


ORIGINAL ARTICLE

Tumor-infiltrating M2 macrophage in pretreatment biopsy sample predicts response to chemotherapy and survival in esophageal cancer

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Abstract

The association between the tumor microenvironment (TME) and treatment response or survival has been a recent focus in several types of cancer. However, most study materials are resected specimens that were completely modified by prior chemotherapy; therefore, the unmodified host immune condition has not yet been clarified. The aim of the present study was to evaluate the relationship between TME assessed in pretherapeutic biopsy samples and chemoresistance in esophageal cancer (EC). A total of 86 endoscopic biopsy samples from EC patients who received neoadjuvant chemotherapy (NAC) prior to surgery were evaluated for the number of intratumoral CD4⁺ lymphocytes (with/without Foxp3 expression), CD8⁺ lymphocytes (with/without PD-1 expression), monocytes (CD14⁺) and macrophages (CD86⁺, CD163⁺ and CD206⁺) by multiplex immunohistochemistry (IHC). The number of tumor-infiltrating CD206⁺ macrophages I significantly correlated with cT, cM, cStage and neutrophil/lymphocyte ratio (NLR), whereas the number of lymphocytes (including expression of Foxp3 and PD-1) was not associated with clinico-pathological features. The high infiltration of CD163⁺ or CD206⁺ macrophages was significantly associated with poor pathological response to NAC ($P = 0.0057$ and 0.0196 , respectively). Expression of arginase-1 in CD163⁺ macrophages tended to be higher in non-responders (29.4% vs 18.2%, $P = 0.17$). In addition, patients with high infiltration of M2 macrophages exhibited unfavorable overall survival compared to those without high infiltration of M2 macrophages (5-year overall survival 57.2% vs 71.0%, $P = 0.0498$). Thus, a comprehensive analysis of TME using multiplex IHC revealed that M2 macrophage infiltration would be useful in predicting the response to NAC and long-term survival in EC patients.

KEYWORDS

biopsy, esophageal cancer, M2 macrophage, multiplex immunohistochemistry, neoadjuvant chemotherapy

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1 | INTRODUCTION

Neoadjuvant chemotherapy (NAC) is widely used to treat locally advanced esophageal cancer (EC) and to eradicate micro-metastases prior to reduction of the primary tumor by surgical resection.¹ However, NAC non-responders experience severe adverse effects and end up facing expensive medical costs without any survival benefit.² Therefore, it is clinically urgent, although challenging, to accurately predict the treatment response before induction of chemotherapy and to determine the optimal treatment strategy for each patient.

In addition to the biological malignancy of tumor cells and the influence of genetic characteristics on sensitivity to therapeutic agents, the immune condition of the tumor microenvironment (TME), consisting of varieties of immune cells, was recently recognized to modulate the efficiency of chemotherapy.³ Among the components of the TME, tumor-infiltrating macrophages (TAM) have been reported to contribute to chemoresistance by modulating the cytotoxicity of tumor-infiltrating T cells.^{4,5} We previously reported the association between TAM and chemotherapy efficacy in EC patients by analyzing the immunohistochemistry (IHC) of resected specimens.⁶ However, as found in other reports, an evaluation of tumor-infiltrating immune cells in resected specimens does not necessarily represent the unmodified immune microenvironment in cancer tissue because of significant modification of both cancer and immune cells from prior chemotherapy.

Recently, multiplex IHC has emerged as a useful technique for evaluating multiple immune parameters on a single slide.⁷ Compared to traditional single-color IHC, multiplex IHC is more efficient and contains many more information sets, which enable us to identify the quantitative and spatial relationships of immune cells in the TME.⁸ The present study aimed to investigate the pre-therapeutic host immune condition to clarify factors associated with chemoresistance or patient survival in EC by performing multiplex IHC of various immune cells, including lymphocytes and macrophages, in pre-therapeutic biopsy samples.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

This study included a total of 86 EC patients admitted for neoadjuvant chemotherapy followed by surgical resection between 2010 and 2015 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University. Tissue samples were obtained through endoscopic biopsies before the administration of NAC in all 86 patients confirmed to have squamous cell carcinoma by histopathological examination. These patients received two cycles of NAC, followed by subtotal esophagectomy with two-field or three-field lymphadenectomy. Clinico-pathological evaluations were performed according to the TMN classification of the Union

for International Cancer Control.⁹ All cancer patients provided written informed consent before tissue sampling according to the guidelines of the Declaration of Helsinki. This study was approved by the Institutional Ethics Committee of Osaka University Hospital (Osaka, Japan).

2.2 | Indication and regimens for neoadjuvant chemotherapy

Our indication for NAC was based on the TMN classification. We considered cT1-3N1-3 an absolute indication, and either cT3N0 or cT4Nany a relative indication, except lesions with massive infiltrations to the bronchus or aorta. The remaining cT4 patients with massive invasion were indicated for preoperative chemoradiotherapy, whereas cT1-2N0 patients were treated by surgery without preoperative therapy. Our hospital used an NAC regimen that comprised two cycles of triplet chemotherapy with 5-FU, cisplatin and docetaxel, as described previously.¹⁰⁻¹² Briefly, cisplatin was administered at 70 mg/m², docetaxel at 70 mg/m² by rapid intravenous infusion on day 1 and 5-FU at 700 mg/m² by continuous intravenous infusion on days 1 to 5. With this regimen, two courses of NAC were used with a 3 to 4-week interval.

