Journal of
Breast Cancer **JBC**

Original Article

Check for updates

Tumor-Associated Macrophages as Potential Prognostic Biomarkers of Invasive Breast Cancer

Hasong Jeong [1], **Ilseon Hwang** [1], **Sun Hee Kang** [2], **Hyeong Chan Shin** [3], **Sun Young Kwon** [1]

[1]Department of Pathology, Keimyung University School of Medicine, Daegu, Korea
[2]Department of General Surgery, Keimyung University School of Medicine, Daegu, Korea
[3]Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

OPEN ACCESS

Correspondence to
Sun Young Kwon
Department of Pathology, Keimyung University School of Medicine, 56 Dalsung-ro, Jung-gu, Daegu 41931, Korea.
E-mail: pathol72@gmail.com

ORCID iDs
Hasong Jeong
https://orcid.org/0000-0002-2205-9586
Ilseon Hwang
https://orcid.org/0000-0002-6122-4417
Sun Hee Kang
https://orcid.org/0000-0002-2155-3785
Hyeong Chan Shin
https://orcid.org/0000-0002-3716-7678
Sun Young Kwon
https://orcid.org/0000-0002-8410-0185

ABSTRACT

Purpose: Tumor-associated macrophages (TAMs) are activated macrophages associated with tumor progression in various cancers. TAMs can polarize M1 or M2 type. M1 has a pro-inflammatory function and kills pathogens. Conversely, M2 shows immunosuppressive action and promotes tumor growth. There are various markers of TAMs. CD11c is considered as a specific marker of M1. CD163 is an optimal marker for M2. CD68 is known as a pan-macrophage marker. We evaluated the relationship between the clinicopathological parameters and immunohistochemical expressions of CD11c, CD163, and CD68 in invasive breast cancer (IBC), and the prognostic value of macrophage localization within the tumor stroma (TS) and tumor nest (TN).

Methods: Immunohistochemistry of CD68, CD11c, and CD163 was analyzed on tissue microarrays of 367 IBCs. The number of CD68+, CD11c+, or CD163+ macrophages in TN vs. TS was counted by 2 pathologists. The correlations between the degree of macrophage (CD68+, CD11c+, or CD163+) infiltration and the clinicopathological parameters were analyzed. We also assessed the impact of macrophages (CD68+, CD11c+, or CD163+) on disease free survival (DFS) and overall survival (OS).

Results: High numbers of macrophages (CD68+, CD11c+, or CD163+) were associated with higher histologic grade, higher Ki-67 proliferating index, estrogen receptor negativity, and progesterone receptor negativity. High numbers of macrophages (CD11c+ or CD163+) in TS were associated with a larger tumor size. Furthermore, CD163+ macrophages in TN were an independent prognostic marker of reduced OS and DFS. Conversely, CD11c+ macrophages in TS were an independent prognostic marker for higher OS and DFS.

Conclusion: TAMs, including M2 type, are associated with tumor progression in IBC. They can also act as a significant unfavorable or favorable prognostic factor. In addition to simply analyzing the degree of TAM infiltration, it is also important to analyze the location of TAMs.

Keywords: Breast neoplasms; CD11c antigen; CD163 antigen; CD68 antigen; Macrophages

Author Contributions
Methodology: Hwang I, Shin HC; Project
administration: Jeong H, Kang SH, Kwon SY;
Writing - original draft: Jeong H, Kwon SY.

INTRODUCTION

Tumor microenvironments play a fundamental role in tumor generation and progression via interaction with tumor cells [1]. Recently, several studies have reported the importance of tumor microenvironment in breast cancer [2-6]. The tumor microenvironment comprises of inflammatory cells, macrophages, extracellular matrix, endothelial cells, and fibroblasts. It affects cancer progression and influences the clinical outcome. Macrophages play an essential role in inflammation, homeostasis, host defense, tissue repair and remodeling, and cancer progression [7]. Tumor-associated macrophages (TAMs) are a major component of the tumor microenvironment and associated with poor prognosis by promoting tumor progression [3,5]. The main role of TAMs is promotion of tumor growth and metastasis [5]. Macrophages can polarize and exhibit M1 or M2 phenotype [8]. M1 macrophages have a pro-inflammatory function and activate T helper type 1 T cells to induce a lethal T-cell response against pathogens, resulting in tumoricidal activity as well as increased hypoxia [9]. M2 macrophages potentially generate anti-inflammatory cytokines [1]. In addition, M2 type macrophages promote tissue repair and wound healing, and are involved in angiogenesis and tumor progression [10,11]. An increased number of polarized M2 macrophages in oral squamous cell carcinoma is associated with lymph node (LN) metastasis [12]. Several immunohistochemical markers are available to evaluate TAMs. CD68 is known as a pan-macrophage marker and facilitates identification of both M1 and M2 macrophages [3]. In breast cancer, a higher degree of CD68+ macrophage infiltration was associated with poor prognosis [5] and was an independent prognostic marker for reduced cancer specific survival in tumor stroma (TS) [3]. CD163 is a scavenger receptor and a highly specific marker for M2 macrophages [13]. Infiltration of CD163+ macrophages into TS of breast cancer is clinically relevant. It is positively correlated with higher histological grade, larger mass/size, Ki-67 proliferating index, hormone receptor negativity, triple-negative/basal-like breast cancer, and inversely correlated with luminal A breast cancer [3]. CD11c is considered a marker of M1 macrophages [14]. To our knowledge, CD11c is yet to be evaluated as a TAM marker in primary breast cancer. In this study, we evaluated the association between the expression of various TAM markers and clinicopathological parameters using tissue microarray (TMA) of 367 invasive breast cancer (IBC) patients. In addition, we evaluated the relationship between clinicopathological parameters and the degree of expression of CD163, CD11c, and CD68 in IBC tissues. Furthermore, the localization of TAMs in TS and tumor nest (TN) in breast cancer was used to determine their prognostic relevance in IBC [3].

