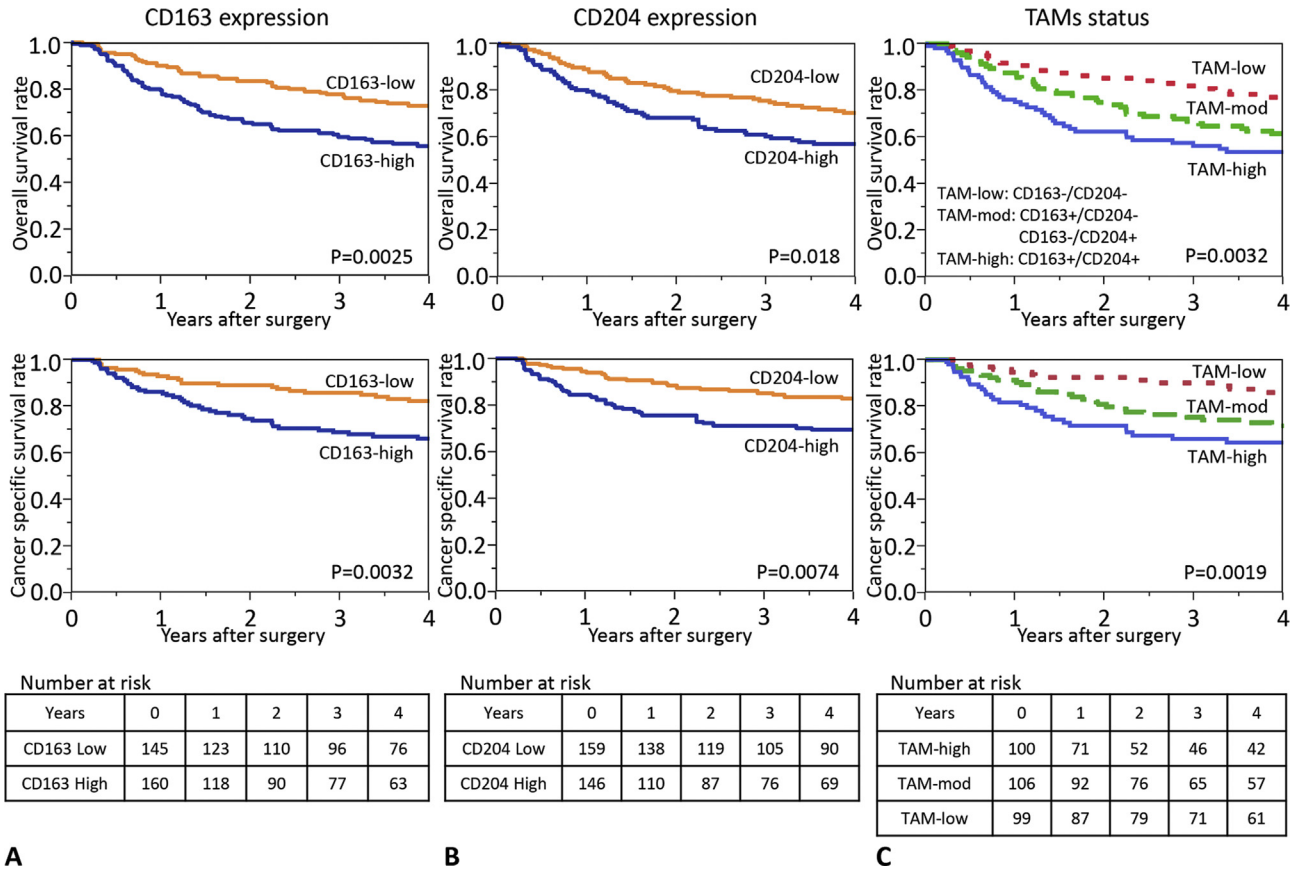

Fig. 2. Kaplan–Meier curves of overall and cancer-specific survivals in patients with oesophageal cancer based on CD163 expression (A), CD204 expression (B) and TAM status (C). In panel C, the TAM-high group strongly expresses both CD163 and CD204, the TAM-moderate group strongly expresses either CD163 or CD204 and the TAM-low group weakly expresses both CD163 and CD204. TAM, tumour-associated macrophage.

