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Impact of the immune molecular profile of the tumor microenvironment on the prognosis of NSCLC

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Abstract. The present study aimed to clarify the association between macrophages, tumor neo-vessels and programmed cell death-ligand 1 (PD-L1) in the tumor microenvironment and the clinicopathological features of patients with non-small cell lung cancer (NSCLC), and to explore the prognostic factors of stromal features in NSCLC. To determine this, tissue microarrays containing samples of 92 patients with NSCLC were studied using immunohistochemistry and immunofluorescence. The quantitative data demonstrated that in tumor islets, the number of CD68$^+$ and CD206$^+$ tumor-associated macrophages (TAMs) was 8-348 (median, 131) and 2-220 (median, 52), respectively (P<0.001). In tumor stroma, the number of CD68$^+$ and CD206$^+$ TAMs was 23-412 (median, 169) and 7-358 (median, 81), respectively (P<0.001). The number of CD68$^+$ TAMs in each location of the tumor islets and tumor stroma was significantly higher than that of CD206$^+$ TAMs, and they were significantly correlated (P<0.0001). The quantitative density of CD105 and PD-L1 in tumor tissues was 19-368 (median, 156) and 9-493 (median, 103), respectively. Survival analysis revealed that a high density of CD68$^+$ TAMs in tumor stroma and islets and a high density of CD206$^+$ TAMs and PD-L1 in tumor stroma were associated with worse prognosis (both P<0.05). Collectively, the survival analysis demonstrated that the high-density group was related to a worse prognosis regardless of combined neo-vessels and PD-L1 expression with the CD68$^+$ TAMs in tumor islets and stroma, or CD206$^+$ TAMs in tumor islets and stroma. To the best of our knowledge, the present study was the first to provide a multi-component combined prognostic survival analysis of different types of macrophages in different regions with tumor neo-vessels and PD-L1, which demonstrated the importance of macrophages in tumor stroma.

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Introduction

Lung cancer is the most common cause of cancer-related death, of which non-small cell lung cancer (NSCLC) comprises 85-90% (1). Despite the development of treatments, the 5-year survival rate is <15% (2). Paget's 'seed-soil' theory demonstrates that the tumor microenvironment (TME) has a vital role in tumor growth and progression (3). The TME is a complex and dynamic community, among which immune infiltrating cells and vascular endothelial cells are the most representative factors (4). Macrophages, important representatives of immune infiltrating cells, act as vital components of the host's defense, antigen-presenting cells and effector cells, and may be classified into the classic M1 type or the alternative M2 type (5,6). M1 type macrophages are a tumor suppressor type that participate in inflammatory response, pathogen removal and antitumor immunity, while M2 type macrophages promote the occurrence and development of tumors by inducing angiogenesis and anti-inflammation (7). Macrophages have different biological properties due to different distributions (8), so the selection of macrophage markers and tissue sites of interest affect the prognostic role of tumor-associated macrophages (TAMs) on NSCLC, to a certain extent. CD68$^+$ is the TAM marker that is commonly considered to be a pan-macrophage marker, but it cannot distinguish between M1 and M2

subtypes (9). M2 macrophages have a variety of surface markers, including CD163, CD204 and CD206. Among them, CD206, which is expressed on the surface of most classes of macrophages and dendritic cell subgroups, is routinely used to identify the M2 subtype (10). Certain studies suggest that a higher stromal TAM density is an independent prognostic factor and leads to poor prognosis (11,12). However, M2 macrophage density is more closely related to poor prognosis than $CD68^+$ TAMs (13,14).

Studies have confirmed that a tumor is only a minor and asymptomatic lesion prior to the formation of angiogenesis (15,16). With the formation of angiogenesis, the tumor size rapidly increases and has an enhanced potential for distant metastasis (17,18). CD105 is an endoglin used to evaluate blood vessels that is considered to only recognize abnormal blood vessels induced by tumors, and it has higher specificity than CD34 (19,20). In addition, studies have indicated that TAMs and cancer cells may promote tumor angiogenesis and metastasis (21-26). Immune checkpoint inhibitors targeting the programmed cell death protein-1 (PD-1)/PD-ligand 1 (PD-L1) pathway have demonstrated an impressive clinical benefit in NSCLC and the expression of PD-L1 may partially predict the treatment effectiveness (27). TAMs are able to secrete vascular endothelial growth factors, such as IL-10, secrete IL-1 β, induce regulatory T cells, increase PD-L1 expression in tumor cells and inhibit the function of effector T cells, leading to infiltration and distant metastasis of cancer cells (28).