METHODS

Patients and clinical data selection

A total of 367 primary IBC patients who underwent surgery at Keimyung University Dongsan Medical Center between 2000 and 2007 were recruited. None of the patients had received preoperative chemotherapy or radiotherapy. All tissues were fixed in 10% buffered formalin and embedded in paraffin. Patient and tumor characteristics, including patient age at the time of diagnosis, tumor size, LN status, histologic grade, and follow-up data, were determined from patients' medical records and pathology reports. Most of the clinicopathological parameters are listed in **Table 1**. Tumor stage was determined according to the 7th American Joint Committee on Cancer criteria [15]. Overall survival (OS) was defined as the time interval between the date of cancer diagnosis and the date of death from any cause or death related to IBC. Disease free survival (DFS) was defined as the number

Table 1. Mean values of macrophages (CD68+, CD11c+, or CD163+) in TS and TN and relevant clinicopathologic features of the patients

Clinicopathologic features	n=367	CD68				CD11c				CD163			
		TS		TN		TS		TN		TS		TN	
		Mean±SD	p-value*	Mean±SD	p-value*	Mean±SD	p-value*	Mean±SD	p-value*	Mean±SD	p-value*	Mean±SD	p-value*
Age (yr)			0.902		0.950		0.880		0.855		0.364		0.860
<50	185	57.50±38.54		14.56±18.20		57.12±35.61		10.96±14.31		48.89±32.95		8.45±14.46	
≥50	182	57.97±34.06		14.68±18.77		57.68±36.11		11.26±17.64		45.77±32.68		8.19±13.36	
Histologic grade			<0.001		0.003		<0.001		0.007		<0.001		<0.001
Grade 1–2	133	43.13±27.54		11.21±12.38		40.14±27.01		8.44±27.01		32.07±25.22		4.84±8.72	
Grade 3	232	66.45±38.01		16.14±19.64		67.16±36.48		12.46±18.16		56.33±33.45		10.06±15.32	
NA	2												
Tumor size (mm)			0.088		0.453		0.018		0.809		0.003		0.237
≤20	144	53.71±36.61		13.72±18.94		51.92±36.16		11.36±17.62		40.94±32.15		7.26±11.90	
>20	223	60.33±36.00		15.20±18.16		60.93±35.22		10.95±14.95		51.48±32.63		9.02±15.05	
LN metastasis			0.791		0.875		0.859		0.394		0.887		0.161
Absent	183	58.23±38.89		14.47±19.85		57.06±37.98		11.83±15.17		47.59±35.29		9.35±15.06	
Present	184	57.23±33.71		14.77±17.01		57.73±33.63		10.40±16.85		47.10±30.24		7.31±12.62	
Ki-67 (%)			<0.001		0.002		<0.001		0.001		<0.001		<0.001
<14	169	45.33±28.92		11.42±14.29		46.11±32.61		8.12±11.25		36.95±28.41		5.43±10.96	
≥14	198	68.32±38.67		17.35±21.03		67.03±35.68		13.66±18.85		56.21±33.76		10.80±15.60	
ER status			<0.001		0.003		<0.001		0.017		<0.001		0.001
Negative	115	73.70±41.99		19.73±25.16		70.75±37.06		14.82±22.62		63.25±36.04		12.78±18.46	
Positive	252	50.44±30.89		12.29±13.85		51.30±33.58		9.42±11.54		40.08±28.48		6.29±10.68	
PR status			<0.001		0.002		<0.001		0.017		<0.001		0.003
Negative	108	74.09±42.51		20.41±25.84		69.25±36.45		15.01±22.57		61.52±36.96		12.41±18.91	
Positive	259	50.91±31.06		12.21±13.65		52.45±34.43		9.48±12.01		41.43±29.01		6.62±10.78	
HER2 status			0.261		0.631		0.618		0.255		0.248		0.161
Negative	285	56.59±36.54		14.87±19.75		56.89±36.27		11.62±17.14		46.28±32.91		8.74±15.07	
Positive	82	61.71±35.56		13.76±13.06		59.14±34.35		9.33±11.30		51.03±32.39		6.90±8.66	

TS = tumor stroma; TN = tumor nest; SD = standard deviation; LN = lymph node; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epithelial growth factor receptor 2; NA = not available.
*Independent t-test.

of months from surgical resection to the development of documented relapse, including recurrence or distant metastasis. TMA blocks were obtained from Keimyung University Human Bio-resource Bank, Korea. The requirement for informed consent from the patients was waived by the ethics committee; this study was approved by the Institutional Review Board at Keimyung University Dongsan Medical Center (DSMC No. 2018-02-007).

TMA construction
Before TMA construction, all tissues were histopathologically studied on hematoxylin and eosin stained slides by a breast pathologist, based on the 2012 World Health Organization Classification [16]. After reviewing the slides, TMAs were constructed using 2 representative cores, each measuring 2.0 mm in diameter, taken from paraffin blocks, and were arrayed in a new recipient block using a manual device (Unitma, Seoul, Korea). In general, the cores were selected from the peripheral aspect of the tumor without hemorrhage or necrosis.

Immunohistochemistry
Immmunohistochemistry (IHC) staining was performed using the automated Benchmark platform (Ventana Medical Systems, Tucson, USA), according to the manufacturer's instructions. Four-micrometer-thick TMA sections were mounted onto slides and deparaffinized followed by antigen retrieval using cell conditioning solution and stained with the UltraView™ Universal DAB detection kit (Ventana Medical Systems). The following primary antibodies were used in this study: estrogen receptor (ER; pre-diluted; Ventana Medical Systems), progesterone receptor (PR; pre-diluted; Ventana Medical Systems),

human epidermal growth factor receptor 2 (HER2; pre-diluted; Ventana Medical Systems), Ki-67 (1:200; DAKO Co., Carpinteria, USA), CD68 (1:400; Santa Cruz Biotechnology, Dallas, USA), CD11c (1:100; Abcam, Cambridge, USA), and CD163 (1:200; Thermo Fisher Scientific, Waltham, USA).