2.3 | Assessment of immune cells by multiplex immunohistochemistry

Fluorescent multi-labeling was performed with formalin-fixed paraffin-embedded (FFPE) specimens from all 86 EC patients using the Opal Fluorescent IHC Kit (PerkinElmer, Waltham) according to the manufacturer's instructions. Tissue sections were antigen-retrieved, stained with primary and secondary antibodies, and stained with tyramide signal amplification conjugated to a fluorochrome label. This was followed by heat-mediated antigen stripping to remove the primary antibody in order to label the other antigens with another primary antibody. The primary antibodies were CD4 (clone 4B12, DAKO, Glostrup, Denmark), CD8 (clone C8/144b, DAKO), Foxp3 (clone 236A/E7, Abcam, Cambridge, UK), PD-1 (clone J116, Abcam) and CD14 (clone C-2, Santa Cruz, Texas, US) for one section from FFPE specimens, and CD86 (clone EP1158Y, Abcam), CD163 (clone SP96, Spring Bio, Pleasanton, US), CD206 (clone D-1, Santa Cruz) and arginase-1 (clone C-2, Santa Cruz) for the other serial section from the same specimen. Co-localized signals were detected and captured using the Vectra automated quantitative pathology imaging system (PerkinElmer). For quantitative analysis, the number of fluorescent signal-positive cells in the 670 μm \times 500 μm field was automatically counted using the InForm software (PerkinElmer) along the programmed cell-count algorithm. During microscopic evaluation of the biopsy specimen, the mean cell number in two fields was calculated.

Representative pictures of multiplex IHC for each molecule are shown in Figure 1. We identified the expression pattern of CD4⁺

lymphocytes with/without Foxp3 expression, CD8⁺ lymphocytes with/without PD-1 expression, CD14⁺ cells, CD86⁺ macrophages, and CD163⁺ and CD206⁺ macrophages with/without arginase-1 expression in tissues with esophageal cancer. All of the cells detected within cancer cell nests or stroma at the invasive front were defined as tumor-infiltrating immune cells. All 86 EC patients were classified into two groups: high and low expression groups based on the median number of each type of tumor-infiltrating immune cell. The median cell counts in the 670 μm × 500 μm field were as follows: 189 for CD4⁺ lymphocytes, 73 for Foxp3⁺CD4⁺ lymphocytes, 204 for CD8⁺ lymphocytes, 15 for PD-1⁺CD8⁺ lymphocytes, 557 for CD14⁺ cells, 18.5 for CD86⁺ macrophages, 322 for CD163⁺ macrophages and 142 for CD206⁺ macrophages. In this study, CD86 was used as a marker for M1 macrophages, whereas CD163-positive cells and/or CD206-positive cells were regarded as M2 macrophages; therefore, the total cell count of M2 macrophages was defined as the sum of CD163-positive cells and CD206-positive cells. The median number of M2 macrophages in the field was 376.

2.4 | Histological response to chemotherapy

The histopathological response was evaluated by our hospital pathologists using surgical excision specimens. The histopathological response was categorized according to the criteria of the Japanese Society for Esophageal Diseases.¹³ Depending on the viable residual tumor cells in the entire cancer tissue, five categories were specified: grade 3, no viable residual tumor cells; grade 2, few residual tumor cells; grade 1b, less than two-thirds residual tumor cells; grade 1a, more than two-thirds residual tumor cells; and grade 0, no response to chemotherapy. In the present study, patients with grade 0, 1a and 1b were classified as non-responders, whereas those with grade 2 and 3 were considered responders.¹⁴⁻¹⁶ The patient background in each group is shown in Table S1.

2.5 | Statistical analysis

Relationships between clinico-pathological features and IHC results were analyzed by Mann-Whitney *U* test and Pearson's χ^2 test. The median cell count for each immune cell was used to categorize patients into two groups: high vs low. A logistic regression model was used to evaluate odds ratios related to the poor histopathological response to chemotherapy. Variables with $P < 0.05$ in the univariate analysis were subjected to the multivariate model. Survival curves were estimated using the Kaplan-Meier method and differences in survival were compared using the log-rank test. All statistical analyses were performed using JMP Pro 14 statistical Discovery (SAS Institute). Data are expressed as median (interquartile range) and $P < 0.05$ is considered significant.

3 | RESULTS

3.1 | Tumor-infiltrating immune cells in esophageal cancer and their correlation with clinico-pathological features

1 summarizes the relationship between the infiltration of immune cells (including CD4⁺ or CD8⁺ lymphocytes, CD14⁺ monocytes, and CD163⁺ or CD206⁺ macrophages) and immuno-pathological parameters. None of the background parameters were significantly associated with the number of tumor-infiltrating CD4⁺ or CD8⁺ lymphocytes and CD14⁺ monocytes. In addition, the number of infiltrating PD-1⁺CD8 and Foxp3⁺CD4 lymphocytes did not correlate with immuno-pathological features (data not shown). However, the high number of infiltrating CD86⁺ macrophages was significantly associated with the more advanced cN stage, whereas the high infiltration of CD206⁺ macrophages exhibited a significant association with malignant tumor phenotypes, including the more advanced cT, cM and high NLR. In contrast, we found no significant relationship between the infiltration of CD163⁺ macrophages and immuno-pathological features (1).

