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Role of tumor-associated macrophages at the invasive front in human colorectal cancer progression

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Abstract

Macrophages are an essential component of antitumor activity; however, the role of tumor-associated macrophages (TAMs) in colorectal cancer (CRC) remains controversial. Here, we elucidated the role of TAMs in CRC progression, especially at the early stage. We assessed the TAM number, phenotype, and distribution in 53 patients with colorectal neoplasia, including intramucosal neoplasia, submucosal invasive colorectal cancer (SM-CRC), and advanced cancer, using double immunofluorescence for CD68 and CD163. Next, we focused on the invasive front in SM-CRC and association between TAMs and clinicopathological features including lymph node metastasis, which were evaluated in 87 SM-CRC clinical specimens. The number of M2 macrophages increased with tumor progression and dynamic changes were observed with respect to the number and phenotype of TAMs at the invasive front, especially at the stage of submucosal invasion. A high M2 macrophage count at the invasive front was correlated with lymphovascular invasion, low histological differentiation, and lymph node metastasis; a low M1 macrophage count at the invasive front was correlated with lymph node metastasis. Furthermore, receiver operating characteristic curve analysis revealed that the M2/M1 ratio was a better predictor of the risk of lymph node metastasis than the pan-, M1, or M2 macrophage counts at the invasive front. These results suggested that TAMs at the invasive front might play a role in CRC progression, especially at the early stages. Therefore, evaluating the TAM phenotype, number, and distribution may be a potential predictor of metastasis, including lymph node metastasis, and TAMs may be a potential CRC therapeutic target.

KEYWORDS

colorectal neoplasms, epithelial-mesenchymal transition, lymphatic metastasis polarization, neoplasm metastasis, tumor-associated macrophages

Abbreviations: AD, advanced cancer; AUC, area under the curve; CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; IM, intramucosal neoplasia; ROC, receiver operating characteristic; SM-CRC, submucosal invasive colorectal cancer; TAM, tumor-associated macrophage; TME, tumor microenvironment.

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers and the third most common cause of cancer-related death.[1] The tumor microenvironment (TME) comprises tumor cells and non-malignant cells, such as immune cells, fibroblasts, adipocytes, and vascular endothelial cells. Immune cells, such as lymphocytes, neutrophils, and macrophages, infiltrate into the TME,[2] with macrophages representing one of the most abundant immune cells, especially in the colon.[3] Macrophages found in the TME are often referred to as TAMs, which include a mixture of tissue-resident and exudative macrophages, with varying proportions depending on the tumor type, location, and stage.[4-6] Macrophages are mainly classified as M1- or M2-polarized macrophages, with M1 macrophages being involved in host defense against viral and microbial infections, and antitumor activity, and M2 macrophages in debris scavenging, angiogenesis, remodeling and repair of damaged tissues, and tumor progression.[7-10] TAMs often exhibit the M2 phenotype[9,11] and are associated with poor prognosis in many types of cancers, such as melanoma[12,13] and breast cancer.[14] Most studies using clinical specimens have reported an association between a high density of TAMs and favorable prognosis in CRC[15-21]; however, recent studies using clinical specimens have reported that a high density of M2 macrophages is associated with an unfavorable prognosis.[22-24] Particularly, animal studies reported that TAMs are associated with malignancy and tumor progression.[22,25-28] Moreover, although it is known that M1 macrophages are involved in mediating antitumor activity, their role in CRC remains unclear because few studies have examined the association between M1 macrophages and CRC prognosis using clinical specimens.[24] Therefore, the role of TAMs in CRC progression is controversial. Moreover, TAM distribution during CRC progression and the role of macrophages in early stage CRC have not been revealed.

To elucidate the distribution and role of TAMs in CRC progression, especially in the early stages, we first evaluated the phenotype and number of TAMs using double immunofluorescence for CD68 (a pan-macrophage marker) and CD163 (an M2 macrophage marker) in 53 patients with colorectal neoplasia, including intramucosal neoplasia (IM), submucosal invasive colorectal cancer (SM-CRC), and advanced cancer (AD). Due to the heterogeneity of macrophage infiltration, the number and phenotype of TAMs were evaluated at multiple sites in the tumor tissues to evaluate TAM distribution. Next, we evaluated the association between TAMs at the invasive front and clinicopathological features, including lymph node metastasis, in 87 SM-CRC clinical specimens. Moreover, the predictive potential of TAMs for the risk of lymph node metastasis was evaluated. Our findings may form a basis for the potential development of TAMs as a predictor of metastasis risk and a therapeutic target in CRC.

2 | MATERIALS AND METHODS

2.1 | Patients

First, 53 patients with colorectal neoplasia (20 with IM (IM group), 20 with SM-CRC (SM group), and 13 with AD (AD group)) treated at Hiroshima University Hospital (Hiroshima, Japan) from 2011 to 2018 were randomly selected. Second, from 132 consecutive patients with SM-CRC undergoing surgical resection with lymph node dissection at the beginning or additional surgical resection with lymph node dissection after an endoscopic resection at Hiroshima University Hospital from March 2013 and June 2019, we excluded 45 patients for the following reasons: history of previous or synchronous cancer, insufficient pathological evaluation, or treated with piecemeal resection, and then the 87 patients were selected. All clinical data were obtained by retrospectively reviewing the patient records. The use of clinical specimens and patient data was approved by the Institutional Review Board of Hiroshima University (No. 2675). This study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. According to the ethical guidelines for medical and health research involving human subjects issued by the Japanese Ministry of Health, Labour, and Welfare, opting-out was used instead of informed consent because of the single-center, retrospective, and observational nature of the study.

2.2 | Histological evaluation

All specimens were evaluated by pathologists (YK or FS) according to the latest World Health Organization classification.[29] A gastrointestinal pathologist (FS) re-evaluated and diagnosed the SM-CRC specimens, specifically the pathology of the invasive front, as described previously.[30]

2.3 | Assessment of the phenotype, number, and distribution of TAMs

The phenotype, number, and distribution of TAMs were assessed using double immunofluorescence for CD68 and CD163 (Figure 1A). M1 macrophages were defined as $CD68^+$ and $CD163^-$, while M2 macrophages were defined as $CD68^+$ and $CD163^+$. TAM distribution was evaluated by dividing the tumor tissue into 4 parts: the center, lateral periphery, and invasive front of the tumor, in addition to the non-neoplastic mucosa adjacent to the tumor, in which the macrophage number and phenotype were evaluated (Figure 1B). The specimens were reviewed under a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan). For each sample, 5 high-power fields (1 high-power field = 0.0988 mm^2)