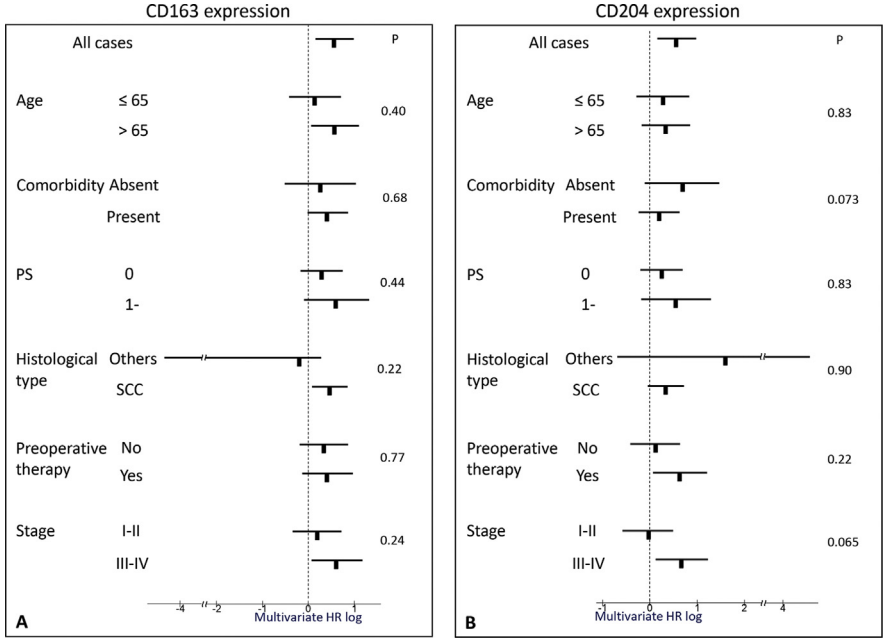

Fig. 3. Relationship between CD163 (A) and CD204 (B) expression in oesophageal cancer and overall survival. Shown are the multivariate HR plots of overall survival rate in groups expressing high and low levels of CD163 or CD204. SCC, squamous cell carcinoma; PS, performance status; HR, hazard ratio.