Assessment of IHC staining

Two pathologists (H.S.J. and S.Y.K) analyzed the IHC stained slides in a blinded manner. In case of any discrepancy, the analysis was discussed, and the histological examination was repeated. IHC staining of ER and PR was evaluated according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines. The guidelines recommend classification of all cases with at least 1% receptor-positive cells [17]. HER2 expression was also scored according to the ASCO/CAP guidelines [18], which include 4 scores ranging from 0 to 3: no staining or incomplete, faint/barely perceptible membrane staining in ≤10% of invasive tumor cells (score 0); incomplete, faint/barely perceptible membrane staining in >10% of invasive tumor cells (score 1); incomplete and/or weak to moderate circumferential membrane staining in >10% of invasive tumor cells or complete, intense, circumferential membrane staining in ≤ 10% of invasive tumor cells (score 2); and complete, intense, and circumferential membrane staining in > 10% of invasive tumor cells (score 3). A score of 3 for HER2 was considered as positive. HER2 status of equivocal (score 2) cases was determined using silver *in situ* hybridization. Quantification of Ki-67 was performed by counting nuclear stained cells among 1000 tumor cells [19]. We set a 14% cut-off of Ki-67 for all statistical analyses. In terms of distribution of the intrinsic subtypes of IBC according to IHC, IBC was classified into 5 subtypes: luminal A (ER+ and/or PR+, HER2−, Ki-67 <14%); luminal B, HER2(−) (ER+ and/or PR+, HER2−, and Ki-67 ≥14%); luminal B, HER2 (+) (ER+ and/or PR+, HER2+); HER2-enriched (ER−, PR−, and HER2+); and triple-negative (ER−, PR−, and HER2−) [20]. The expression of CD68, CD11c, and CD163 was determined by counting the number of positive macrophages. After reviewing TMA slides containing cores from each case, a field of maximum intensity of expression was selected. In each case, 3 visual fields with the highest infiltration density of positive macrophages were selected using ImageScope (Aperio Technologies, Vista, USA), yielding a final magnification of 400× (1 mm^2). Number of stained macrophages was counted in the 3 visual fields to obtain an average. Only cells with monocytoid/macrophage-like morphology were counted. For statistical evaluation, the number of positive cells was divided into lower and higher groups based on the cut-off points. CD68+, CD11c+, or CD163+ macrophages were counted in the TN and TS, separately. We analyzed the correlations between number of macrophages (CD68+, CD11c+, or CD163+) and the clinicopathological parameters. Additionally, we assessed the impact of CD68+, CD11c+, or CD163+ macrophages in TS and TN in IBC.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, USA) or R 3.4.3 (R Development Core Team, Vienna, Austria, https://www.R-project.org). The independent *t*-test was used to identify the correlation between the number of macrophages (CD68+, CD11c+, or CD163+) and clinicopathological parameters. The independent *t*-test was also used to identify association between the number of macrophages (CD68+, CD11c+, or CD163+) and molecular subtypes. MaxStat (MaxStat Software, Jever, Germany), a maximal χ^2 method in R 3.4.3 was used to identify optimal cut-off points for the number of CD68+, CD11c+, and CD163+ macrophages. Optimal cut-off points of the number of CD68+, CD11c+, and CD163+ macrophages in OS were as follows: CD68 in TS: 17.8, CD68 in TN: 33, CD11c in TS: 75, CD11c in TN: 1, CD163 in TS: 21, and CD163 in TN: 1.67. The cut-off points of the

number of CD68+, CD11c+, and CD163+ macrophages in DFS were as follows: CD68 in TS: 17.8, CD68 in TN: 33, CD11c in TS: 75, CD11c in TN: 10, CD163 in TS: 20, and CD163 in TN: 1.67. Kaplan-Meier analysis and log rank tests were used to demonstrate differences in DFS and OS based on CD68, CD11c, and CD163 expression. Cox proportional hazard models were used to determine the hazard ratios (HR) for death from breast cancer or other causes based on CD68, CD11c, and CD163 expression in both uni- and multivariable analysis.

RESULTS

Clinical characteristics

Among 367 patients, 319 (86.9%) were invasive ductal carcinoma, not otherwise specified; 11 (3.0%) were invasive lobular carcinoma; and 37 (10.1%) were a variety of other histological types of carcinomas (5 mucinous, 5 micropapillary, 4 apocrine, 4 metaplastic, 4 medullary, 3 neuroendocrine, 2 cribriform, 2 papillary, 2 solid papillary, 2 tubular, and 4 mixed carcinoma). We classified 134 patients (36.5%) as luminal A, 97 patients (26.4%) as luminal B (HER2−), 53 patients (14.4%) as luminal B (HER2+), 29 patients (7.9%) as HER2-enriched, and 54 patients (14.7%) as triple-negative based on the results of IHC. Median age was 49 years (range 24–84 years). One hundred five patients (28.6%) received mastectomy and 262 patients (71.4%) underwent breast conserving surgery. After surgery, 281 patients (76.6%) received chemotherapy and 153 patients (41.7%) underwent radiation therapy. Of the 256 patients (69.6%) receiving endocrine therapy, 99 patients (26.9%) were treated with tamoxifen only. During the follow-up, 61 patients (16.6%) died and 76 patients (20.7%) had recurrence or metastasis. The median follow-up time was 9.3 years (range 0.1–17.1 years) for all patients.

Clinical significance of infiltration

The CD68+, CD11c+, and CD163+ expression in TN and TS was determined for all the 367 samples. CD68+, CD11c+, and CD163+ macrophages were detected in both TS and TN of IBC (**Figure 1**). The relationship between the number of macrophages (CD68+, CD11c+, or CD163+) and clinicopathological features is shown in **Table 1**. High numbers of TAMs (CD68+, CD11c+, or CD163+) in both TS and TN were associated with higher histological grade, higher Ki-67 proliferating index, ER negativity, and PR negativity. High numbers of macrophages (CD11c+ or CD163+) in TS were associated with a larger tumor size. Age, LN metastasis, and HER2 status showed no significant differences.