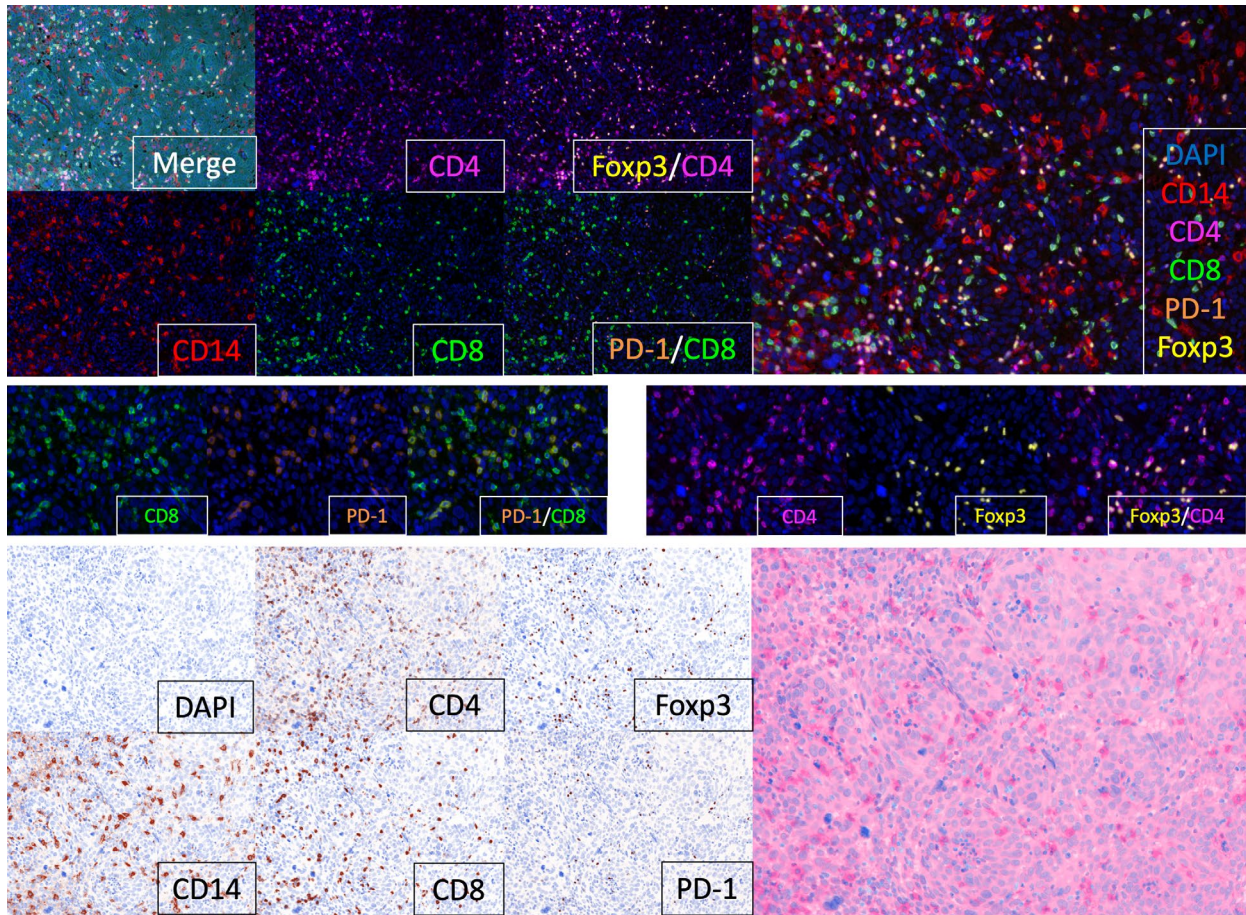
3.2 | High infiltration of M2 macrophages associated with poorer histological response to neoadjuvant chemotherapy

Next, we examined the association between the NAC response and tumor infiltration of various immune cells. The number of tumor-infiltrating CD163⁺ ($P = 0.0057$) and CD206⁺ ($P = 0.0196$) macrophages was significantly higher in non-responders (histological grade 0-1b, $n = 39$) compared to responders (histological grade 2-3, $n = 47$), whereas the number of CD14⁺, CD4⁺, CD8⁺ and CD86⁺ cells did not correlate with the NAC response ($P = 0.91$, $P = 0.34$, $P = 0.86$ and $P = 0.54$, respectively; Figure 2A). The ratio of Foxp3⁺ CD4⁺ lymphocytes tended to be higher in responders than non-responders but with no significance (52.7% vs 42.9%, $P = 0.058$). The ratio of PD-1⁺ CD8⁺ lymphocytes was similar between the two groups (Figure 2B).

Univariate analysis of predictors of the histological response to NAC showed that cT and high infiltration of M2 macrophages, defined as high infiltration of CD163⁺ or CD206⁺ macrophages, were significant. Multivariate analysis further identified the infiltration of M2 macrophages as the only independent predictor of the NAC response (OR = 3.0, 95% confidence interval [CI] = 1.2-7.3, $P = 0.019$; Table 2).

The relationship between the clinical response as assessed by FDG-PET (cutoff: Δ SUVmax reduction of 70%)¹⁷ and the infiltration of M2 macrophages was also evaluated. However, we found no relationship between the clinical response (responders vs non-responders) and M2 macrophage infiltration (the median cell counts for M2 macrophages; 376 vs 619, $P = 0.48$).