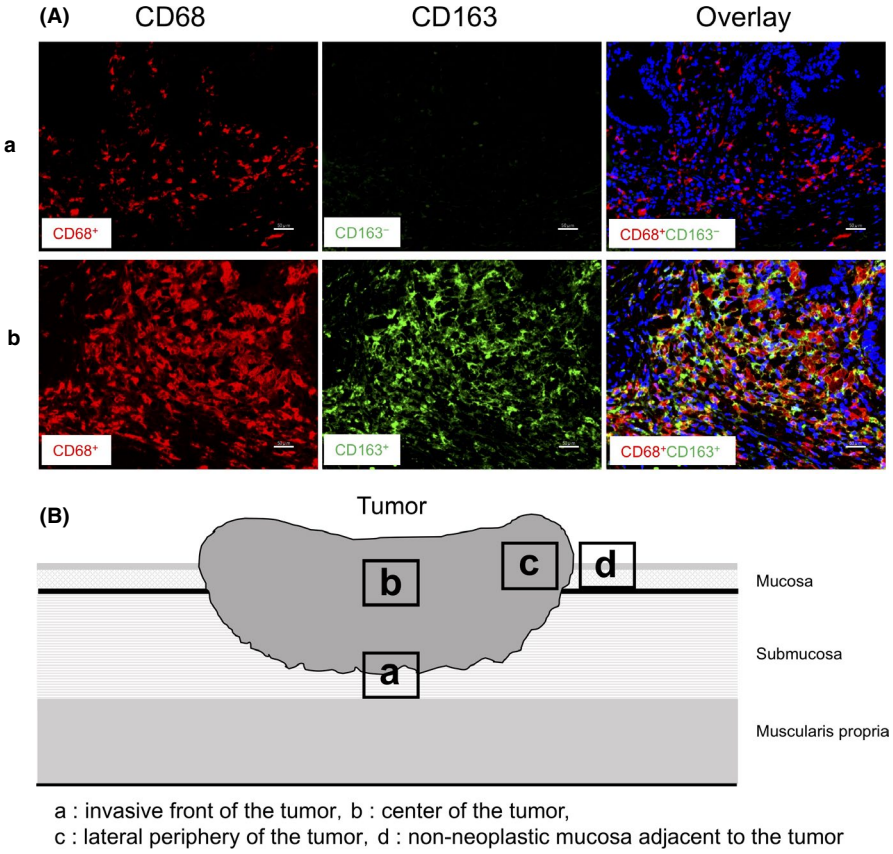

FIGURE 1 Phenotype and distribution of tumor-associated macrophages. A, Double immunofluorescence for CD68 (red) and CD163 (green). Nuclei were stained with DAPI (blue), and the overlay depicts fluorescence detected by all channels. a: $CD68^+CD163^-$ cells (entirely red). M1 macrophages were defined as $CD68^+CD163^-$ cells. b: $CD68^+CD163^+$ cells (the center is red and edge is yellow). M2 macrophages were defined as $CD68^+CD163^+$ cells. Scale bars, 50 μm. B, Schema of the tumor. The number of macrophages was counted in 4 different regions: (a) the invasive front of the tumor, (b) the center of the tumor, (c) the lateral periphery of the tumor, and (d) the non-neoplastic mucosa adjacent to the tumor

showing substantial macrophage infiltration were selected and photographed. With each captured image, the numbers of pan-, M1, and M2 macrophages were counted using an image analyzer (WinRoof software; Olympus).

2.4 | Immunofluorescence staining

Double immunofluorescence was performed using the Opal 4-color manual immunohistochemistry (IHC) kit (NEL810001KT; PerkinElmer). All specimens were fixed in 10% formaldehyde and embedded in paraffin in accordance with the routine procedures at the Department of Clinical Pathology, Hiroshima Universal Hospital. A 4-μm section from each specimen was cut and deparaffinized, followed by heat-induced antigen retrieval for 15 min using a microwave after pretreatment of tissues with 0.3% H_2O_2. The sections were then probed with an anti-CD68 antibody (1:400; 76437; Cell Signaling Technology) for 1 h at 37°C. Next, the sections were washed and incubated with secondary antibodies included in the kit for 15 min at 37°C, followed by washing and incubation at room temperature for 10 min with the Opal 570 reagent provided in the kit. After another round of heat-induced antigen retrieval, the sections were incubated with an anti-CD163 antibody (1:50; NCL-L-CD163; Leica Biosystems) overnight at 4°C. The sections were then washed and incubated with secondary antibodies for 15 min at 37°C, followed by washing and incubation at room temperature for 10 min with the Opal 520 reagent. After washing, the sections were counterstained with DAPI (1:500) for 5 min and mounted in mounting medium (Fluoromount; Diagnostic BioSystems).

2.5 | IHC

IHC was performed using an EnVision™ kit (K5007; Dako). After deparaffinization of the tissues, heat-induced antigen retrieval was performed for 15 min, followed by incubation with the primary antibody anti-E-cadherin (1:100; sc7870; Santa Cruz Biotechnology) overnight at 4°C after pretreatment of tissues with 0.3% H_2O_2. The sections were then washed and incubated with the secondary antibodies included in the kit for 30 min at 37°C, after which the sections were washed and incubated for 7 min at room temperature with 3,3′-diaminobenzidine provided in the kit. The sections were counterstained with hematoxylin and eosin and mounted after dehydration and attainment of transparency.

2.6 | Statistical analysis

Quantitative data are presented as the mean ± SD or percentage. The Shapiro-Wilk test was used to determine whether datasets were normally distributed. Inter-group differences were evaluated using Student *t* test or Mann-Whitney *U* test for quantitative data and a chi-square test or Fisher exact test for categorical data. For multiple comparisons, one-way ANOVA Kruskal-Wallis test, or chi-square

test with a Bonferroni post hoc test was used, as appropriate. All tests were two-tailed. a *P*-value < .05 was considered significant, whereas $P < .0167$ or $P < .0083$ was considered significant for multiple comparisons using a Bonferroni post hoc test. ROC curve analysis with maximal Youden index values was used to identify the optimal cut-off values and evaluate the predictive potential of TAMs for the presence of lymph node metastasis. JMP Pro software (v.14.0.0; SAS Institute) was used for all statistical analyses.

3 | RESULTS

3.1 | Changes in the number, phenotype, and distribution of TAMs in the progression of colorectal neoplasia

Patient samples were classified into 3 groups based on the invasive depth of tumor cells, ie, the IM, SM, and AD groups. Clinicopathological characteristics of each group are summarized in Table 1. In 70% of the patients in the SM group, tumor cells had deeply invaded the submucosal layer (depth ≥ 1000 μm). In the AD group, 46% of the patients had T2 stage cancer (invasion into the muscle layer) and 54% patients had T3 stage cancer (invasion into the subserosa).