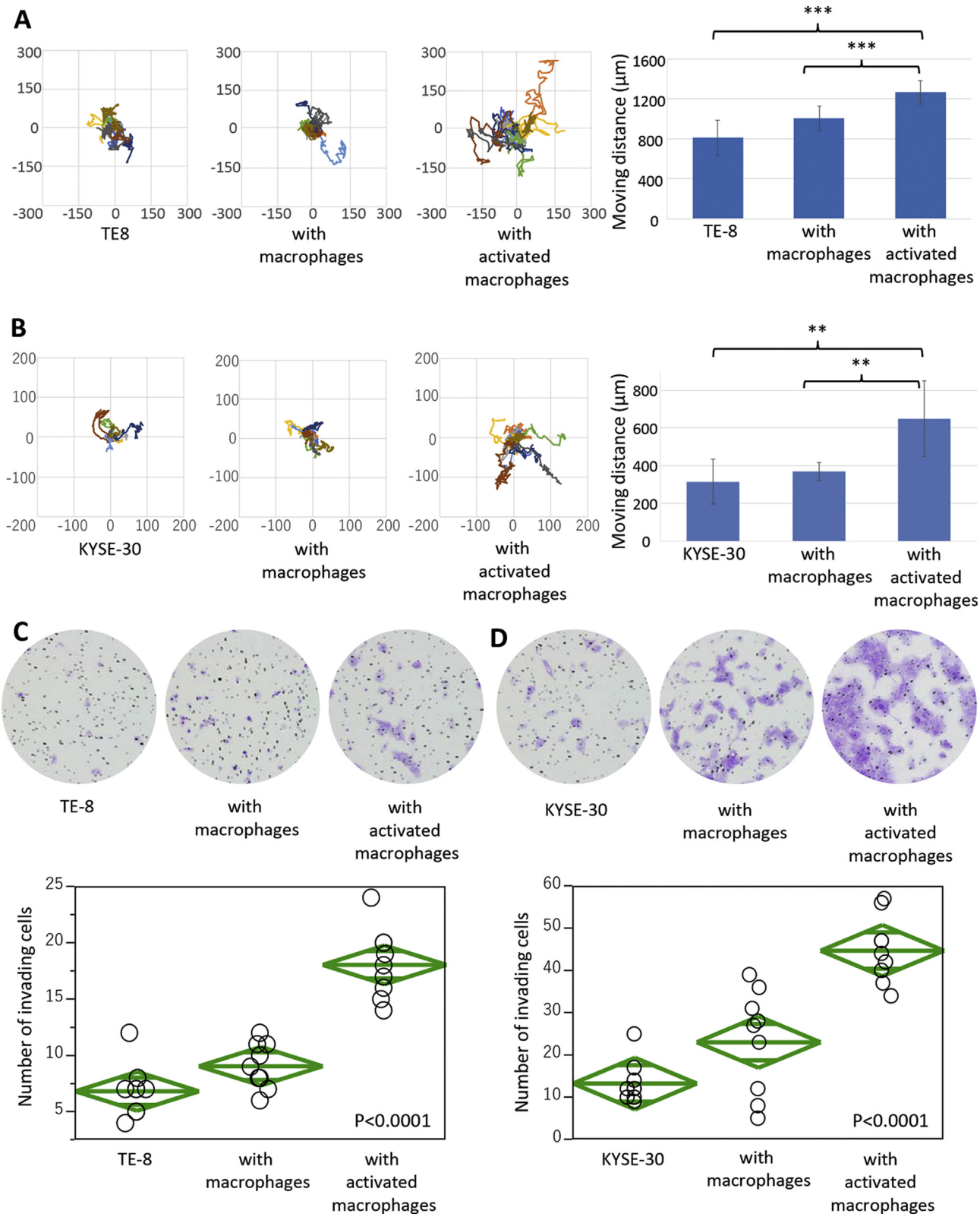

Fig. 4. Activated macrophages elevate the motility of oesophageal cancer cells on Matrigel. All experiments were performed on three cell groups: cell line only, coculture with macrophages and coculture with activated macrophages. Migration patterns and moving distances of TE-8 (A) and KYSE-30 cells (B) (n = 10). Photographs were taken every 10 min for 24 h. Invasion assay was performed on TE-8 (C) and KYSE-30 cells (D). The number of invading cells was counted in three predetermined fields (total magnification, × 200). $**P < 0.01$; $***P < 0.001$ (χ^2 test).