The present study focused on the temporal-spatial distribution and quantitative expression of $CD68^+$ and $CD206^+$ TAMs in two intra-tumor areas, and CD105 and PD-L1 in the TME, to investigate the heterogenic molecular profile of the TME, in an attempt to provide a guide for improving the individual treatment strategy for patients with NSCLC.

Materials and methods

Patients and samples. A total of 92 paraffin-embedded NSCLC samples were collected from Zhejiang Cancer Hospital (Hangzhou, China) between April 2008 and January 2014. Using these samples, 6 tissue microarrays (TMAs) were constructed, as previously described (29). For construction, a 3-mm core was taken from each representative tumor tissue. The study protocol was approved by The Ethics Committee of Zhejiang Cancer Hospital (Hangzhou, China) and the patients provided written informed consent regarding the use of their tissues. The main patient inclusion criteria were as follows: i) Histologically confirmed primary NSCLC; ii) patient underwent curative radical surgery. Patients who had received other anticancer treatment prior to surgery were excluded. Tumor staging was based on the 8th Tumor-Node-Metastasis classification system of the American Joint Committee on Cancer staging criteria (30). The last follow-up date was April 2017, at which point all patients had died. Overall survival (OS) time was defined as the interval from the date of surgery to the date of death. Disease-free survival (DFS) time was defined as the interval from the date of surgery to the date of disease progression.

Immunohistochemistry (IHC) and multiplexed immunofluorescence. IHC staining for macrophages marked by CD68 and CD206, and tumor neo-vessels marked by CD105 and PD-L1, was performed. In brief, TMA slides were treated by deparaffinization in xylene, hydration with graded alcohols and subjected to antigen retrieval (98˚C, 20 min). The slides were then placed in 3% hydrogen peroxide for 10 min at room temperature to inactivate endogenous peroxidases. After washing three times in PBS, the slides were blocked with 2% BSA (cat. no. B2064; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature, followed by incubation with primary antibodies against CD68 (1:200 dilution; cat. no. ab125212; Abcam), CD206 (1:200; cat. no. 60143-1-Ig; ProteinTech Group, Inc.), CD105 (1:500; cat. no. ab2529; Abcam) and PD-L1 (1:100; cat. no. ab205921; Abcam) at 4˚C overnight. After washing in PBS, the slides were incubated with secondary antibody (1:200; Goat Anti-Rabbit IgG (horseradish peroxidase, cat. no. ab150077; Abcam) for 60 min at 37˚C. The slides were visualized using Dako REAL EnVision™ (DAB; cat. no. PW017; Sangon Biotech, Co., Ltd.) and counterstained with haematoxylin for 2 min at room temperature.

Multiplexed immunofluorescence staining for CD68 and CD105 was also performed. The primary antibodies against CD68 and CD105 were mixed. Similar to IHC, after the secondary immunofluorescent antibody (1:2,000; Goat Anti-Rabbit IgG and Goat Anti-Mouse IgG (horseradish peroxidase), cat. nos. ab6721 and ab6789; Abcam) was incubated with the slides (37˚C, 60 min), all slides were covered by Fluoroshield containing DAPI (Abcam) for 10 min at room temperature to identify nuclei.

Quantification of IHC and immunofluorescent staining. All slides were examined under an Olympus BX51 fluorescence microscope equipped with an Olympus DP72 camera (Olympus Corporation). Positive staining was indicated by brownish granules. The macrophages marked by CD68 and CD206 were counted in three high-power fields selected at the tumor islets and tumor stroma, and the mean number of $CD68^+$ and $CD206^+$ cells in these three fields was documented. Tumor islets were defined as areas where tumor cells accounted for >70% of the total cells, and tumor stroma as areas where tumor stromal cells accounted for >70% of the total cells (12). Tumor neo-vessels marked by CD105 and tumor cells expressing PD-L1 were counted in six high-power fields selected at the tumor site and the mean cell counts were documented. Pathologists defined positively expressed cells according to the Hue, Saturation, Intensity (HSI) color selection system (H=0-30; S=0-255; I=0-255), specified cell size and filtered non-specific positive color rendering noise <50 pixels, after which the software automatically counted. Positive cell counting was completed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc). The cut-off value according to the median of each group was used to determine the density of infiltrating macrophages, tumor neo-vessel density and PD-L1 expression. Two independent pathologists who were blinded to the clinicopathological characteristics of all tissue specimens participated in the data evaluation.