(A)



(B)

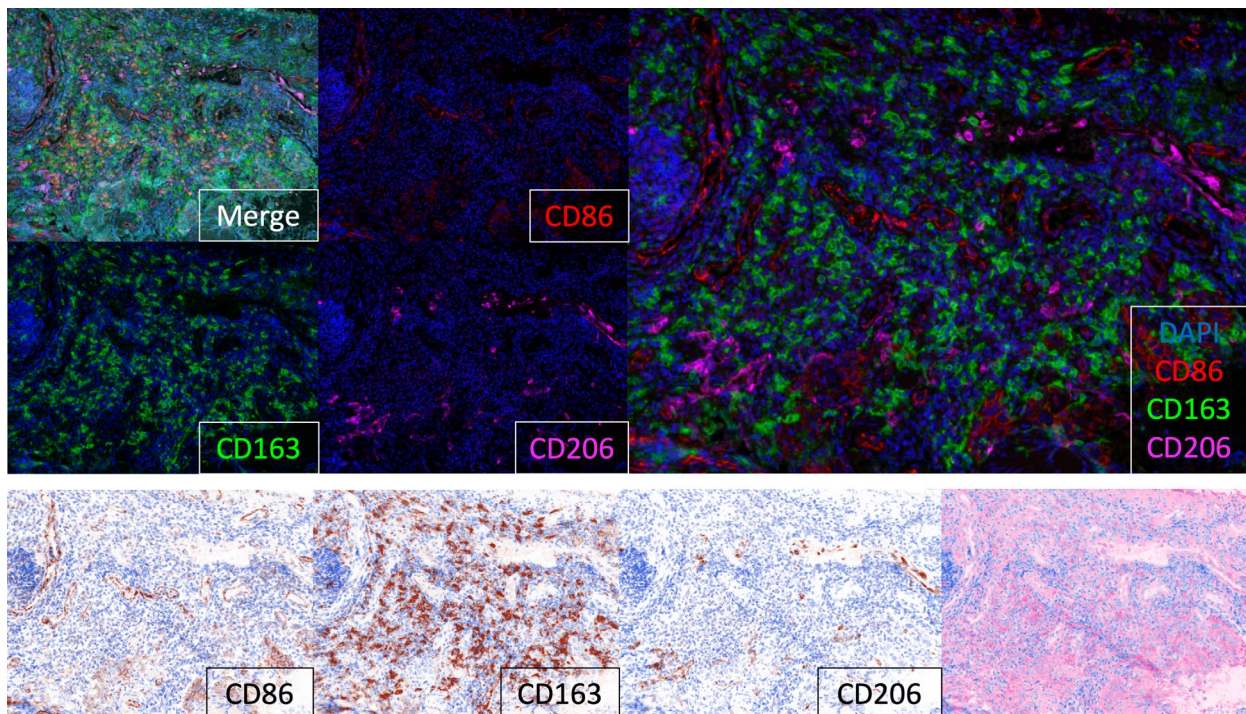


FIGURE 1 Multiplex fluorescent immunohistochemistry of esophageal cancer biopsies (200× fields). A, CD14, CD4, CD8, Foxp3, PD-1, DAPI and cytokeratin. B, CD86, CD163, CD206, DAPI and cytokeratin. In each panel: top, fluorescence imaging; bottom left, bright color imaging; bottom right, pseudocolor H&E imaging

TABLE 1 Tumor-infiltrating immune cells in esophageal squamous-cell carcinoma and their correlation with clinico-pathological features