In the IM group, macrophages infiltrated into the tumor tissues sparsely and homogenously, with most of the macrophages exhibiting a shift toward the M1 polarization (Figure 2A). In contrast, the total number of macrophages in the SM and AD groups was significantly higher compared with that in the IM group, with M2 macrophages identified in each part (Figure 2B,C). Specifically, we observed dense accumulation of pan-macrophages and M2 macrophages at the invasive front. The number of pan-macrophages and M2 macrophages, and the M2/M1 ratio were higher in the AD group relative to those in the SM group (Figure 3A). In addition, these differences were most significant at the invasive front in each sample, with the number of pan-macrophages and M2 macrophages and the M2/M1 ratio at the invasive front significantly higher in the AD group compared with those in the SM group ($P < .001$ for all groups). Furthermore, in the IM group, the number of M2 macrophages at the invasive front was low in most cases (Figure 3B), whereas in the AD group, it was high in most cases. In contrast, in the SM group, there was a mix of cases with high and low numbers of M2 macrophages at the invasive front. These results indicated that dynamic changes in the number and phenotype of TAMs at the invasive front might occur during CRC progression, especially at the time of submucosal invasion.

3.2 | Clinicopathological characteristics of patients with SM-CRC

Based on the above findings, we aimed to elucidate the role of TAMs at the invasive front during the stage of submucosal

TABLE 1 Clinicopathological characteristics of patients with colorectal neoplasia

	IM	SM	AD
Characteristics	**n = 20**	**n = 20**	**n = 13**
Age (years) Mean ± SD	65.3 ± 8.4	68.9 ± 11.4	65.4 ± 10.9
Gender			
Male	12 (60)	13 (65)	6 (46)
Female	8 (40)	7 (35)	7 (54)
Tumor size (mm) Mean ± SD	15.1 ± 3.9	16.5 ± 4.9	29.4 ± 12.5
Localization			
Right colon	8 (40)	9 (45)	5 (38)
Left colon	6 (30)	8 (40)	7 (54)
Rectum	6 (30)	3 (15)	1 (8)
Morphology			
Flat/Depressed	10 (50)	12 (60)	0 (0)
Elevated	10 (50)	8 (40)	13 (100)
Dominant histological type			
Dysplasia			
Low grade	10 (50)	0 (0)	0 (0)
High grade	10 (50)	0 (0)	0 (0)
Adenocarcinoma			
tub/pap	0 (0)	20 (100)	13 (100)
por/sig/muc	0 (0)	0 (0)	0 (0)
Depth of tumor`			
Intramucosa	20 (100)	0 (0)	0 (0)
T1			
< SM invasion depth 1000 μm	0 (0)	6 (30)	0 (0)
≥ SM invasion depth 1000 μm	0 (0)	14 (70)	0 (0)
T2	0 (0)	0 (0)	6 (46)
T3	0 (0)	0 (0)	7 (54)
T4	0 (0)	0 (0)	0 (0)
Vascular invasion positive	0 (0)	2 (10)	7 (53)
Lymphatic invasion positive	0 (0)	2 (10)	9 (69)
Lymph node metastasis positive	0 (0)	0 (0)	5 (38)
Distant metastasis positive	0 (0)	0 (0)	1 (8)
Recurrence	0 (0)	0 (0)	1 (8)

Note: Abbreviations: muc, mucinous adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; SD, standard deviation; sig, signet-ring cell carcinoma; SM, submucosal; tub, tubular adenocarcinoma.