Statistical analysis. Statistical analyses were performed with SPSS 25.0 (IBM Corp.) and R 4.2.2 (R Development Core Team). For categorical data, the χ^2 test was performed. Spearman's rank correlation analysis was used to analyze

Table I. Relationship between $CD68^+$ TAMs, $CD206^+$ TAMs and the clinicopathological features of patients with non-small cell lung cancer.

		$CD68^+$ TAMs[a]						$CD206^+$ TAMs					
		Tumor islets			Tumor stroma			Tumor islets			Tumor stroma		
Clinicopathological feature	Total number of patients	Low n=46	High n=45	P-value	Low n=46	High n=45	P-value	Low n=47	High n=45	P-value	Low n=46	High n=46	P-value
Sex				0.193			0.759			0.390			0.804
Female	21	8 (38.1)	13 (61.9)		10 (47.6)	11 (52.4)		9 (42.9)	12 (57.1)		11 (52.4)	10 (47.6)	
Male	71	38 (54.3)	32 (45.7)		36 (51.4)	34 (48.6)		38 (53.5)	33 (46.5)		35 (49.3)	36 (50.7)	
Age, years				0.908			0.760			0.982			0.294
≤60	41	21 (51.2)	20 (48.8)		20 (48.8)	21 (51.2)		21 (51.2)	20 (48.8)		23 (56.1)	18 (43.9)	
>60	51	25 (50.0)	25 (50.0)		26 (52.0)	24 (48.0)		26 (51.0)	25 (49.0)		23 (45.1)	28 (54.9)	
Smoking status				0.320			0.691			0.552			1.000
Never smoker	26	11 (42.3)	15 (57.7)		14 (53.8)	12 (46.2)		12 (46.2)	14 (53.8)		13 (50.0)	13 (50.0)	
Current/former	66	35 (53.8)	30 (46.2)		32 (49.2)	33 (50.8)		35 (53.0)	31 (47.0)		33 (50.0)	33 (50.0)	
Histology				0.607			0.107			0.549			1.000
Adenocarcinoma	54	28 (52.8)	25 (47.2)		23 (43.4)	30 (56.6)		29 (53.7)	25 (46.3)		27 (50.0)	27 (50.0)	
Non-adenocarcinoma	38	18 (50.5)	20 (52.6)		23 (60.5)	15 (39.5)		18 (47.4)	20 (52.6)		19 (50.0)	19 (50.0)	
Tumor size, cm				0.423			0.606			0.879			0.381
≤5	60	28 (47.5)	31 (52.5)		31 (52.5)	28 (47.5)		31 (51.7)	29 (48.3)		32 (53.3)	28 (46.7)	
>5	32	18 (56.3)	14 (43.8)		15 (46.9)	17 (53.1)		16 (50.0%	16 (50.0)		14 (43.8)	18 (56.3)	
Differentiation[b]				0.413			0.144			0.371			0.631
Low	16	5 (33.3)	10 (66.7)		6 (40.0)	9 (60.0)		11 (68.8)	5 (31.3)		10 (62.5)	6 (37.5)	
Moderate	34	18 (52.9)	16 (47.1)		13 (38.2)	21 (61.8)		17 (50.0)	17 (50.0)		17 (50.0)	17 (50.0)	
High	31	16 (51.6)	15 (48.4)		19 (61.3)	12 (38.7)		15 (48.4)	16 (51.6)		15 (48.4)	16 (51.6)	
Lymph node metastasis				0.603			0.941			0.038			0.026
Negative	30	14 (46.7)	16 (53.3)		15 (50.0)	15 (50.0)		20 (66.7)	10 (33.3)		20 (66.7)	26 (41.9)	
Positive	62	32 (52.5)	29 (47.5)		31 (50.8)	30 (49.2)		27 (43.5)	35 (56.5)		10 (33.3)	36 (58.1)	
Stage				0.072			0.168			0.820			0.209
I, II	42	25 (61.0)	16 (39.0)		24 (58.5)	17 (41.5)		22 (52.4)	20 (47.6)		24 (57.1)	18 (42.9)	
III	50	21 (42.0)	29 (58.0)		22 (44.0)	28 (56.0)		25 (50.0)	25 (50.0)		22 (44.0)	28 (56.0)	

[a]91 cases were detected. Values are expressed as n (%). TAM, tumor-associated macrophages. [b]81 cases recorded differentiation.

Table II. Relationship between CD105, PD-L1 and the clinicopathological features of non-small cell lung cancer.