(A)	CD4 ⁺ cells			CD8 ⁺ cells			CD14 ⁺ cells		
	High n = 43	Low n = 43	P-value	High n = 43	Low n = 43	P-value	High n = 43	Low n = 43	P-value
(n = 86)	67 (50-77)	66 (47-79)	0.49	68 (50-79)	66 (47-78)	0.83	66 (48-79)	68 (47-78)	0.97
Age, y	38/5 (88.4%/11.6%)	37/6 (86.0%/14.0%)	0.75	38/5 (88.4%/11.6%)	37/6 (86.1%/13.9%)	0.75	38/5 (88.4%/11.6%)	37/6 (86.1%/13.9%)	0.75
Sex, n (%)	20.2 (15.4-28.4)	21.6 (15.5-27.2)	0.12	20.2 (15.4-28.4)	20.9 (15.5-26.9)	0.61	20.4 (15.4-26.6)	20.4 (15.5/28.4)	0.80
BMI, kg/m ²	8/17/18 (18.6%/39.5%/41.9%)	6/26/11 (14.0%/60.5%/25.6%)	0.15	10/19/14 (23.3%/44.2%/32.6%)	4/24/15 (9.3%/55.8%/34.9%)	0.20	8/18/17 (18.6%/41.9%/39.5%)	6/25/12 (13.9%/ 58.1%/27.9%)	0.32
Location, U/M/L, n (%)	9/34 (20.9%/79.1%)	9/34 (20.9%/79.1%)	1.0	8/35 (18.6%/81.4%)	10/33 (23.3%/76.7%)	0.60	6/37 (14.0%/86.0%)	12/31 (27.9%/72.1%)	0.11
cT, 1 + 2/3 + 4, n (%)	29/14 (67.4%/32.6%)	27/16 (62.8%/37.2%)	0.65	28/15 (65.1%/34.9%)	28/15 (65.1%/34.9%)	1.0	28/15 (65.1%/34.9%)	28/15 (65.1%/34.9%)	1.0
cN, 0 + 1/2 + 3, n (%)	39/4 (90.7%/9.3%)	37/6 (86.1%/13.9%)	0.50	39/4 (90.7%/9.3%)	37/6 (86.1%/13.9%)	0.50	39/4 (90.7%/9.3%)	37/6 (86.1%/13.9%)	0.50
cM, 0/1, n (%)	10/33 (23.3%/76.7%)	11/32 (25.6%/74.4%)	0.80	9/34 (20.9%/79.1%)	12/31 (27.9%/72.1%)	0.45	8/35 (18.6%/81.4%)	13/30 (30.2%/69.8%)	0.21
cStage, 1 + 2/3 + 4, n (%)	3.7 (2.9-4.5)	3.7 (2.4-4.3)	0.46	3.7 (2.4-4.5)	3.8 (2.9-4.3)	0.26	3.7 (2.9-4.5)	3.7 (2.4-4.3)	0.58
Alb, g/dL	0.2 (0-5.9)	0.4 (0-4.2)	0.38	0.3 (0-5.8)	0.2 (0-5.9)	0.41	0.3 (0-5.8)	0.1 (0-5.9)	0.12
CRP, mg/dL	2.9 (1.3-10.8)	3.0 (1.1-12.7)	.53	3.0 (1.5-10.8)	2.9 (1.1-12.7)	0.59	3.0 (1.3-12.7)	2.9 (1.1-7.3)	0.47
(B)	CD86 ⁺ cells			CD163 ⁺ cells			CD206 ⁺ cells		
(n = 86)	High n = 43	Low n = 43	P-value	High n = 43	Low n = 43	P-value	High n = 43	Low n = 43	P-value
Age, y	67 (47-79)	67 (48-78)	0.99	66 (56-79)	67 (47-78)	0.33	68 (47-77)	66 (50-79)	0.87
Sex, n (%)	35/8 (81.4%/18.6%)	40/3 (93.0%/7.0%)	0.11	40/3 (93.0%/7.0%)	35/8 (81.4%/18.6%)	0.11	38/5 (88.4%/11.6%)	37/6 (86.0%/14.0%)	0.75
BMI, kg/m ²	20.1 (15.5-28.4)	21.6 (15.4-27.2)	0.037*	20.4 (15.4-28.4)	20.8 (15.5-27.2)	0.63	20.4 (15.4-28.4)	20.4 (15.5-27.2)	0.63
Location, U/M/L, n (%)	8/16/19 (18.6%/37.2%/44.2%)	6/27/10 (13.9%/62.8%/23.3%)	0.053	8/20/15 (18.6%/46.5%/34.9%)	6/23/14 (13.9%/53.5%/32.6%)	0.77	6/20/17 (14.0%/46.5%/39.5%)	8/23/12 (18.6%/53.5%/27.9%)	0.51
cT, 1 + 2/3 + 4, n (%)	8/35 (18.6%/81.4%)	10/33 (23.3%/76.7%)	0.60	7/36 (16.3%/83.7%)	11/32 (25.6%/74.4%)	0.29	4/39 (9.3%/90.7%)	14/29 (32.6%/67.4%)	.008*
cN, 0 + 1/2 + 3, n (%)	23/20 (53.5%/46.5%)	33/10 (76.7%/23.3%)	0.024*	28/15 (65.1%/34.9%)	28/15 (65.1%/34.9%)	1.0	27/16 (62.8%/37.2%)	29/14 (67.4%/32.6%)	0.65
cM, 0/1, n (%)	36/7 (83.7%/16.3%)	40/3 (93.0%/7.0%)	0.18	38/5 (88.4%/11.6%)	38/5 (88.4%/11.6%)	1.0	35/8 (81.4%/18.6%)	41/2 (95.4%/4.6%)	0.044*
cStage, 1 + 2/3 + 4, n (%)	8/35 (18.6%/81.4%)	13/30 (30.2%/69.8%)	0.21	10/33 (23.3%/76.7%)	11/32 (25.6%/74.4%)	0.80	7/36 (16.3%/83.7%)	14/29 (32.6%/67.4%)	0.079
Alb, g/dL	3.7 (2.9-4.4)	3.7 (2.4-4.5)	0.30	3.7 (2.9-4.4)	3.8 (2.4-4.5)	0.44	3.7 (2.4-4.4)	3.7 (2.9-4.5)	0.46
CRP, mg/dl	0.3 (0-5.9)	0.1 (0-5.8)	0.46	0.3 (0-5.9)	0.1 (0-5.8)	0.09	0.3 (0-5.9)	0.1 (0-5.8)	0.084
NLR	3.0 (1.1-8.1)	2.9 (1.3-12.7)	0.61	3.3 (1.3-12.7)	2.8 (1.1-7.3)	0.056	3.2 (1.1-12.7)	2.8 (1.3-6.1)	0.042*

Note: The median value of each cell count was used to divide the patients into high and low expression groups. Data were analyzed using the Mann-Whitney U test and Pearson's χ^2 test. * $P < 0.05$ was considered significant

3.3 | Relationship between M2 macrophages and regulatory T cells and significance of arginase-1 expression in M2 macrophages

We evaluated the association between the tumor infiltration of M2 macrophages and Foxp3⁺CD4⁺ lymphocytes. However, no significant correlation was identified between CD163⁺ or CD206⁺

macrophages and Foxp3⁺CD4⁺ lymphocytes (Figure S1; $P = 0.36$ and $P = 0.94$, respectively). Next, the expression of arginase-1 in CD163⁺ and CD206⁺ macrophages was analyzed (Figure 3A). The positivity of arginase-1 in CD163⁺ macrophages and CD206⁺ macrophages is 23.6% and 14.7%, respectively. The ratio of arginase-1⁺ CD163⁺ macrophages tended to be higher in non-responders compared to responders, but this difference was not significant (29.4% vs 18.2%,