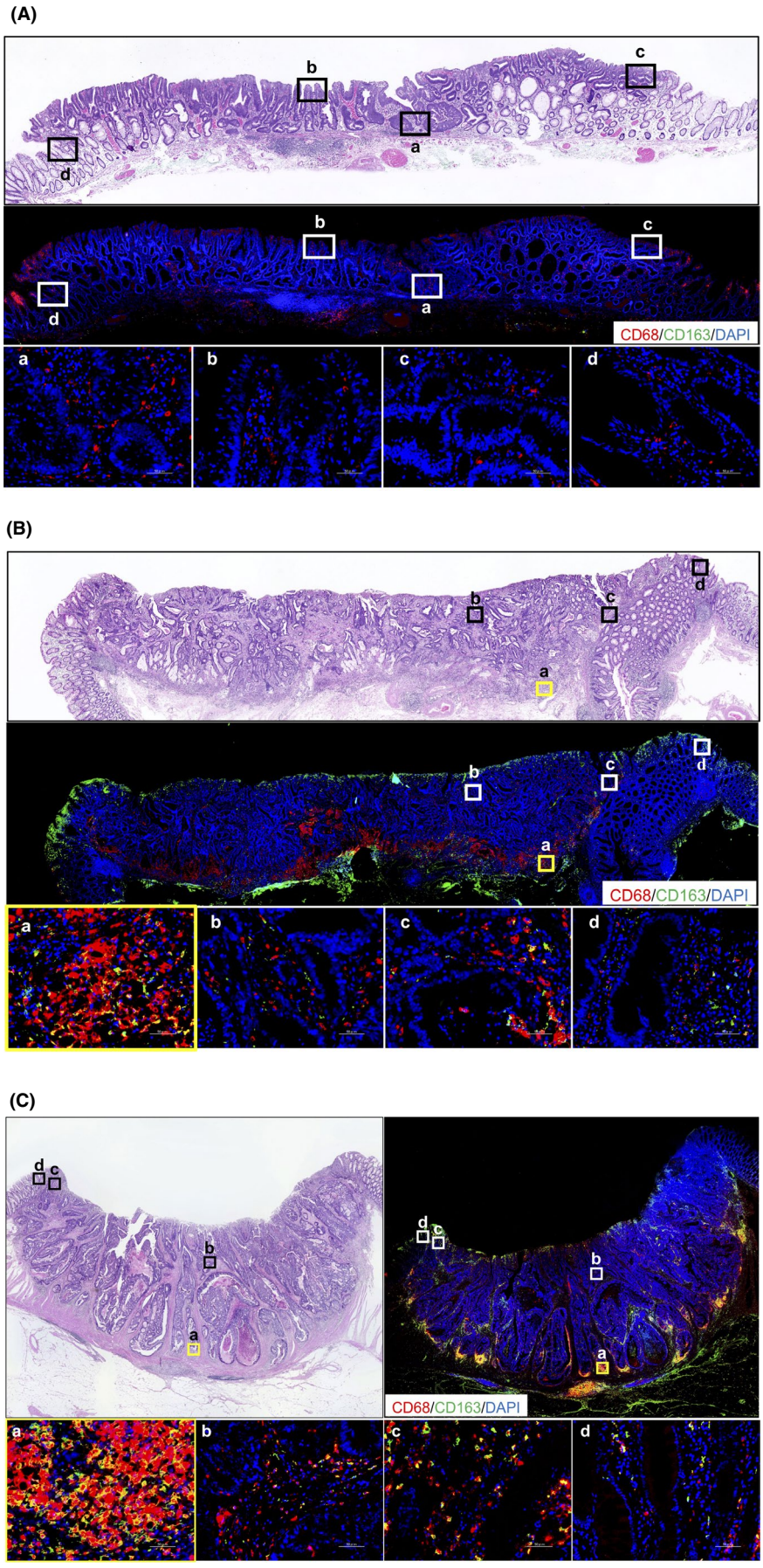

FIGURE 2 Immunofluorescence in specimens from patients with colorectal neoplasia. A, Representative images of immunofluorescence in the IM group (20 patients with IM). A low number of macrophages homogenously infiltrated into the tumor stroma, with most of them being M1 macrophages. Images are of the sigmoid colon with high-grade dysplasia [type 0-IIa (12 × 10 mm)]. B, Representative images of immunofluorescence in the SM group (20 patients with SM-CRC). In the SM group, the number of pan-macrophages was higher compared with that in the IM group, and M2 macrophages were present in each region. Pan-macrophages were abundant, especially at the invasive front. Images are of the ascending colon with moderately differentiated adenocarcinoma [type 0-IIa+IIc, pT1 (submucosal invasion depth: 2000 μm), 15 × 10 mm]. C, Representative images of immunofluorescence in the AD group (13 patients with AD). In the AD group, the number of M2 macrophages was higher compared with that in the SM group, with a dense accumulation of M2 macrophages detected at the invasive front. Images are of the ascending colon with moderately differentiated adenocarcinoma [type 2, pT2 (20 × 15 mm)]. Scale bars, 50 μm. AD, advanced cancer; IM, intramucosal neoplasia; SM-CRC, submucosal invasive colorectal cancer

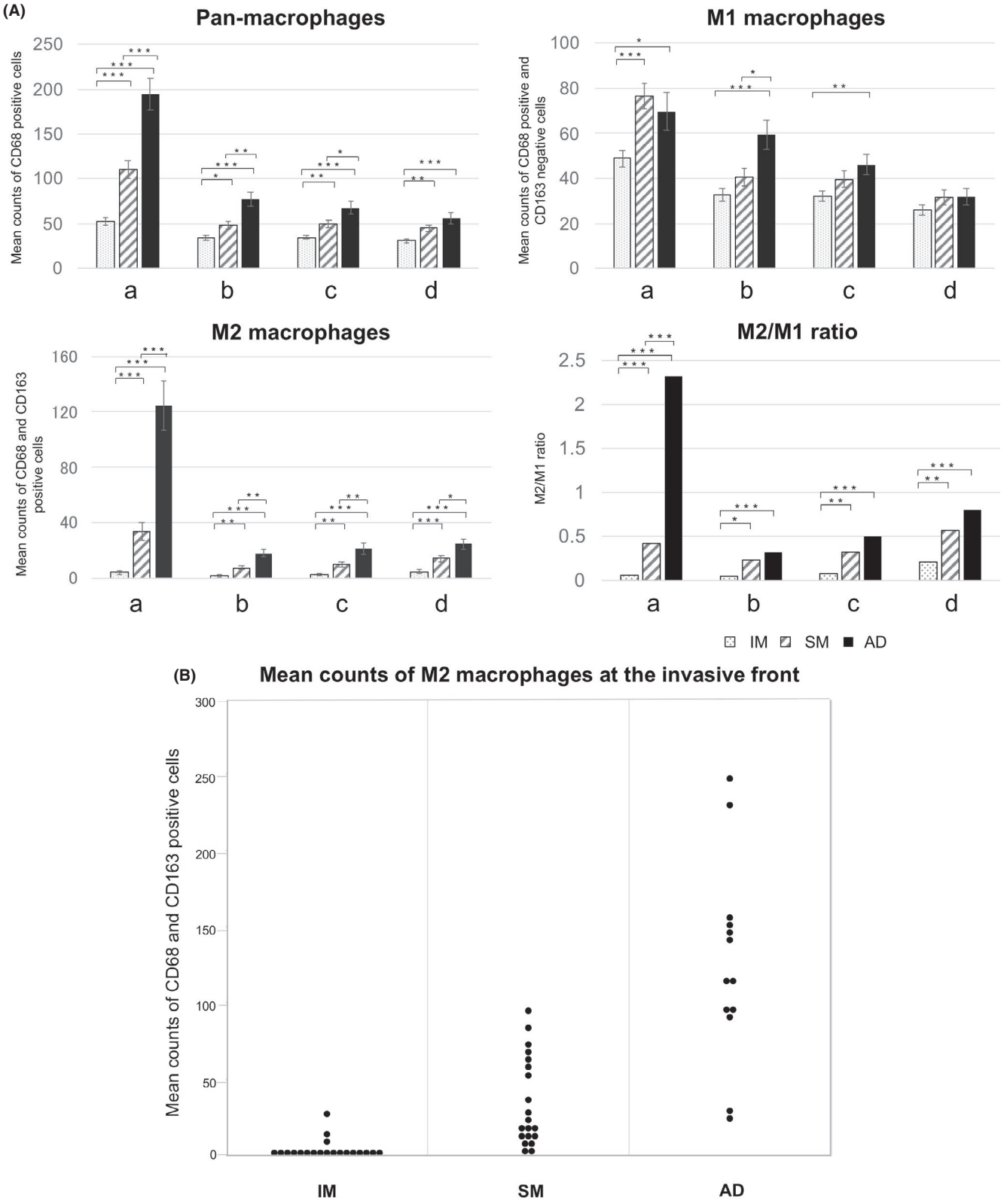

FIGURE 3 Comparison of the number of tumor-associated macrophages in the IM, SM, and AD groups. A, The numbers of pan-macrophages and M2 macrophages, and the M2/M1 ratio in each tumor region were higher in the SM group relative to those in the IM group and in the AD group relative to those in the SM group, with these differences most significant at the invasive front. *$P < .05$, **$P < .01$, ***$P < .001$. Significant predictive variables: $P < .0167$. B, Mean counts of M2 macrophages at the invasive front were measured in each group. In the IM group, the number of M2 macrophages at the invasive front was low in most cases. In the SM group, there was a mix of cases with high and low numbers of M2 macrophages at the invasive front. In the AD group, the number was high in most cases. AD, advanced cancer; IM, intramucosal neoplasia; SM-CRC, submucosal invasive colorectal cancer

invasion. We evaluated the association between TAMs at the invasive front and clinicopathological characteristics, including lymph node metastasis, in clinical SM-CRC specimens. The clinicopathological characteristics are summarized in Table 2. Eight percent of the patients were positive for lymph node metastasis. In 94% patients, tumor cells had deeply invaded the submucosal layer (depth ≥ 1000 μm).