		CD105-positive cells			PD-L1-positive cells		
Clinicopathological feature	Total number of patients	Low n=46	High n=46	P-value	Low n=46	High n=46	P-value
Sex				0.804			0.082
Female	21	11 (52.4)	10 (47.6)		14 (66.7)	7 (33.3)	
Male	71	35 (49.3)	36 (50.7)		32 (45.1)	39 (54.9)	
Age, years				0.294			0.529
≤60	41	18 (43.9)	23 (56.1)		19 (46.3)	22 (53.7)	
>60	51	28 (54.9)	23 (45.1)		27 (52.9)	24 (47.1)	
Smoking status				0.643			0.552
Never smoked	26	12 (46.2)	14 (53.8)		16 (61.5)	10 (38.5)	
Current/former smoker	66	34 (51.5)	32 (48.5)		30 (45.5)	36 (47.0)	
Histology				0.672			0.165
Adenocarcinoma	54	26 (48.1)	28 (51.9)		25 (46.3)	29 (54.5)	
Non-adenocarcinoma	38	20 (52.6)	18 (47.4)		21 (55.3)	17 (44.7)	
Tumor size, cm				0.381			0.662
≤5	60	28 (46.7)	32 (53.5)		31 (51.7)	29 (48.3)	
>5	32	18 (56.3)	14 (43.8)		15 (46.9)	17 (53.1)	
Differentiation[a]				0.691			0.399
Low	16	9 (56.3)	7 (43.8)		7 (43.8)	9 (56.3)	
Moderate	34	15 (44.1)	19 (55.9)		16 (47.1)	18 (52.9)	
High	31	16 (51.6)	15 (48.4)		19 (61.3)	12 (38.7)	
Lymph node metastasis				0.656			0.656
Negative	30	14 (46.7)	16 (53.3)		14 (46.7)	16 (53.3)	
Positive	62	32 (51.6)	30 (48.4)		32 (51.6)	30 (48.4)	
Stage				0.675			0.209
I, II	42	20 (47.6)	22 (52.4)		24 (57.1)	18 (42.9)	
III	50	26 (52.0)	24 (48.0)		22 (44.0)	28 (56.0)	

Values are expressed as n (%). PD-L1, programmed cell death-ligand 1. [a]81 cases recorded differentiation.

the correlation between macrophages, tumor neo-vessels and PD-L1 expression. Differences in the CD68+ and CD206+ TAMs among the groups were analyzed by the Mann-Whitney U-test. The Kaplan-Meier method was used to estimate the survival curve for OS time, and the log-rank or two-stage tests were used to assess the differences in survival between groups. The Cox regression model was used to assess the influence of the binary factors in univariate and multivariate analyses. The factors with P<0.2 in the univariate analysis were included in the multivariate analysis, and the relationship between TME-related markers and prognosis was examined. A two-tailed P<0.05 was considered to indicate statistical significance.

Results

Major clinicopathological features of the 92 NSCLC cases and heterogenic TME in TMAs. Among the 92 NSCLC cases included in the present study, 71 (77.2%) were male and 21 (22.8%) were female, and they were aged 39-75 (median, 61) years. With regards to staging, there was 1 (1.1%) case of IA, 9 (9.8%) cases of IB, 7 (7.6%) of IIA, 23 (25%) of IIB, 44 (47.8%) of IIIA and 8 (8.7%) of IIIB. A total of 58 patients underwent adjuvant therapy, 40 of which received adjuvant chemotherapy, 12 received adjuvant radiotherapy and chemotherapy, 5 received adjuvant radiotherapy and 1 received tyrosine kinase inhibitor treatment. A total of 16 patients did not receive any adjuvant therapy and the therapy regimen of the remaining 18 patients was unknown. The two-stage test demonstrated that there was no significant difference in OS and DFS times between the treatment and no-treatment groups (median OS, 30 vs. 23 months, respectively; P=0.571; median DFS, 11 vs. 13 months, respectively; P=0.844; Fig. S1). There were 73 known progression or recurrence events, and the treatments of 51 patients were known. Among these 51 patients, 30 (58.8%) received two or more combined treatments, 19 (37.3%) received one treatment strategy and 2 (3.9%) did not receive any treatment. These treatments had no impact on the OS time between single treatment, multiple treatments and no-treatment (median OS, 33 vs. 36 vs. 6 months, respectively; P=0.289;