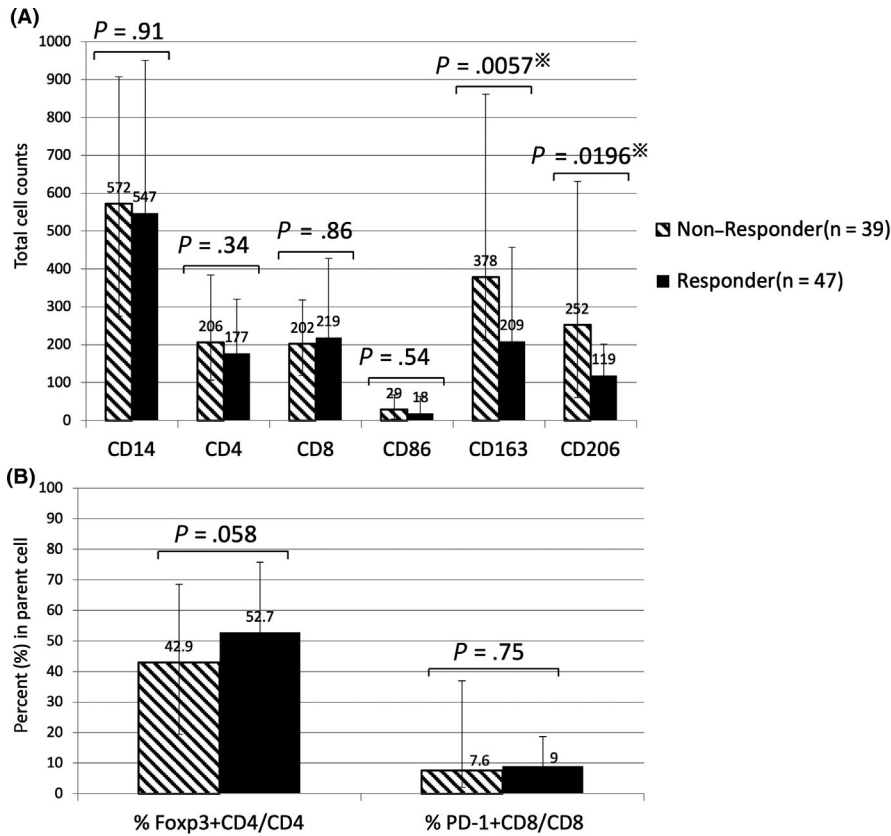


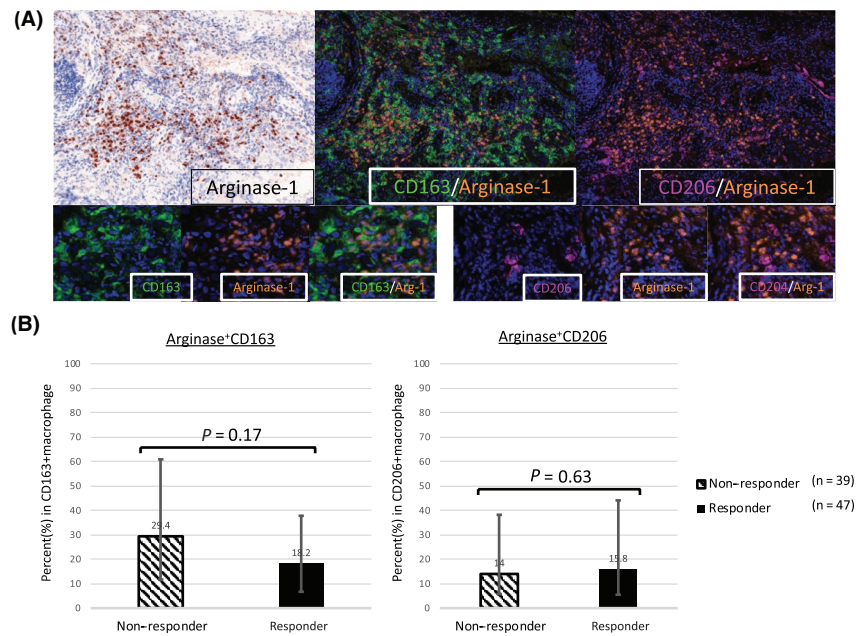
FIGURE 2 The association between the histopathological response to chemotherapy and the number of tumor-infiltrating immune cells. A, CD14, CD4, CD8, CD86, CD163 and CD206 cells in non-responders and responders. B, The ratio of Foxp3⁺CD4⁺ in CD4⁺ lymphocytes and PD-1⁺CD8⁺ in CD8⁺ lymphocytes as a percentage of the parent populations

TABLE 2 Univariate and multivariate analysis of poor histopathological response to chemotherapy

(n = 86)	Univariate analysis			Multivariate analysis		
	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Age (≥ 65 y)	0.6	[0.24-1.5]	0.26			
Sex (male)	1.5	[0.43-5.4]	0.51			
cT (3, 4)	3.0	[1.1-9.1]	0.047*	2.5	[0.77-7.9]	0.13
cN (2, 3)	1.4	[0.57-3.4]	0.47			
cM (1)	2.1	[0.50-8.7]	0.31			
NLR (high)	1.3	[0.57-3.1]	0.52			
CD8/CD4 (high)	1.3	[0.57-3.1]	0.52			
% Foxp3 ⁺ CD4/CD4 (high)	0.52	[0.22-1.2]	0.13			
% PD-1 ⁺ CD8/CD8 (high)	0.91	[0.39-2.1]	0.83			
CD86 ⁺ macrophage (high)	1.3	[0.57-3.1]	0.52			
M2 macrophage (high)	2.9	[1.3-7.8]	0.010*	3.0	[1.2-7.3]	0.019*

Note: Clinico-pathological features and the numbers of tumor-infiltrating immune cells were analyzed using a logistic regression model to clarify the factors related to a poor histopathological response to chemotherapy. Total cell count of M2 macrophages was defined as the sum of CD163-positive cells and CD206-positive cells. The median M2 macrophage count was used to divide the patients into high and low infiltrating groups (n = 42 vs 44, respectively). CI, confidence interval * $P < 0.05$.

FIGURE 3 Difference in arginase-1 expression in M2 macrophages among each therapeutic response. A, Representative immunohistochemical images of M2 macrophages. B, The ratio of arginase-1⁺CD163⁺ and arginase-1⁺CD206⁺ macrophages for each therapeutic response. $P < 0.05$ (Mann-Whitney *U* test) was considered significant



$P = 0.17$; Figure 3B). In addition, no significant difference was found in the ratio of arginase-1⁺ CD206⁺ macrophages between responders and non-responders ($P = 0.63$).