TABLE 2 Clinicopathological characteristics of patients with SM-CRC

Characteristics	n = 87
Age (years) Mean ± SD	67.8 ± 10.8
Gender	
Male	53 (61)
Female	34 (39)
Tumor size (mm) Mean ± SD	26.4 ± 15.9
Localization	
Right colon	29 (33)
Left colon	42 (48)
Rectum	16 (19)
Morphology	
Flat/Depressed	23 (26)
Elevated	64 (74)
Treatment	
Surgery alone	42 (48)
Surgery after endoscopic resection	45 (52)
Dominant histological type	
tub/pap	87 (100)
por/sig/muc	0 (0)
Histological type at invasive front	
W/Mw/pap	44 (51)
Mp/por/sig/muc	43 (49))
SM invasion depth	
Distance (μm) mean ± SD	3563.6 ± 2227.9
< 1000 μm	5 (6)
≥ 1000 μm	82 (94)
Vascular invasion positive	27 (31)
Lymphatic invasion positive	36 (41)
Budding grade 2/3	40 (46)
Lymph node metastasis positive	7 (8)
Distant metastasis positive	0 (0)
Recurrence	0 (0)

Note: Abbreviations: muc, mucinous adenocarcinoma; Mw, moderately well differentiated adenocarcinoma; Mp, moderately poorly differentiated adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; SD, standard deviation; sig, signet-ring cell carcinoma; SM, submucosal; tub, tubular adenocarcinoma; W, well differentiated adenocarcinoma.

3.3 | Association between TAMs at the invasive front and the clinicopathological characteristics

The patients were stratified based on the optimal cut-off values, ie, pan-macrophage-high (Pan-M-high group; pan-macrophage number ≥cut-off) and pan-macrophage-low groups (Pan-M-low group; pan-macrophage number <cut-off); M1 macrophage-high (M1-high group; M1 macrophage number ≥cut-off) and M1 macrophage-low groups (M1-low group; M1 macrophage number <cut-off); and M2 macrophage-high (M2-high group; M2 macrophage number ≥cut-off) and M2 macrophage-low groups (M2-low group; M2 macrophage number <cut-off). ROC curve analysis was used to identify the optimal cut-off values for the pan-macrophage, M1 macrophage, and M2 macrophage numbers at the invasive front. We found that the optimal cut-off was 101.6 for the pan-macrophage number [sensitivity = 71.4%, specificity = 73.8%, AUC = 0.6357 (95% CI: 0.4223-0.8064)], 49.2 for the M1 macrophage number [sensitivity = 100%, specificity = 71.3%, AUC = 0.7804 (95% CI: 0.6675-0.8628)], and 54.8 for the M2 macrophage number [sensitivity = 71.4%, specificity = 93.6%, AUC = 0.8696 (95% CI: 0.6742-0.9556)] in accordance with the maximization of the Youden index. Next, the clinicopathological characteristics were compared between the 2 groups (Table 3). The ratio of lymph node metastasis-positive tumors was significantly higher in the Pan-M-high group compared with that in the Pan-M-low group ($P = .0231$). The ratios of lymphatic invasion-positive and lymph node metastasis-positive tumors were significantly higher in the M1-low group compared with that in the M1-high group ($P = .0004$ and $P = .005$, respectively). In contrast, the ratios of flat or depressed tumor type, moderately poorly differentiated/poorly differentiated/signet-ring cell/mucinous carcinoma at the invasive front, and lymphatic invasion-positive, vascular invasion-positive, and lymph node metastasis-positive tumors were significantly higher in the M2-high group compared with those in the M2-low group ($P = .0028$, $P = .0049$, $P = .0013$, $P = .0011$ and $P = .0001$, respectively). Pathological analyses at the invasive front revealed that the TAMs were distributed around the cancer gland ducts (Figure 4G, K), and there was a mix of areas with high and low numbers of TAMs at the invasive front (Figure 4C, G, K). In the area where the number of TAMs was low at the invasive front (M-low area), the structure of the cancer gland ducts was preserved, tumor cells were histologically differentiated, and E-cadherin expression was not decreased (Figure 4A-D). However, in the area where the number of M2 macrophages was high (M2-high area), the structure of the cancer gland ducts collapsed, tumor cells were poorly differentiated, the morphology of tumor cells transformed from epithelioid to fibroid, and E-cadherin expression was decreased (Figure 4E-H). Moreover, in the area where the number of M1 macrophages was high (M1-high area), TAMs infiltrated destructively and the glandular structure of the tumor was destroyed (Figure 4I-K).

TABLE 3 Association between the number and phenotype of TAMs at the invasive front and clinicopathological characteristics

	Pan-M			M1			M2		
	Low	High		Low	High		Low	High	
Characteristics	n = 61	n = 26	*P*-value	n = 29	n = 58	*P*-value	n = 77	n = 10	*P*-value
Age (years) Mean ± SD	67.3 ± 10.6	69.0 ± 11.4	.5029	68.7 ± 9.0	67.4 ± 11.7	.6217	67.8 ± 10.8	68.2 ± 11.7	.9115
Gender									
Male	38 (62)	15 (58)	.811	21 (72)	32 (55)	.1628	46 (60)	7 (70)	.7339
Female	23 (38)	11 (42)		8 (28)	26 (45)		31 (40)	3 (30)	
Tumor size (mm) Mean ± SD	26.3 ± 15.0	26.8 ± 18.4	.8883	24.5 ± 14.0	27.4 ± 16.9	.4304	27.4 ± 16.6	19.1 ± 4.9	.1226
Localization									
Right colon	18 (30)	11 (42)	.4395	7 (24)	22 (38)	.4256	27 (35)	2 (20)	.494
Left colon	32 (52)	10 (39)		16 (55)	26 (45)		37 (48)	5 (50)	
Rectum	11 (18)	5 (19)		6 (21)	10 (17)		13 (17)	3 (30)	
Morphology									
Flat/Depressed	13 (21)	10 (38)	.1156	9 (31)	14 (24)	.607	16 (21)	7 (70)	.0028*
Elevated	48 (79)	16 (62)		20 (69)	44 (76)		61 (79)	3 (30)	
Dominant histological type									
tub/pap	61 (100)	26 (100)	1	29 (100)	58 (100)	1	77 (100)	10 (100)	1
por/sig/muc	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Histological type at invasive front									
W/Mw/pap	27 (44)	8 (31)	.3398	10 (34)	25 (43)	.493	35 (45)	0 (0)	.0049*
Mp/por/sig/muc	34 (56)	18 (69)		19 (66)	33 (57)		42 (55)	10 (100)	
SM invasion depth									
Distance (μm) Mean ± SD	3438.2 ± 2463.0	3857.7 ± 1544.8	.4246	3005.2 ± 1857.0	3842.8 ± 2347.2	.0986	3600.0 ± 2328.8	3280.0 ± 1236.3	.6713
< 1000 μm	5 (8)	0 (0)	.3162	3 (10)	2 (3)	.328	5 (6)	0 (0)	1
≥ 1000 μm	56 (92)	26 (100)		26 (90)	56 (97)		72 (94)	10 (100)	
Lymphatic invasion positive	24 (39)	12 (46)	.6368	20 (69)	16 (28)	.0004*	27 (35)	9 (90)	.0013*
Vascular invasion positive	17 (28)	10 (38)	.4479	13 (45)	14 (24)	.0839	19 (25)	8 (80)	.0011*
Budding grade 2/3	28 (46)	12 (46)	1	15 (52)	25 (43)	.4986	33 (43)	7 (70)	.176
Lymph node metastasis positive	2 (3)	5 (19)	.0231*	6 (21)	1 (2)	.005*	2 (3)	5 (50)	.0001*