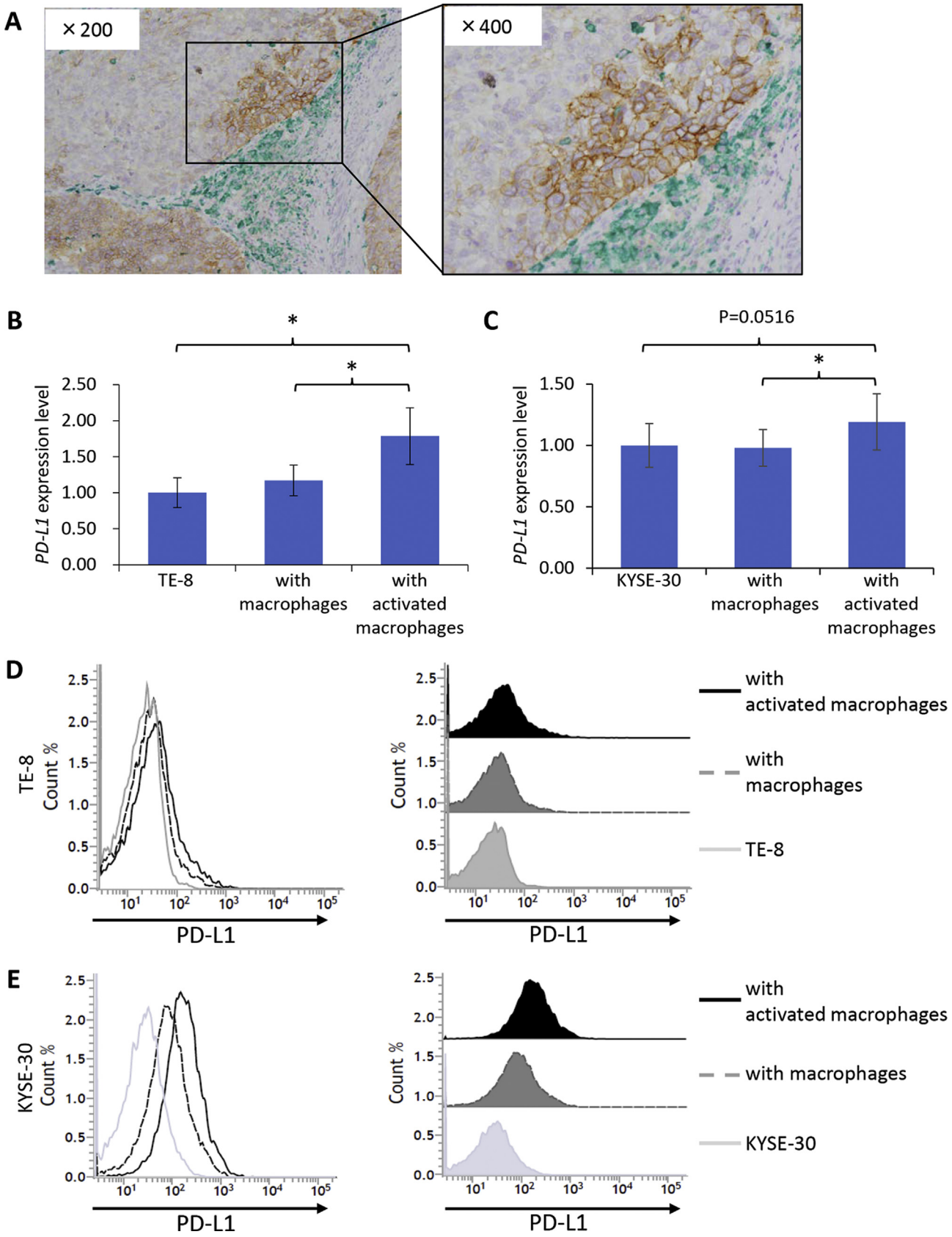

Fig. 5. Double immunostaining of PD-L1 and CD163 and coculture assay. The *in vitro* experiment was conducted on three cell groups: cell line only, coculture with macrophages and coculture with activated macrophages. Brown areas indicate PD-L1 expression and the green spots are CD163-expressing macrophages (A). PD-L1 expression levels in TE-8 (B) and KYSE-30 cells (C) evaluated by qRT-PCR and in TE-8 (D) and KYSE-30 cells (E) analysed by FACS. $*P < 0.05$; (χ^2 test). PD-L1, programmed death ligand 1.

2.8. Flow cytometry

The cell concentration was adjusted to 2×10^5 cells/ml in phosphate-buffered solution with 2% FBS. Cell suspensions were incubated with antibodies for 30 min at 4 °C. A PE-conjugated CD274 antibody (clone 29E.2A3) was obtained from BioLegend. Dead cells were removed with 7-amino-actinomycin D (7-AAD) (BD Biosciences). The flow cytometry was analysed with a FACSVerse instrument (BD Biosciences). The flow cytometry data were analysed using FlowJo 3.3 software (Tree Star).

2.9. Migration assay

The TE-8 and KYSE-30 cells were cocultured with macrophages or activated macrophages for 24 h, then seeded on Matrigel-coated 6-well plates at 5.0×10^4 cells/well. The plates were imaged in a BZ-X700 all-in-one fluorescence microscope equipped with a CO_2- and temperature-controlled chamber and a time-lapse tracking system (KEYENCE). Phase contrast images were taken every 10 min for 24 h and converted to movie files by a BZ-X Analyzer (KEYENCE). The movies were analysed for cell migration using the video editing analysis software VW-H2MA (KEYENCE). The x-y coordinates, velocities and distances of the migrated cells were then determined by processing the tracking data in Microsoft Excel 2010 (Microsoft).

2.10. Invasion assay

The cell invasion was assayed *in vitro* in a BD BioCoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's protocol. In brief, this assay determines the invasion rate of the tumour cells migrating through the Transwell inserts (8-μm pore size) in a uniform layer of BD Matrigel basement membrane matrix. After coculture assay, the TE-8 (2.5×10^4 cells) and KYSE-30 (5.0×10^4 cells) were seeded into the upper chamber of the insert in 500 μl of serum-free medium and in the lower well in 750 μl of 10% FBS-containing medium. After 22-h incubation (37 °C, 5% CO_2), the invading cells were fixed and stained. The numbers of invading cells in three predetermined fields (200 × total magnification) were counted under the microscope by independent investigators. Each group was cultured in triplicate.