3.4 | Impact of M2 macrophage infiltration on esophageal cancer prognosis

The 2-year and 5-year overall survival rates in all cases were 74.9 and 64.5%, respectively. NAC non-responders had a significantly worse prognosis than responders (2-year overall survival: 67.4% vs 84.1%, 5-year overall survival: 56.9% vs 73.7%, $P = 0.036$; Figure 4). Notably, patients with high tumor infiltration of M2 macrophages had a significantly worse prognosis than those with low infiltration (2-year overall survival: 68.1% vs 81.3%, 5-year overall survival: 57.2% vs 71.0%, $P = 0.0498$). This tendency was more remarkable in stage 3 + 4 patients (2-year overall survival: 62.2% vs 80.7%, 5-year overall survival: 47.9% vs 67.7%, $P = 0.0504$), but the survival difference was not significant in stage 1 + 2 patients (2-year overall survival: 88.9% vs 83.3%, 5-year overall survival: 88.9% vs 83.3%, $P = 0.73$).

4 | DISCUSSION

In the present study, the high infiltration of CD206⁺ macrophages in pre-therapeutic endoscopic biopsies from a total of 86 EC patients who received NAC followed by surgery was associated with malignant tumor phenotypes, including more advanced cT and cM, and high NLR. We are the first to demonstrate that, among several types of immune cells, the high tumor infiltration of M2 macrophages (defined as either CD163⁺ or 206⁺ macrophages) in pre-therapeutic TME is an independent predictor of the NAC response. The ratio of arginase-1⁺CD163⁺ macrophages tended to be higher in

non-responders than responders. In addition, high infiltration of M2 macrophages was associated with poor prognosis in EC, especially advanced cases.

There are two classic TAM phenotypes: pro-inflammatory M1 macrophages with antitumor function and anti-inflammatory M2 macrophages with tumor supportive function.¹⁸ Recent studies have revealed that M2 macrophages consist of three different subpopulations (M2a, M2b and M2c) that act through distinct signal pathways, but the function of each subpopulation is not fully understood.¹⁹ In this study, we focused on M1/M2 macrophage classification and reported that high infiltration of CD206⁺ macrophages correlates with malignant tumor phenotypes. We previously reported a significant correlation between CD163⁺ macrophages and advanced cT, and positive lymphatic and blood vessel invasion in EC patients.⁶ Shigeoka et al also reported high infiltration of CD204⁺ macrophage-mediated angiogenesis with upregulation of vascular endothelial growth factor-A mRNA and significant association with more malignant phenotypes in EC, including depth of tumor invasion, lymph and blood vessel invasion, lymph node metastasis, and clinical stages.²⁰ Our results are consistent with most published data on the relationship with tumor progression and infiltration of M2 macrophages.²¹ As for markers of TAM, CD163 and CD206, the haptoglobin-hemoglobin scavenger receptor and the macrophage mannose receptor 1, respectively, are not exclusive markers of the M2 phenotype, as CD163 is expressed on some monocyte subsets and CD206 is expressed on immature dendritic cells other than M2 macrophages.²¹ In previous reports, CD163 and CD206 exhibited mutually exclusive induction, and M2 macrophages have partially overlapping expression patterns for CD163 and CD206.^{22,23} The recent study revealed an inverse relationship between CD163 and CD206 expression on TAM as they near the cancer nests, defining tumor-associated CD163⁺CD206⁻, CD163⁺CD206⁺ and CD163⁻CD206⁺ macrophages as M2-like macrophages.²⁴ Therefore, considering this background, we defined