Note: Abbreviations: muc, mucinous adenocarcinoma; Mw, moderately well differentiated adenocarcinoma; Mp, moderately poorly differentiated adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; SD, standard deviation; sig, signet-ring cell carcinoma; SM, submucosal; tub, tubular adenocarcinoma; W, well differentiated adenocarcinoma.

*Significant predictive variables: $P < .05$.

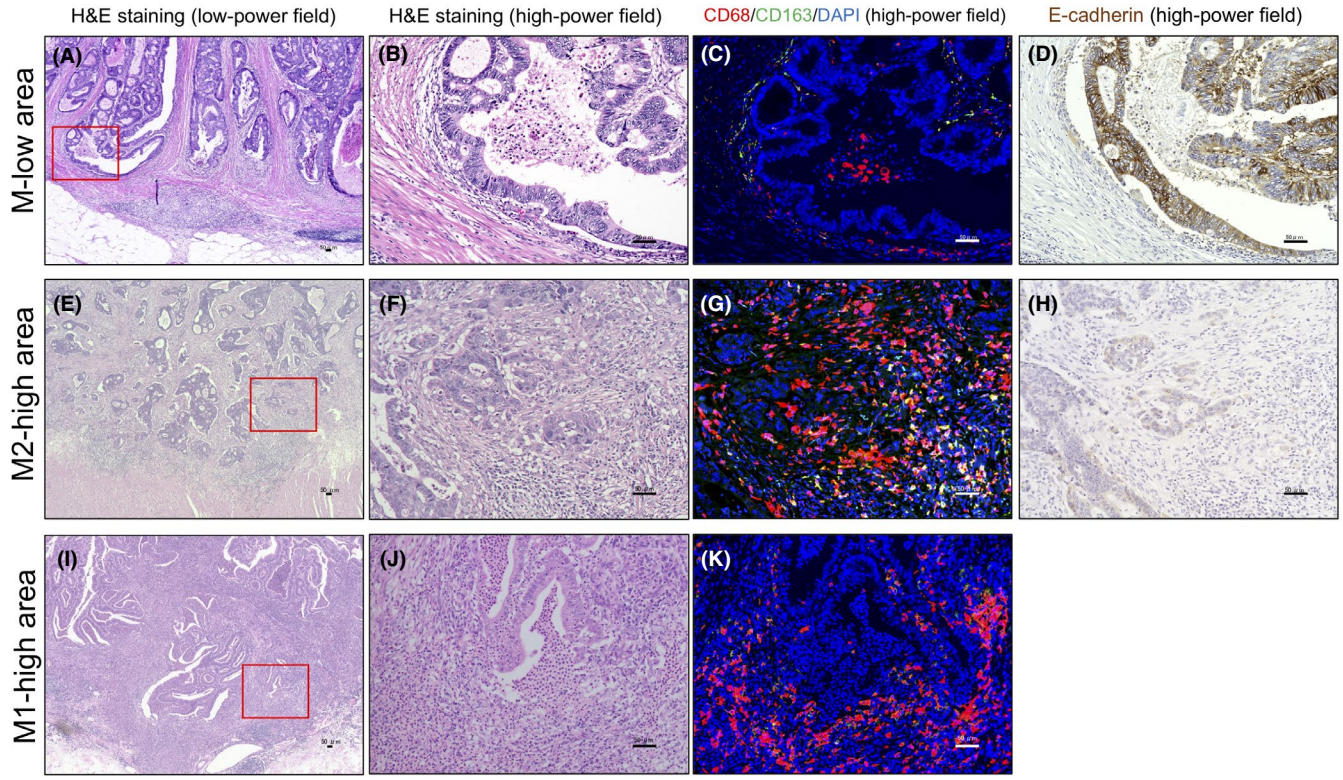

FIGURE 4 Areas with high and low numbers of TAMs at the invasive front. A-D, An invasive front in AD; representative images of the area with a low TAM number (M-low area). A, Hematoxylin and eosin (H&E) staining (low-power field). B, H&E staining (high-power field). C, Double immunofluorescence for CD68 (red) and CD163 (green) (high-power field). D, Immunohistochemistry (IHC) for E-cadherin (high-power field). In the M-low area, the glandular structure of the tumor was preserved, cell differentiation was observed, and E-cadherin expression was not decreased. E-H. An invasive front in SM-CRC; representative images of the area with a high M2 macrophage number (M2-high area). E, H&E staining (low-power field). F, H&E staining (high-power field). G, Double immunofluorescence for CD68 (red) and CD163 (green) (high-power field). H, IHC for E-cadherin (high-power field). In the M2-high area, cancer cells were poorly differentiated, morphology of cancer cells changed from epithelioid to fibroid, and E-cadherin expression in cancer cells was decreased. I-K, An invasive front in SM-CRC; representative images of the area with a high M1 macrophage number (M1-high area). I, H&E staining (low-power field). J, H&E staining (high-power field). K, Double immunofluorescence for CD68 (red) and CD163 (green) (high-power field). In the M1-high area, TAMs infiltrated destructively and the glandular structure of the tumor collapsed and was destroyed. Scale bars, 50 μm. AD, advanced cancer; IM, intramucosal neoplasia; SM-CRC, submucosal invasive colorectal cancer; TAMs, tumor-associated macrophages

3.4 | Prediction of the risk of lymph node metastasis by TAMs at the invasive front