Infiltration densities of all the TAM markers were significantly different among different molecular subtypes (**Figure 2**). In TS, the luminal A showed significantly lower number of CD68+ macrophages (42.72 ± 27.69) than all other molecular subtypes ($p < 0.05$) (**Figure 2A**). In TN, the triple-negative showed significantly higher number of CD68+ macrophages (26.67 ± 33.10) than all other molecular subtypes ($p < 0.05$) (**Figure 2B**). In TS, luminal A showed significantly lower number of CD11c+ macrophages (44.63 ± 31.74) than luminal B (HER2−) (62.24 ± 36.04, $p < 0.001$), HER2-enriched (67.56 ± 33.53, $p = 0.001$), and triple-negative (77.74 ± 35.94, $p < 0.001$). The number of positive cells in triple-negative (77.74 ± 35.94) was significantly higher than luminal B (HER2−) (62.24 ± 36.04, $p = 0.012$) and luminal B (HER2+) (54.53 ± 34.23, $p = 0.001$) (**Figure 2C**). In TN, triple-negative showed significantly higher number of CD11c+ macrophages (20.11 ± 29.14) than luminal A (7.51 ± 9.49, $p = 0.003$), luminal B (HER2+) (10.08 ± 12.23, $p = 0.023$), and HER2-enriched (7.97 ± 9.40, $p = 0.007$). The number of positive cells in luminal A (7.51 ± 9.49) was significantly lower than luminal B (HER2−) (12.57 ± 14.50,
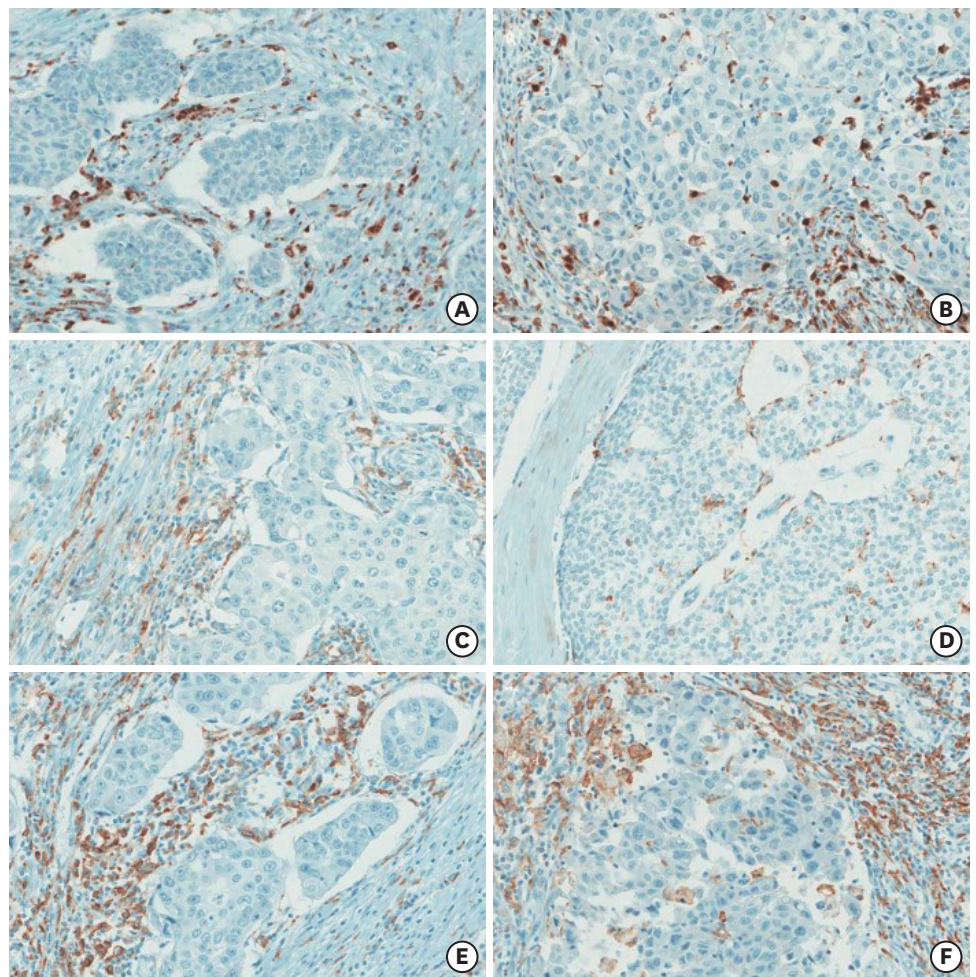
Figure 1. Representative images of IHC analysis of TAM markers in IBC. (A) CD68+ macrophages are mostly present in TS (IHC for CD68, 400× magnification). (B) CD68+ macrophages are present in both TS and TN (IHC for CD68, 400× magnification). (C) CD11c+ macrophages are mainly present in TS (IHC for CD11c, 400× magnification). (D) CD11c+ macrophages are mostly present in TN (IHC for CD11c, 400× magnification). (E) CD163+ macrophages are chiefly present in TS (IHC for CD163, 400× magnification). (F) CD163+ macrophages are present in both TS and TN (IHC for CD163, 400× magnification).
IBC = invasive breast cancer; IHC = immunohistochemistry; TAM = tumor-associated macrophage; TS = tumor stroma; TN = tumor nest.

$p = 0.003$) (**Figure 2D**). In TS, luminal A showed a significantly lower number of CD163+ macrophages (33.36 ± 25.78) than all other molecular subtypes ($p < 0.05$) (**Figure 2E**). In TN, the triple-negative showed a significantly higher number of CD163+ macrophages (17.72 ± 23.97) than all other molecular subtypes ($p < 0.05$) (**Figure 2F**).

Prognostic significance of CD68+, CD11c+, and CD163+ macrophages

We found that infiltration of higher number of CD68+ macrophages in TS was not correlated with OS, but infiltration in TN was correlated with unfavorable OS (**Figure 3A and B**). Infiltration of higher number of CD11c+ macrophages in TS was correlated with favorable OS, but infiltration in TN was not correlated with OS (**Figure 3C and D**). Like CD68+ macrophages, infiltration of higher number of CD163+ macrophages in TS was not correlated with OS, but infiltration in TN was correlated with unfavorable OS (**Figure 3E and F**).
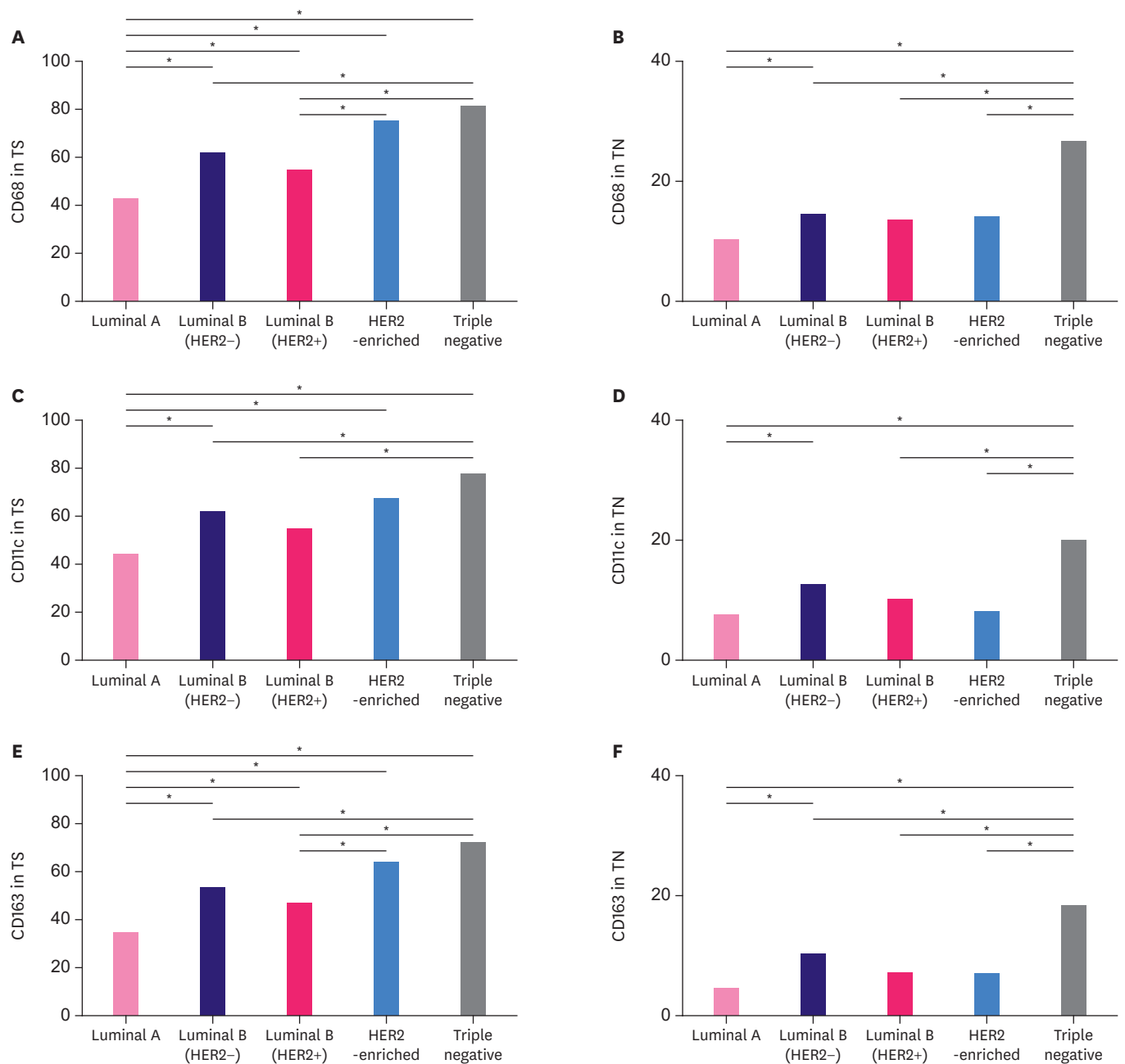
Figure 2. Mean of the macrophages (CD68+, CD11c+, or CD163+) according to each molecular subtype. (A) CD68 in TS, (B) CD68 in TN, (C) CD11c in TS, (D) CD11c in TN, (E) CD163 in TS, and (F) CD163 in TN.
HER2 = human epithelial growth factor receptor 2; TS = tumor stroma; TN = tumor nest.
*$p < 0.05$.