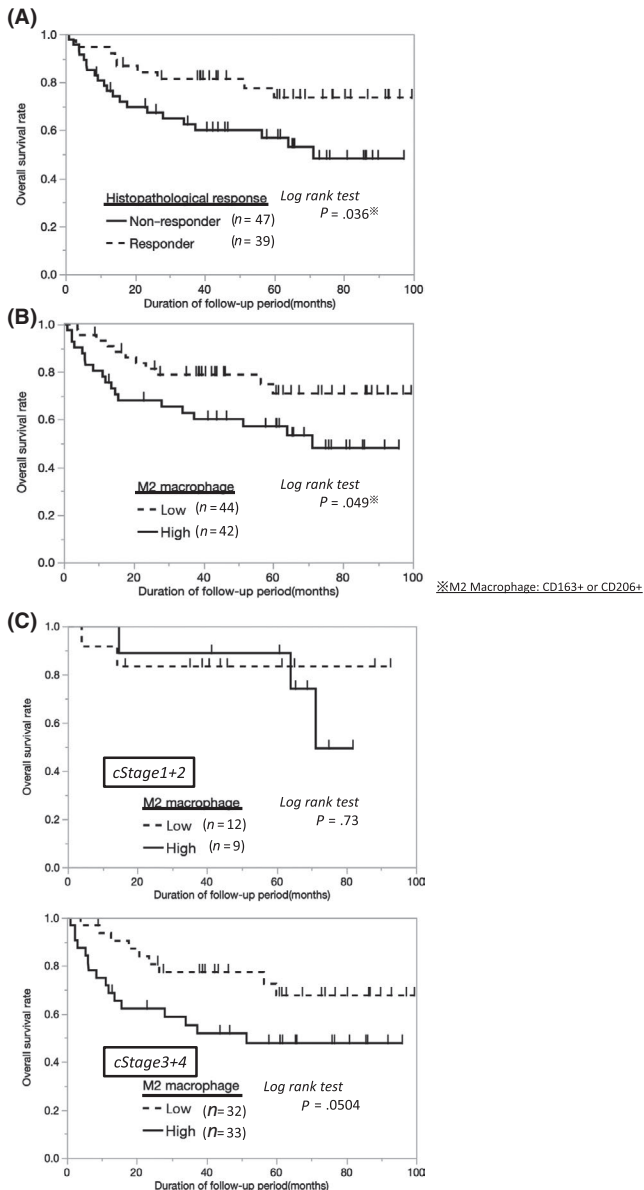


FIGURE 4 Kaplan-Meier curves of overall survival. A, According to the histopathological response to chemotherapy. B, According to the infiltration of M2 macrophages (CD163⁺ or CD206⁺). C, Patients in stage 1/2 and stage 3/4 according to the infiltration of M2 macrophages

CD163-positive cells and CD206-positive cells as M2 macrophages in this study. Nevertheless, because macrophages comprise several subtypes and are heterogeneously activated into TAM depending on the TME and organ-specific microenvironment, recent studies have shown the insufficiency of conventional classification (ie, M1/M2) to precisely distinguish macrophage phenotypes.^{19,21,25} Further studies should be conducted to clarify significant prognostic markers of TAM in EC.

In an analysis of pre-therapeutic biopsy specimens that should represent unmodified host immune conditions, we demonstrated that infiltration of M2 macrophages negatively impacts the efficacy of chemotherapy in addition to long-term survival in EC patients.

High infiltration of TAM was previously reported to be associated with a progressive tumor state and poor prognosis, mediating the proliferation of tumor cells and angiogenesis.¹⁹ However, only a few reports have clarified the relationship between TAM and chemoresistance. In particular, in EC, we previously reported the association of TAM with NAC efficacy through immunochemical examination of CD8⁺ lymphocytes and CD68⁺ and CD163⁺ macrophages.⁶ However, these analyses were performed with resected specimens that had been significantly modified by prior chemotherapy. In breast cancer, patients with high amounts of TAM and low amounts of cytotoxic T cells had a limited response to NAC.⁵ In contrast, in pancreatic adenocarcinoma, high amounts of CD68⁺ TAM before treatment are associated with improved prognosis only in patients who receive adjuvant gemcitabine-based chemotherapy, and not in untreated patients.²⁶ M2 macrophages express arginase-1, which has been reported to dysregulate the T cell receptor signal and subsequently induce CD8⁺ T cell dysfunction in TME.²⁷ The ratio of arginase-1⁺ CD163⁺ macrophages (one of the immunosuppressive factors) may be closely associated with chemoresistance and tended to be higher in non-responders in the present study.

M2 macrophages recruit regulatory T cells (Tregs) in the TME via a CCL1-CCR1 signal, contributing to immunosuppression.²⁸ However, in the present study, no significant association was identified between the infiltration of M2 macrophages and Tregs in the TME. Saito et al report difficulties distinguishing the suppressive function of tumor-infiltrating Tregs by only the expression of Foxp3, because Tregs consist of functionally distinct subpopulations of naïve Tregs, effector Tregs and non-suppressive Tregs.²⁹ Considering that tumor-infiltrating Tregs do not always have a suppressive function, we cannot conclude that tumor-infiltrating Tregs do not affect chemoresistance and concomitant infiltration of M2 macrophages. In contrast to the immune-suppressive function of Tregs, the ratio of Foxp3⁺ CD4⁺ lymphocytes was higher in responders than non-responders in the present analysis. Oda et al³⁰ and Lee et al³¹ previously reported that high infiltration of Foxp3⁺ T cells in pretreatment biopsy specimens was significantly correlated with a higher rate of pathological complete response to NAC in breast cancer patients. Considering that Tregs are predominantly eradicated by chemotherapy agents in a murine model,³² it was speculated that the depletion of Tregs by chemotherapy agents might reduce Treg-dependent immune suppression, resulting in reinvigoration of the host antitumor immunity and leading to significant responses to NAC.

This study potentially has several limitations, starting from the problem of a relatively small sample size. Considering the heterogeneous distribution of immune cells, the tissue samples obtained only from the tumor surface by endoscopic biopsy do not necessarily represent the TME. As the biopsy samples are small in size, the biopsy-based assessment does not necessarily represent the status of a whole tumor tissue, particularly for tumors with heterogeneous distribution of immune cells. However, the validity of pre-therapeutic biopsy samples with a TME comparable to that of

the surgically resected specimen has been reported in terms of the CD3/CD8 distribution in colorectal cancer.^{33,34} It may still be useful to predict the response to NAC and poor prognosis in advance by evaluating M2 macrophages in endoscopic biopsy samples. Given that the poor response to NAC was predicted, concurrent radiotherapy and conversion to neoadjuvant immunotherapy would be additional treatment options leading to personalized medicine.

M2 macrophages are a promising therapeutic target.^{3,35} CCR2 or CSF-1R inhibition has been considered to prevent recruitment of M2 macrophages to the TME or proliferation of TAM.³⁶ Several clinical trials have been conducted, and remarkable synergic efficacy with chemotherapeutic agents has been reported but limited efficiency as a single agent.³⁷ Macrophage-targeting therapy is expected to improve the therapeutic efficacy of existing conventional chemotherapy and radiotherapy through activation of anti-cancer immunity.

In conclusion, the present study confirmed that pre-therapeutic M2 macrophage infiltration would be a useful biomarker for predicting the response to NAC among a variety of immune cells in EC patients. Targeting M2 macrophages alongside NAC agents may be a promising treatment option for advanced EC.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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