2.11. Statistical methods

All statistical calculations were performed in JMP version 10 (SAS Institute) and Excel 2016 (Microsoft) software. All P values were two-sided. The means were compared using Student's t-tests, or χ^2 or Fisher's exact tests. In the survival analysis, the survival time distribution was evaluated by the Kaplan–Meier method and comparisons were made by the log-rank test. To compute the hazard ratio (HR) for each TAM expression status, we constructed a multivariate Cox proportional hazard model including the potential clinical and pathological factors related to clinical outcomes. Significant variables in the univariate analysis ($P < 0.05$) were inserted into the multivariate model by a stepwise backward elimination procedure with a threshold P value of 0.05.

3. Results

3.1. CD163 and CD204 expressions and clinicopathological features

Among the 305 oesophageal cancer cases, 160 (52.4%) and 146 (47.8%) expressed high levels of CD163 and CD204, respectively. The clinical and pathological features of all examined cases are summarised in Table 1. Both CD163 and CD204 expressions were significantly associated with advanced tumour stage, positive PD-L1 status, vascular invasion and lymphoduct invasion. We classified the cases into three groups based on their CD163 and CD204 expression statuses: the TAM-high group (high density of both CD163 and CD204), the TAM-moderate group (high density of either CD163 or CD204) and the TAM-low group (low density of both CD163 and CD204). The proportions of the TAM-high, TAM-moderate and TAM-low types were 33% (n = 100), 35% (n = 106) and 32% (n = 99), respectively. The TAM-high and TAM-moderate groups were significantly associated with the histological type, advanced tumour stage, vascular/lymphoduct invasion and positive PD-L1 status (Supplementary Table 1).

3.2. CD163 and CD204 expression and survival

Within the study period, there were 122 deaths among the 305 patients with oesophageal cancer (including 72 oesophageal cancer-specific deaths) and 104 recurrences of oesophageal cancer. The median follow-up time for censored patients was 4.3 years. According to the Kaplan–Meier analysis, patients in the high CD163 and CD204 expression group showed significantly shorter overall survival (log rank $P = 0.0025$ and 0.018, respectively) and cancer-specific survival ($P = 0.0032$ and 0.0074, respectively) than those in the low CD163 and CD204 expression groups (Fig. 2A and B). Interestingly, we found a significant difference in overall survival ($P = 0.0032$) and cancer-specific survival ($P = 0.0019$) among the three TAM status groups (Fig. 2C).

3.3. Survival analyses of multivariate hazard ratio between the TAM status and other variables

We next investigated whether the influence of CD163 and CD204 expression on overall survival was affected by any of the clinical, pathological or epidemiological

Table 1
CD163 and CD204 expressions in patients presenting with various clinicopathological features.

Clinicopathological feature	Total N	CD163 expression		*P* value	CD204 expression		*P* value
		Low	High		Low	High	
All cases	305	145	160		159	146	
Age (years), median ± SD	66 ± 9.2	64 ± 8.9	67 ± 9.4	0.10	65 ± 9.2	67 ± 9.1	0.033
Gender				0.0070			0.30
Male	272	122	150		139	133	
Female	33	23	10		20	13	
Body mass index, median ± SD	21.7 ± 2.9	21.8 ± 2.8	21.7 ± 3.1	0.79	21.6 ± 2.7	21.9 ± 3.2	0.25
Performance status				0.098			0.34
0	237	119	118		127	110	
1+	68	26	42		32	36	
Tobacco use				0.58			0.060
Yes	255	123	132		139	116	
No	50	22	28		20	30	
Alcohol use				0.97			0.97
Yes	264	124	140		139	125	
No	41	21	20		20	21	
Comorbidity				0.19			0.60
Present	215	97	118		110	105	
Absent	90	48	42		49	41	
Tumour location				0.37			0.16
Upper	49	26	23		30	19	
Lower	256	119	137		129	127	
Histological type				0.088			0.038
Squamous cell carcinoma	279	137	142		150	129	
Adenocarcinoma	15	3	12		3	12	
Others	11	5	6		6	5	
Preoperative treatment				0.16			0.061
Present	109	46	63		49	60	
Absent	196	99	97		110	86	
Pathological stage				<0.0001			0.011
I	123	77	46		77	46	
II	78	30	48		36	42	
III	104	38	66		46	58	
Vascular invasion				0.0001			0.0003
Present	149	54	95		62	87	
Absent	156	91	65		97	59	
Lymph vessel invasion				0.0002			0.0044
Present	160	60	100		71	89	
Absent	145	85	60		88	57	
PD-L1 status				0.002			0.0088
Positive	53	15	38		19	34	
Negative	252	130	122	0.13	140	112	
Postoperative treatment							0.28
Present	75	30	45		35	40	
Absent	230	115	115		124	106	

SD, standard deviation.

variables. The effects of CD163 and CD204 were not significantly modified by age, comorbidity, performance status (PS), histological type, preoperative treatment or pathological stage ($P > 0.05$ for all interactions) (Fig. 3A and B). We also determined the overall survival of patients in different TAM status groups (TAM-high and TAM-moderate group vs. TAM-low group). The overall survivals in each group were not significantly modified by age, comorbidity, PS, histological type, preoperative treatment or pathological stage (Supplementary Figure 2).