Additionally, the infiltration of higher number of CD68+ macrophages in TS was not correlated with DFS, but infiltration in TN was correlated with unfavorable DFS (**Figure 4A and B**). Infiltration of higher number of CD11c+ macrophages in TS was correlated with favorable DFS, but infiltration in TN was not correlated with OS (**Figure 4C and D**). Like CD68+ macrophages, infiltration of higher number of CD163+ macrophages in TS was not correlated with DFS, but infiltration in TN was correlated with unfavorable DFS (**Figure 4E and F**).
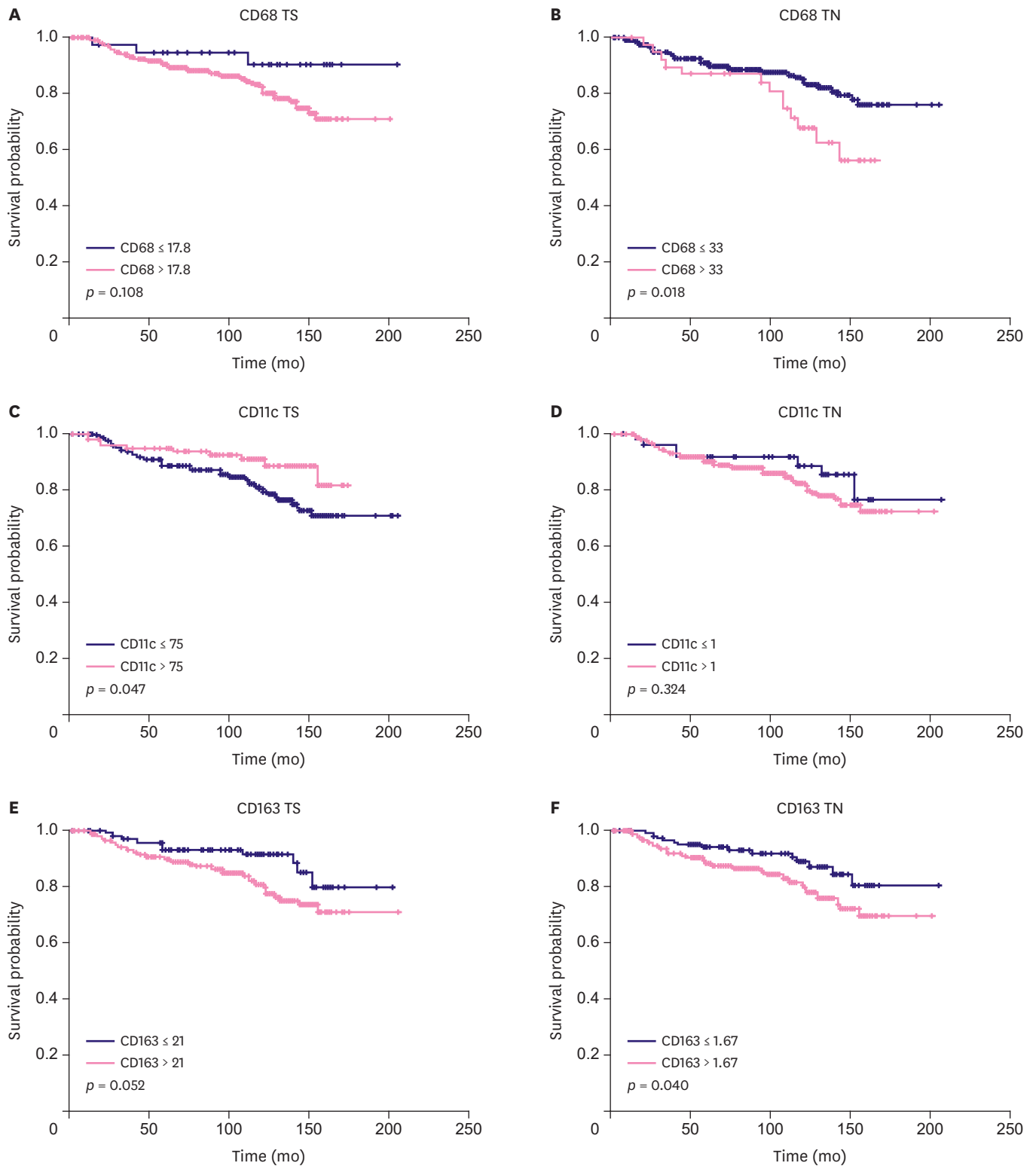
Figure 3. The OS curves according to the infiltration density of CD68+ macrophages in TS (A) and TN (B), CD11+ macrophages in TS (C) and TN (D), and CD163+ macrophages in TS (E) and TN (F) in IBC.
OS = overall survival; TS = tumor stroma; TN = tumor nest; IBC = invasive breast cancer.
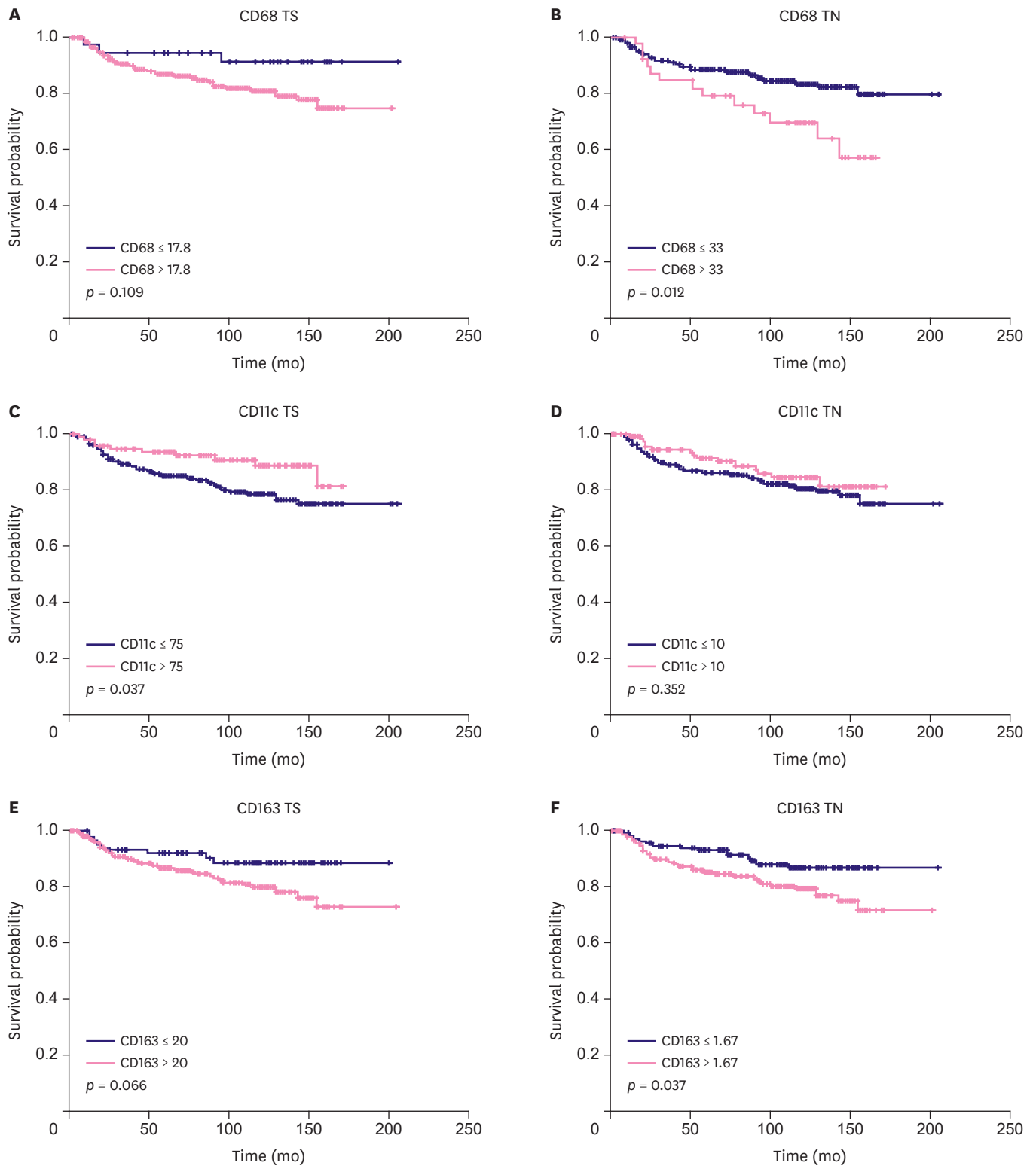
Figure 4. Disease free curves based on the infiltration density of CD68+ macrophages in TS (A) and TN (B), CD11+ macrophages in TS (C) and TN (D), and CD163+ macrophages in TS (E) and TN (F) in IBC.
TS = tumor stroma; TN = tumor nest; IBC = invasive breast cancer.