The 87 patients were stratified into the following groups: lymph node metastasis-positive (LN+ group) and lymph node metastasis-negative groups (LN− group) based on the presence of lymph node metastasis, and the phenotype and number of TAMs at the invasive front were compared between these 2 groups. There was no significant difference in the number of pan-macrophages between the 2 groups, but the number of M1 macrophages was significantly lower and the number of M2 macrophages and M2/M1 ratio were significantly higher in the LN+ group compared with those in the LN− group ($P = .0199$, $P < .0001$, and $P < .0001$, respectively) (Figure 5A). We compared the ROC curves for predicting the presence of lymph node metastasis based on the pan-macrophage, M1 macrophage, and M2 macrophage numbers, and M2/M1 ratio at the invasive front. Figure 5B shows that the AUCs of pan-macrophage, M1 macrophage, and M2 macrophage numbers, and M2 /M1 ratio were 0.64, 0.78, 0.87, and 0.90, respectively. The AUCs of the M2 macrophage number (AUC = 0.8696, 95% CI: 0.6742-0.9556) and M2/M1 ratio (AUC = 0.9018, 95% CI: 0.7594-0.9639) were significantly higher compared with that of the pan-macrophage number (AUC = 0.6357, 95% CI: 0.4223-0.8064) ($P < .0001$, and $P < .0001$, respectively). Furthermore, the AUC of the M2/M1 ratio was the highest among the 4 parameters.

4 | DISCUSSION

To elucidate the role of TAMs in CRC, double immunofluorescence for CD68 (a pan-macrophage marker) and CD163 (an M2 macrophage marker) was performed. Because TAMs were distributed heterogeneously in the tumors, 4 regions of the tumor tissues were selected, and the distribution and phenotype of TAMs were evaluated. We found that TAMs at the invasive front underwent dramatic changes at the stage of submucosal invasion. Therefore, we narrowed down to the invasive front in SM-CRC.

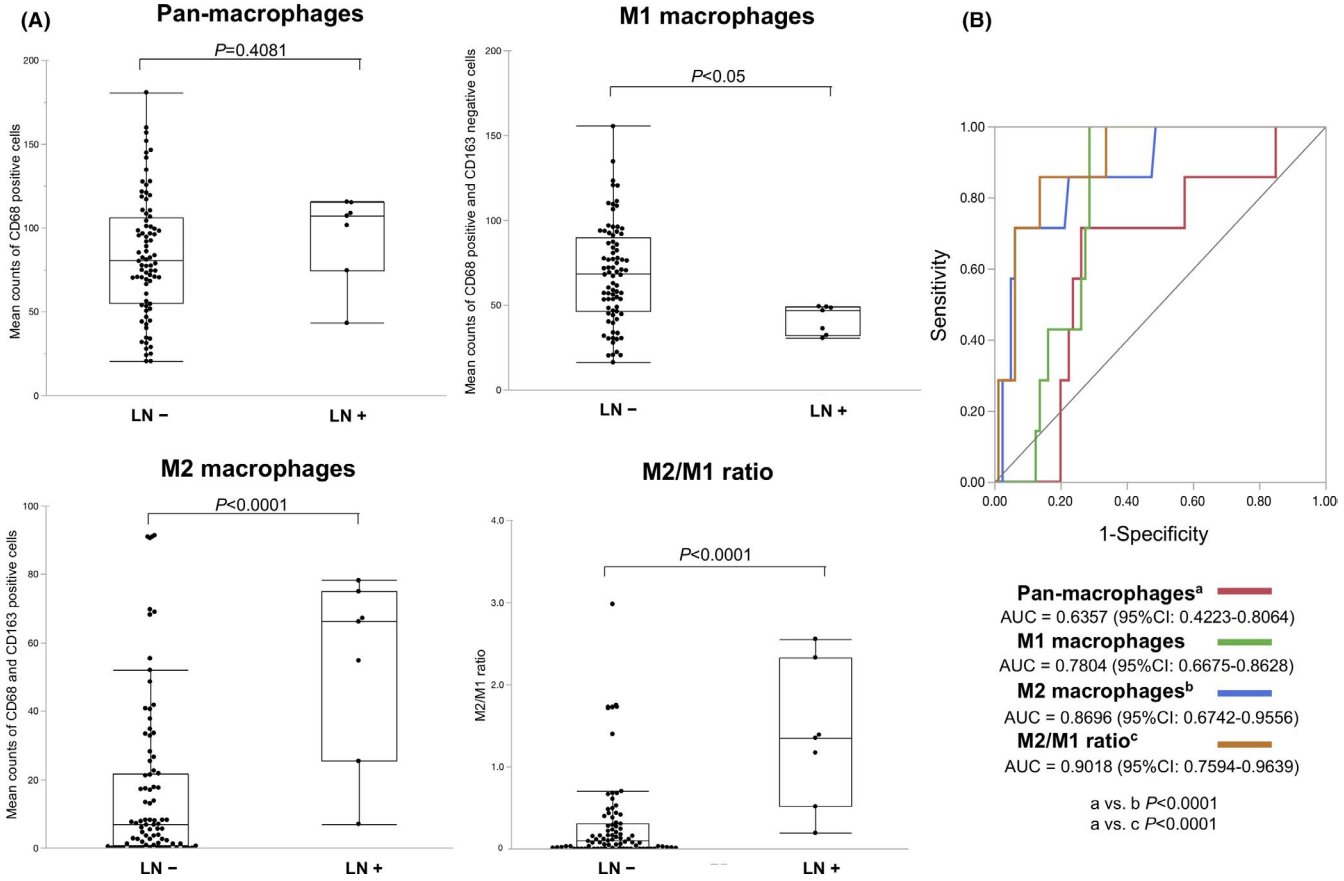

FIGURE 5 Association of TAMs at the invasive front with lymph node metastasis. A, Association between the number and phenotype of TAMs at the invasive front and the presence of lymph node metastasis. There was no significant difference in the number of pan-macrophages between the lymph node metastasis-positive (LN+) and metastasis-negative (LN−) groups, but the number of M1 macrophages was significantly lower and the number of M2 macrophages and M2/M1 ratio were significantly higher in the LN+ group compared with those in the LN− group. Significant predictive variables: $P < .05$. B, Lymph node metastasis prediction based on TAMs at the invasive front. We compared the receiver operating characteristic curves for predicting lymph node metastasis based on the pan-macrophage, M1 macrophage, and M2 macrophage numbers, and M2/M1 ratio at the invasive front. The AUCs of the pan-macrophage, M1 macrophage, and M2 macrophage numbers, and M2/M1 ratio were 0.64, 0.78, 0.87, and 0.90, respectively. The AUCs of the M2 macrophage number and M2/M1 ratio were significantly higher compared with those of the pan-macrophage number; the AUC of the M2/M1 ratio was the highest among the 4 parameters. Significant predictive variables: $P < .0083$. AUC, area under the curve; CI, confidence interval; TAMs, tumor-associated macrophages