3.4. TAMs and patient survival in oesophageal SCC

SCC is the predominant type of oesophageal cancer in the East, including Japan. Therefore, we also analysed the survival of patients with SCC (n = 279). Overall and cancer-specific survival differed significantly among the three TAM statuses in this group (log rank P = 0.0036 and 0.0012, respectively); univariate analysis revealed a higher overall mortality in the TAM-high and TAM-moderate groups than in the TAM-low group (HR = 1.76, 95% CI 1.17–2.73, P = 0.0063). Similar

results were obtained for cancer-specific survival and disease recurrence rates.

3.5. Multivariate cox regression analysis

In the model including PS, preoperative treatment and tumour stage, the TAM status was associated with significantly higher overall mortality (multivariate HR: 1.57; 95% CI: 1.03–2.38; $P = 0.037$) (Supplementary Table 2). In this model, the overall survival was reduced in patients with advanced the tumour stage (multivariate HR: 2.88; 95% CI: 1.97–4.21; $P < 0.0001$), worse PS (multivariate HR: 2.78; 95% CI: 1.76–4.29; $P < 0.0001$) and preoperative treatment (multivariate HR: 1.51; CI 1.03–2.20; $P = 0.035$).

3.6. PD-L1 expression levels in oesophageal cancer cell lines

We first performed an *in vitro* assay using oesophageal cancer cell lines. The mRNA levels of *PD-L1* in ten oesophageal cancer cell lines were determined by RT-qPCR (Supplementary Figure 3). We selected TE-8 and KYSE-30 cells as low and moderate expression of *PD-L1* mRNA, respectively.

3.7. Influence of TAMs on malignant behaviour

To clarify the mechanism by which TAMs affect the malignant phenotype, we cocultured TAMs with macrophages or activated macrophages and examined the change in migration and invasion ability. Coculturing with macrophages did not increase the motilities of TE-8 and KYSE-30 cells but did increase their moving distances (Fig. 4A and B). In the invasion assay, the number of invading cells was significantly higher when cocultured with activated macrophages (Fig. 4C and D). These results suggest that TAMs contribute to the upregulation of migration and invasion activity.

3.8. Influence of TAMs on PD-L1 expression in tumour cells

We first performed double immunostaining for CD163 and PD-L1. PD-L1 expression in the cancer cells was strong where the periphery contacted with TAMs, suggesting that TAMs contribute to PD-L1 expression (Fig. 5A). Next, we performed an *in vitro* coculture assay with macrophages or activated macrophages. Coculture with activated macrophages increased the PD-L1 expression in both TE-8 and KYSE-30 cells (qRT-PCR analysis, Fig. 5B and C). Flow cytometry analysis also confirmed a tendency for higher PD-L1 expression in cocultures with activated macrophages than in other cultures (Fig. 5D and E). These results suggest that TAMs can upregulate the PD-L1 expression level in oesophageal cancer cells.

4. Discussion

We examined the relationship among TAM density, PD-L1 expression and clinical outcomes in 305 patients who had undergone resection of oesophageal cancer and confirmed the extracted data in *in vitro* assays. Given the current recognition of TAMs as an attractive target in cancer therapy, a better understanding of the TAM status in human tumours is increasingly important. To our knowledge, we present the first evaluation of the prognostic features of CD163 and CD204 expression in more than 300 cases of resected oesophageal cancer. These findings may be clinically valuable but must be confirmed in independent cohorts in future. In addition, further studies are necessary to examine whether these findings can be applied to a Western population in which the vast majority of patients have adenocarcinoma. We also found a positive relationship between TAM density and PD-L1 expression. Because immune checkpoint inhibitors such as anti–PD-1 agents have delivered promising clinical trial data and will likely be developed as next-generation treatments for several types of cancer, these findings should have considerable clinical implications.