Table 2. Univariate Cox regression analyses for OS and DFS

Variables	OS			DFS		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (<50 vs. ≥50)	1.015	0.992–1.039	0.206	1.012	0.989–1.035	0.313
ER status (− vs. +)	0.843	0.490–1.450	0.536	0.876	0.509–1.506	0.631
PR status (− vs. +)	0.574	0.342–0.964	0.036	0.597	0.355–1.002	0.051
HER2 status (− vs. +)	3.125	1.879–5.197	<0.001	3.176	1.909–5.283	<0.001
Ki-67, % (<14 vs. ≥14)	1.769	1.047–2.987	0.033	1.706	1.011–2.880	0.045
Tumor size, mm (≤20 vs. >20)	2.748	1.488–5.075	0.001	2.801	1.516–5.173	0.001
LN metastasis (− vs. +)	3.288	1.835–5.891	<0.001	3.504	1.955–6.279	<0.001
HG (1–2 vs. 3)	1.604	0.916–2.807	0.098	1.594	0.911–2.790	0.102
CD68 in TS	2.507	0.785–8.005	0.121	2.501	0.783–7.983	0.122
CD68 in TN	2.066	1.119–3.814	0.020	2.153	1.167–3.975	0.014
CD11c in TS	0.510	0.259–1.005	0.052	0.494	0.251–0.974	0.042
CD11c in TN	1.483	0.674–3.263	0.327	0.759	0.424–1.359	0.353
CD163 in TS	1.937	0.983–3.817	0.056	1.920	0.946–3.897	0.071
CD163 in TN	1.851	1.019–3.362	0.043	1.870	1.029–3.396	0.040

OS = overall survival; DFS = disease free survival; HR = hazard ratio; CI = confidence interval; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; LN = lymph node; HG = histologic grade; TS = tumor stroma; TN = tumor nest.