TAMs, which often exhibit the M2 phenotype,[9,11] have been reported to affect virtually almost every step of tumor cell metastasis, including invasion, vascularization, intravasation, extravasation, establishment of pre-metastatic niches, and maintenance of circulating tumor cell survival.[31] In most solid tumors, a high density of TAMs is associated with a significantly poor prognosis.[12-14] However, the role of TAMs and the association between TAMs and clinical prognosis remains controversial in CRC. Some experimental studies on CRC have reported that TAMs are associated with tumor progression by inducing tumor growth, EMT, invasion, migration, neovascularization, and matrix remodeling.[22,25,26,28,32] In contrast, most studies using clinical specimens from patients with CRC have demonstrated an association between a high density of TAMs and a favorable prognosis.[15-21] In recent years, it has been reported that a high density of M2 macrophages is associated with poor prognosis in CRC.[22,28] Moreover, it has been suggested that M1 macrophages are involved in mediating antitumor activity,[33] but few studies have examined the association between M1 macrophages and CRC prognosis, and the role of M1 macrophages in CRC is unclear.[24] Concerning the distribution of TAMs in CRC, most of the previous studies assessed the invasive margin or tumor center, and few assessed the distribution in detail.[34] Importantly, previous studies have focused on advanced cancer, and not early stage CRC.

Our findings revealed a dynamic increase in pan-macrophage and M2 macrophage counts, and M2/M1 ratio at the invasive front with tumor progression (Figures 2 and 3). Previous studies have reported that most of the TAMs accumulate in the leading edge and avascular areas, and M2 macrophages become predominant with CRC progression.[31,35] The results of this study were consistent with these previously reported findings and suggested that TAMs at the

invasive front might play an important role in CRC progression. It has been reported that monocytes are recruited to the tumor with tumor progression, and that these polarize into either M1 or M2 macrophages in response to a variety of chemokines and cytokines secreted by stromal and tumor cells in the TME.[6-9,36,37] Therefore, it is suggested that chemokines and cytokines undergo dynamic changes upon interaction with stromal and tumor cells in the TME, resulting in tumor progression and promotion of monocyte recruitment and polarization into M2 instead of M1 macrophage phenotypes at the invasive front as the tumor progresses.

Next, we observed that the histological differentiation at the invasive front was significantly lower and the ratios of lymphovascular invasion-positive and lymph node metastasis-positive tumors were significantly higher in the M2-high group compared with those in the M2-low group. However, the ratios of lymphatic invasion-positive and lymph node metastasis-positive tumors were significantly higher in the M1-low group compared with those in the M1-high group (Table 3). Therefore, patients with a high number of M2 macrophages at the invasive front had pathological findings of high malignancy, while those with a high number of M1 macrophages had pathological findings of low malignancy. As noted above, it is generally known that M1 macrophages exert an antitumor effect and M2 macrophages exert a pro-tumor effect,[31,33] suggesting that TAMs may be involved CRC progression at the invasive front, with M1 macrophages suppressing and the proliferation of M2 macrophages that promote cancer progression.

Macrophages infiltrated heterogeneously at the invasive front even in the same tumor tissue. Therefore, a detailed observation of TAM low- and high-density areas at the invasive front revealed that the glandular structure of the tumor in the M-low area was well preserved, but in the M2-high area, cancer cells were poorly differentiated, morphology of cancer cells changed from epithelioid to fibroid, and E-cadherin expression in cancer cells was decreased (Figure 4). Moreover, TAMs infiltrated destructively and the glandular structure of the tumor collapsed and was destroyed in the M1-high area. It has been reported that M2 macrophages promote tumor progression by inducing EMT,[10,22,28,38] and M1 macrophages exert an antitumor effect by promoting CD8-mediated tumor immunity and secreting TNF-α, IFN-γ, and nitric oxide synthase.[10,33] Therefore it is suggested that, at the invasive front, M2 macrophages may promote tumor progression via EMT, while M1 macrophages may suppress it through CD8-mediated tumor immunity and TNF-α, IFN-γ, and NO production.

We had previously reported that pathological findings, such as differentiation, at the invasive front are important predictors of lymph node metastasis.[30,39] Additionally, tumor budding at the invasive front, as well as unfavorable tumor grade, are predictors of lymph node metastasis in SM-CRC.[40] Here, on comparing the number and phenotype of TAMs at the invasive front between the LN+ and LN− groups, we did not find any significant difference in the number of pan-macrophages between the 2 groups (Figure 5A). However, the number of M1 macrophages in the LN+ group was significantly lower compared with that in the LN− group, and the number of M2 macrophages and M2/M1 ratio in the LN+group was significantly higher compared with that in the LN− group (Figure 5A). Furthermore, comparison of the ROC curves for predicting the presence of lymph node metastasis based on the pan-macrophage, M1 macrophage, and M2 macrophage numbers, and M2/M1 ratio, revealed that the AUC of the M2/M1 ratio, which included both M1 and M2 macrophages, was the highest among the 4 parameters (Figure 5B). Therefore, it is suggested that evaluating the phenotypes of TAMs is important to understand the role of TAMs, and the risk of lymph node metastasis may be predicted by evaluating the number and phenotype of TAMs at the invasive front.

Nonetheless, this study has several limitations. First, this study was a retrospective observational study performed using clinical specimens. Further basic experimental studies are needed to support the present findings; however, there have been some in vivo and in vitro studies reporting that M1 macrophages inhibit tumor growth and M2 macrophages promote it.[6,7,22,28] Second, the sample size was relatively small, and the present study was a single-center study. Additional studies with larger sample sizes and a multi-center and prospective study design are required to confirm our findings. Lastly, the macrophage classification method, ie, M1/M2 classification, may be an oversimplification and debatable. Several studies have shown that macrophages cannot be clearly divided into 2 types based on the existence of more complex phenotypes.[4,41,42] CD68 has been widely used as a pan-macrophage marker in most studies using clinical specimens, however Vakkila et al[43] reported that CD68 can occasionally be expressed in dendric cells, stromal cells, and cancer cells themselves. CD163, CD204, and/or CD206 have been widely recommended as M2 phenotype markers.[9,35,44] In contrast, Barros et al[45] reported that CD163 expression is not exclusive to M2 phenotype macrophages. Therefore, it is necessary to develop an appropriate method to distinguish macrophage phenotypes.

In conclusion, we identified dynamic changes in the number and phenotype of TAMs at the invasive front in CRC, especially at the stage of submucosal invasion, which may be caused by interaction with stromal and tumor cells in the TME. Furthermore, TAMs at the invasive front may play a role in CRC progression, especially in early stage CRC, ie, M1 macrophages at the invasive front may inhibit CRC progression, while M2 macrophages may promote it via EMT. Therefore, a marker comprising the phenotype, number, and distribution of TAMs may serve as a potential predictor of metastasis, including lymph node metastasis, and TAMs may be a potential therapeutic target in CRC.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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