TAMs promote many important features of tumour progression, such as angiogenesis, invasion and proliferation [1,2,34]. For example, the TAM releases growth factors such as vascular endothelial growth factor, platelet-derived growth factor and TGF-β and contributes to angiogenesis [7,35,36]. In addition, the TAM acquires malignant phenotype cells by TNF-α [37]. TAMs are also known to be involved in metastasis through the secretion of interleukin-1α (IL-1α) and epidermal growth factor (EGF) [38,39]. In addition, TAMs respond to cytokines by entering proinflammatory or anti-inflammatory activation states [10]. The TAM exhibits immunoregulatory effects in response to stimulation or education by cytokines (e.g., IL-4, IL-13, CSF-1 and TGF-β) in many solid tumours [35,36]. Conversely, TAMs exhibit immunostimulatory effects in response to IFN-γ and TNF-α [40]. As such, they are attractive targets for recalibrating the immune response within the tumour microenvironment. Mechanistically, tumour cells synthesise CSF-1, which stimulates macrophages to move and produce EGF. In turn, EGF activates migration of the tumour cells [34]. As macrophages and tumour cells move in lock-step, inhibiting either the EGF or CSF-1 signalling pathways inhibits the migration and chemotaxis of both cell types, although the CSF-1 and EGF receptors are restricted to macrophages and tumour cells, respectively [34,41].

There are a number of limitations in this study. For example, some patients of this cohort had received preoperative treatment. Importantly, we found that the prognostic effects of TAMs were not significantly modified

by preoperative treatment. However, it is still uncertain whether preoperative treatment affects the relationship between TAM infiltration and PD-L1 expression. In addition, the number of female cases is limited in this study (n = 33). The relationship between TAM and gender may be important. For example, androgen receptor (AR) may play a role in oesophageal cancer development. AR expression has been inversely correlated with immune cell infiltration in Human Epidermal Growth Factor Receptor Type 2 (HER2)-positive breast cancer [42]. In this study, CD163 expression was significantly higher in female than in male cases (Table 1, P = 0.0070). However, the prognostic effects of CD163 and CD204 were not significantly modified by gender (Supplementary Figure 4). Thus, the relationship among TAM expression, gender and clinical outcomes in oesophageal cancer should be evaluated by independent studies.

Several studies showed that chemotherapy can increase TAM recruitment to tumours, leading to tumour resistance and recurrence after chemotherapy [43–45]. Zhao *et al.* also showed that macrophage infiltration was increased in the presence of several commonly used chemotherapy drugs, including cyclophosphamide, docetaxel, doxorubicin and 5-Fu [46]. In our study, preoperative treatment showed a tendency to influence TAM invasion but the effect was not significant. Further study is needed to clarify the relationship between TAMs and preoperative treatment.

Recently, macrophage-targeting therapies such as CSF-1/CSF-1R blockade have gained traction in cancer research. In several types of solid tumours, CSF-1/CSF-1R blockade reduces the infiltration of M2-like macrophages into tumour tissue and increases the CD8+/CD4+ T-cell ratio [47]. In another report, CSF-1R inhibition added to T-cell checkpoint immunotherapy increased the effectiveness of pancreatic cancer treatment [48]. An ongoing clinical trial is currently evaluating the combination of CSF-1R antagonists and a PD-1/PD-L1 inhibitor (NCT02323191). Therefore, clarifying the relationship between TAMs and PD-1/PD-L1 expression is imperative. Gordon *et al.* [49] showed that blockading PD-1/PD-L1 *in vivo* increases macrophage phagocytosis, reduces tumour growth and prolongs the survival in mouse models of cancer. Harada *et al.* [50] suggested an association between TAM infiltration and upregulated PD-L1 expression in gastric cancer. In our experiments, activated macrophages upregulated the PD-L1 expression in oesophageal tumour cells. These findings suggest that treatment by a CSF-1R antagonist can reduce tumour cell PD-L1 expression and enhance the therapeutic effect of PD-1/PD-L1 blockade.

5. Conclusions

TAMs are a potentially important prognostic biomarker. In addition, TAMs enhance the expression of PD-L1 in tumour cells. Given that cancer immunotherapies targeting these TAMs and PD-1/PD-L1 pathways are actively sought today, the current findings should have considerable implications.

Acknowledgements

The authors thank Leonie Pipe, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Role of funding source

This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, grant numbers 17H04273, 17K19702, and 17KK0195 (to Y.B.).

Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.01.018.

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