Table 3. Multivariate Cox regression analyses for OS and DFS

Variables	OS			DFS		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
PR status (− vs. +)	0.759	0.443–1.301	0.316	NA	NA	NA
HER2 status (− vs. +)	2.552	1.515–4.299	<0.001	3.065	1.817–5.170	<0.001
Ki-67, % (<14 vs. ≥14)	1.246	0.722–2.148	0.430	1.396	0.811–2.401	0.229
Tumor size, mm (≤20 vs. >20)	1.770	0.937–3.343	0.079	2.226	1.188–4.172	0.013
LN metastasis (− vs. +)	2.865	1.574–5.215	0.001	2.938	1.625–5.312	<0.001
CD11c in TS	NA	NA	NA	0.323	0.160–0.654	0.002
CD163 in TN	1.418	0.765–2.627	0.267	1.858	1.001–3.448	0.049

OS = overall survival; DFS = disease free survival; HR = hazard ratio; CI = confidence interval; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; LN = lymph node; TS = tumor stroma; TN = tumor nest; NA = not available.

Univariate Cox regression analyses revealed that PR positivity, HER2 overexpression, higher Ki-67 proliferating index (≥ 14%), larger tumor size, LN metastasis, and CD68+ or CD163+ macrophages in TN were independent prognostic factors for OS. HER2 overexpression, higher Ki-67 proliferating index (≥ 14%), larger tumor size, LN metastasis, CD11c+ macrophages in TS, and CD68+ or CD163+ macrophages in TN were independent prognostic factors for DFS (**Table 2**).

Multivariate analyses revealed that larger tumor size, CD11c+ macrophages in TS, and CD163+ macrophages in TN were an independent prognostic factor for DFS. In addition, HER2 overexpression and LN metastasis were independent prognostic factors for both OS and DFS (**Table 3**).

DISCUSSION

In this study, we found that a high degree of TAM infiltration in IBC was associated with adverse clinical and pathological prognostic parameters, such as high histological grade, large tumor size, ER negativity, PR negativity, and high Ki-67 proliferating index. We found that the high expression of TAMs in breast cancer correlated with negative hormone receptor status. These results were similar to those of previous studies, which reported that high expression of CD68 or CD163 was associated with hormone receptor negativity [2-4,6]. We found no significant association between HER2 status and TAMs. Several studies have

disputed the association between macrophage infiltration and HER2 status. A few studies have reported that TAMs are associated with HER2-positivity [2,4]. Other studies showed no association between TAMs and HER2 status [3,6]. We found that the higher number of TAMs in IBC correlated with a higher expression of tumor proliferation marker, Ki-67, as reported previously [6]. Our study showed that luminal A breast cancer contained fewer CD68+, CD11c+, and CD163+ cells when compared with triple-negative breast cancer. This result corroborated the results of a previous study suggesting that the triple-negative/basal-like breast cancer contained more CD163+ or CD68+ macrophages in TS than in luminal A breast cancer [3].

Programmed cell death 1 (PD-1) is a receptor that regulates the activation and apoptosis of inflammatory cells. PD-ligand 1 (PD-L1) binds to PD-1 leading to a reduced immune response of the tumor cells. In breast cancer, PD-L1 expression is associated with LN metastasis, higher histological grade, ER negativity, and triple-negative breast cancer [21]. PD-L1 expression has been associated with poor prognosis in various human cancers [22]. In triple-negative breast cancer, TAMs regulate the activity of anti-PD-1 and PD-L1 agents by secreting cytokines [23].

Macrophage polarization into M1 or M2 phenotypes leads to varied cytokine secretion and function. M1 macrophages generate interleukin (IL)-12 and tumor necrosis factor with lethal antimicrobial and antitumor effects in cells. M2 macrophages produce cytokines, including IL-10, IL-1 receptor antagonist type II, and IL-1 decoy receptor. They regulate the immune response and adaptive immunity and promote angiogenesis and tissue repair. TAMs are functionally similar to M2 macrophages due to the secretion of IL-10 and transforming growth factor-beta (TGF-β). By expressing characteristics of M2 macrophages, TAMs promote tumor growth and metastasis [8].

CD68 is best known as a pan-macrophage marker and commonly used as a marker for TAMs [5]. Previous studies have reported that higher CD68+ macrophage infiltration in IBC is associated with larger tumor size and unfavorable prognosis [3,5]. It was suggested that IBC interacts with TAMs and induces an immune response that supports tumor growth. Infiltration of CD68+ macrophages correlated with poor prognosis in breast, cervix, and bladder carcinoma, but with favorable prognosis in prostate, lung, and brain tumors [24]. These results are probably due to similar CD68 expression by both M1 and M2 macrophages. In our study, the infiltration of CD68+ macrophages in TN showed poor prognosis and infiltration into TS showed a trend of unfavorable prognosis.

CD163 is the optimal marker for M2 macrophages [13]. High numbers of CD163+ macrophages in breast cancer are associated with rapid proliferation, poor differentiation, ER negativity, and ductal histological type [25]. In this study, the higher numbers of CD163+ macrophages in TS or TN were associated with poor prognosis, high histological grade, larger tumor size, high Ki-67 proliferating index, ER negativity, and PR negativity. In pancreatic cancer, the increased number of CD163+ macrophages within the tumor invasive front was associated with LN metastasis and poor prognosis [26]. High-grade tumor may secrete higher levels of monocyte colony stimulating factor, IL-10, and/or TGF-β, which results in the high number of CD163+ M2 macrophages [25]. Considering that M2 macrophages play a role in tumor progression by producing vascular endothelial growth factor and extracellular matrix remodeling proteins [12], the increased number of CD163+ macrophages might explain an unfavorable prognosis.

Tumor infiltrating mature dendritic cells have been known to be associated with a favorable prognosis; however, immature dendritic cells are not associated with a favorable prognosis [27]. CD11c is overexpressed in myeloid- and monocyte-associated dendritic cells, in natural killer cells, macrophages, and even a few activated B and T cells [28]. In our study, the infiltration of CD11c+ macrophages in both TS and TN was associated with high histological grade, high Ki-67 proliferating index, ER negativity, and PR negativity. Further, the infiltration of CD11c+ macrophages in TS was associated with favorable OS and DFS. Interestingly, the CD11c+ macrophages in TS were associated with large tumor size. However, infiltration of high number of CD11c+ macrophages in TS was an independent predictor of favorable OS or DFS. In gastric cancer, high expression of CD11c reduced the risk of death and relapse compared with patients showing a low expression, except for tumor size [27]. Consequently, CD11c expression may be a potential indicator of tumor size.

In our patients, the infiltration of CD68+ or CD163+ macrophages in TN correlated with clinicopathological features and was associated with unfavorable OS and DFS, while the infiltration of CD11c+ macrophages in TS was associated with favorable OS and DFS. In melanoma, the infiltration of CD68+ TAMs in TN correlated with poor OS and DFS [29]. In endometrial cancer, the infiltration of CD68+ cells in TN showed positive correlation with decreased recurrence [30]. Considering these results, it was suggested that when examining TAMs in malignant tumors, localization should also be considered apart from their mere presence. However, 1 breast cohort study showed that CD68+ macrophages in the TS, but not in the TN, were correlated with clinicopathological features or survival [3]. Although our result showed that the infiltration of TAMs in TN appears to be associated with patient outcomes in IBC, further evaluation is still needed.

We found that increased abundance of TAMs including M2 macrophages are associated with tumor progression in IBC. The infiltration of CD68+ or CD163+ macrophages in TN suggested unfavorable prognosis for breast cancer patients, whereas CD11c+ macrophages in TS lead to favorable prognosis. In addition to simply analyzing the degree of TAM infiltration, it is also important to analyze the location of TAMs as a prognostic marker. Finally, the lack of a reliable M1 macrophage marker appears to be a challenge in studies investigating macrophages.

ACKNOWLEDGEMENTS

REFERENCES

1. Burugu S, Asleh-Aburaya K, Nielsen TO. Immune infiltrates in the breast cancer microenvironment: detection, characterization and clinical implication. Breast Cancer 2017;24:3-15.
   PUBMED | **CROSSREF**

2. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. Tumour-infiltrating macrophages and clinical outcome in breast cancer. J Clin Pathol 2012;65:159-63.
   PUBMED | **CROSSREF**

3. Medrek C, Pontén F, Jirström K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer 2012;12:306.
   PUBMED | **CROSSREF**

4. Gwak JM, Jang MH, Kim DI, Seo AN, Park SY. Prognostic value of tumor-associated macrophages according to histologic locations and hormone receptor status in breast cancer. PLoS One 2015;10:e0125728.
PUBMED | CROSSREF

5. Yang J, Li X, Liu X, Liu Y. The role of tumor-associated macrophages in breast carcinoma invasion and metastasis. Int J Clin Exp Pathol 2015;8:6656-64.
PUBMED

6. Lindsten T, Hedbrant A, Ramberg A, Wijkander J, Solterbeck A, Eriksson M, et al. Effect of macrophages on breast cancer cell proliferation, and on expression of hormone receptors, uPAR and HER-2. Int J Oncol 2017;51:104-14.
PUBMED | CROSSREF

7. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010;32:593-604.
PUBMED | CROSSREF

8. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549-55.
PUBMED | CROSSREF

9. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. Nat Rev Immunol 2009;9:609-17.
PUBMED | CROSSREF

10. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23-35.
PUBMED | CROSSREF

11. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol 2004;4:583-94.
PUBMED | CROSSREF

12. Weber M, Büttner-Herold M, Hyckel P, Moebius P, Distel L, Ries J, et al. Small oral squamous cell carcinomas with nodal lymphogenic metastasis show increased infiltration of M2 polarized macrophages--an immunohistochemical analysis. J Craniomaxillofac Surg 2014;42:1087-94.
PUBMED | CROSSREF

13. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TR, Reedquist KA, et al. Systematic validation of specific phenotypic markers for *in vitro* polarized human macrophages. J Immunol Methods 2012;375:196-206.
PUBMED | CROSSREF

14. Liu C, Li Y, Yu J, Feng L, Hou S, Liu Y, et al. Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. PLoS One 2013;8:e54841.
PUBMED | CROSSREF

15. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC Cancer Staging Manual. New York (NY): Springer; 2010.

16. Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ. WHO Classification of Tumours of the Breast. Lyon: International Agency for Research on Cancer; 2012.

17. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Arch Pathol Lab Med 2010;134:907-22.
PUBMED

18. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 2013;31:3997-4013.
PUBMED | CROSSREF

19. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst 2011;103:1656-64.
PUBMED | CROSSREF

20. Guiu S, Michiels S, André F, Cortes J, Denkert C, Di Leo A, et al. Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 working group statement. Ann Oncol 2012;23:2997-3006.
PUBMED | CROSSREF

21. Zhang M, Sun H, Zhao S, Wang Y, Pu H, Wang Y, et al. Expression of PD-L1 and prognosis in breast cancer: a meta-analysis. Oncotarget 2017;8:31347-54.
PUBMED

22. Bae SB, Cho HD, Oh MH, Lee JH, Jang SH, Hong SA, et al. Expression of programmed death receptor ligand 1 with high tumor-infiltrating lymphocytes is associated with better prognosis in breast cancer. J Breast Cancer 2016;19:242-51.
   PUBMED | CROSSREF

23. Santoni M, Romagnoli E, Saladino T, Foghini L, Guarino S, Capponi M, et al. Triple negative breast cancer: key role of tumor-associated macrophages in regulating the activity of anti-PD-1/PD-L1 agents. Biochim Biophys Acta Rev Cancer 2018;1869:78-84.
   PUBMED | CROSSREF

24. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 2002;196:254-65.
   PUBMED | CROSSREF

25. Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Mönkkönen J, et al. Human breast cancer cells educate macrophages toward the M2 activation status. Breast Cancer Res 2015;17:101.
   PUBMED | CROSSREF

26. Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, Kubo F, et al. Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J Surg Res 2011;167:e211-9.
   PUBMED | CROSSREF

27. Wang Y, Xu B, Hu WW, Chen LJ, Wu CP, Lu BF, et al. High expression of CD11c indicates favorable prognosis in patients with gastric cancer. World J Gastroenterol 2015;21:9403-12.
   PUBMED | CROSSREF

28. Castro FV, Tutt AL, White AL, Teeling JL, James S, French RR, et al. CD11c provides an effective immunotarget for the generation of both CD4 and CD8 T cell responses. Eur J Immunol 2008;38:2263-73.
   PUBMED | CROSSREF

29. Jensen TO, Schmidt H, Møller HJ, Høyer M, Maniecki MB, Sjoegren P, et al. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J Clin Oncol 2009;27:3330-7.
   PUBMED | CROSSREF

30. Ohno S, Ohno Y, Suzuki N, Kamei T, Koike K, Inagawa H, et al. Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. Anticancer Res 2004;24:3335-42.
   PUBMED