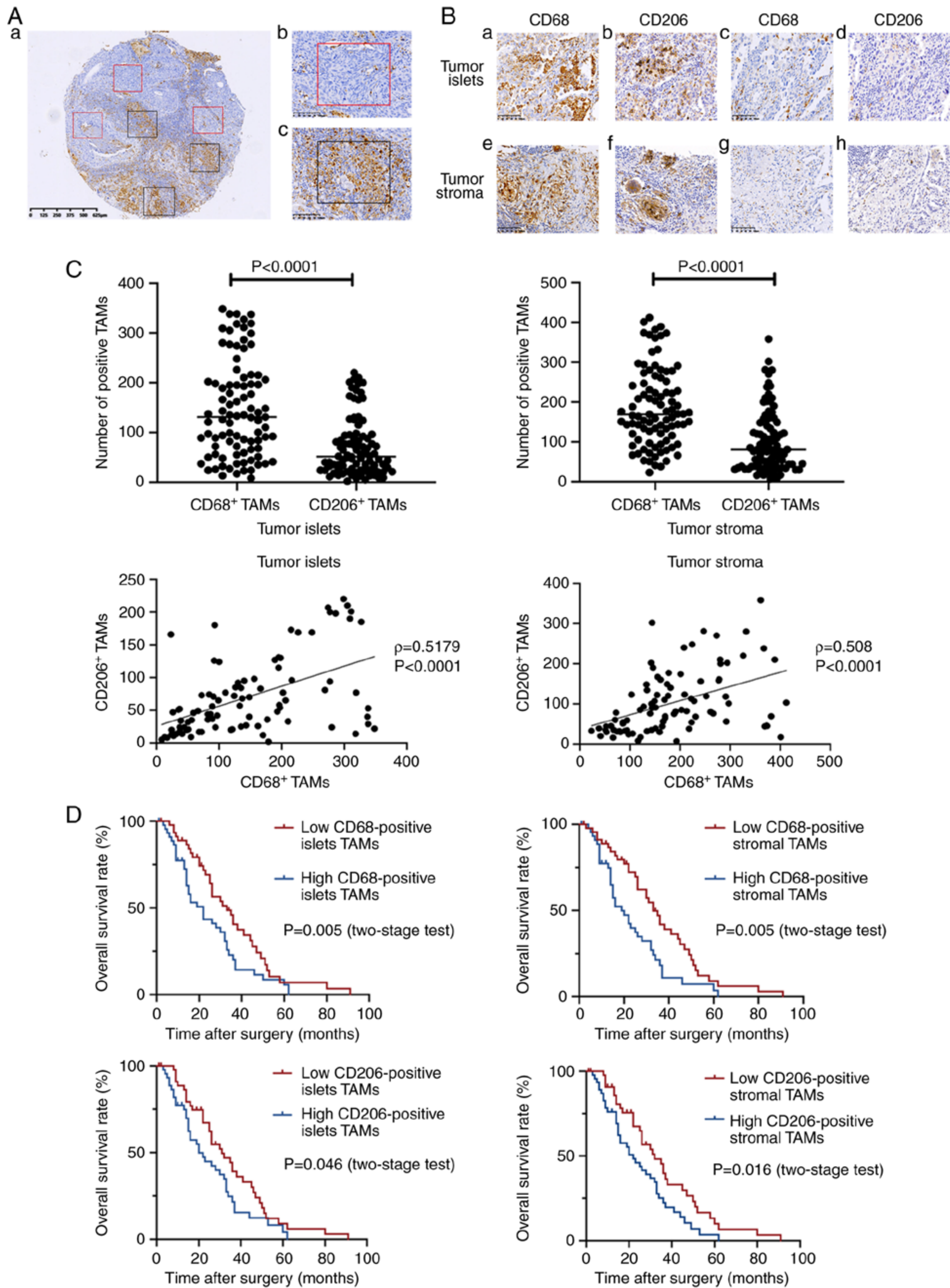

Figure 1. Distribution and quantitative data of $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma. (A) The digital image of $CD68^+$ expression with immunohistochemical staining scanned by a pathology digital imaging system at (a) x4 magnification. For each tissue microarray core, three representative 0.1-mm^2 fields were separately selected [tumor islets are marked with a (b) red frame and tumor stroma marked with a (c) black frame] at x20 magnification. (B) Representative images of $CD68^+$ and $CD206^+$ expression in tumor islets and stroma. (a and b) Case with high expression of $CD68^+$ and $CD206^+$ TAMs in tumor islets, respectively. (c and d) Case with low expression of $CD68^+$ and $CD206^+$ TAMs in tumor islets, respectively. (e and f) Case with high expression of $CD68^+$ and $CD206^+$ TAMs in tumor stroma, respectively. (g and h) Case with low expression of $CD68^+$ and $CD206^+$ TAMs in tumor stroma, respectively (x20 magnification). (C) Quantitative data of $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma (above). The correlations between $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma (below). (D) Survival analysis of $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma. TAMs, tumor-associated macrophages.

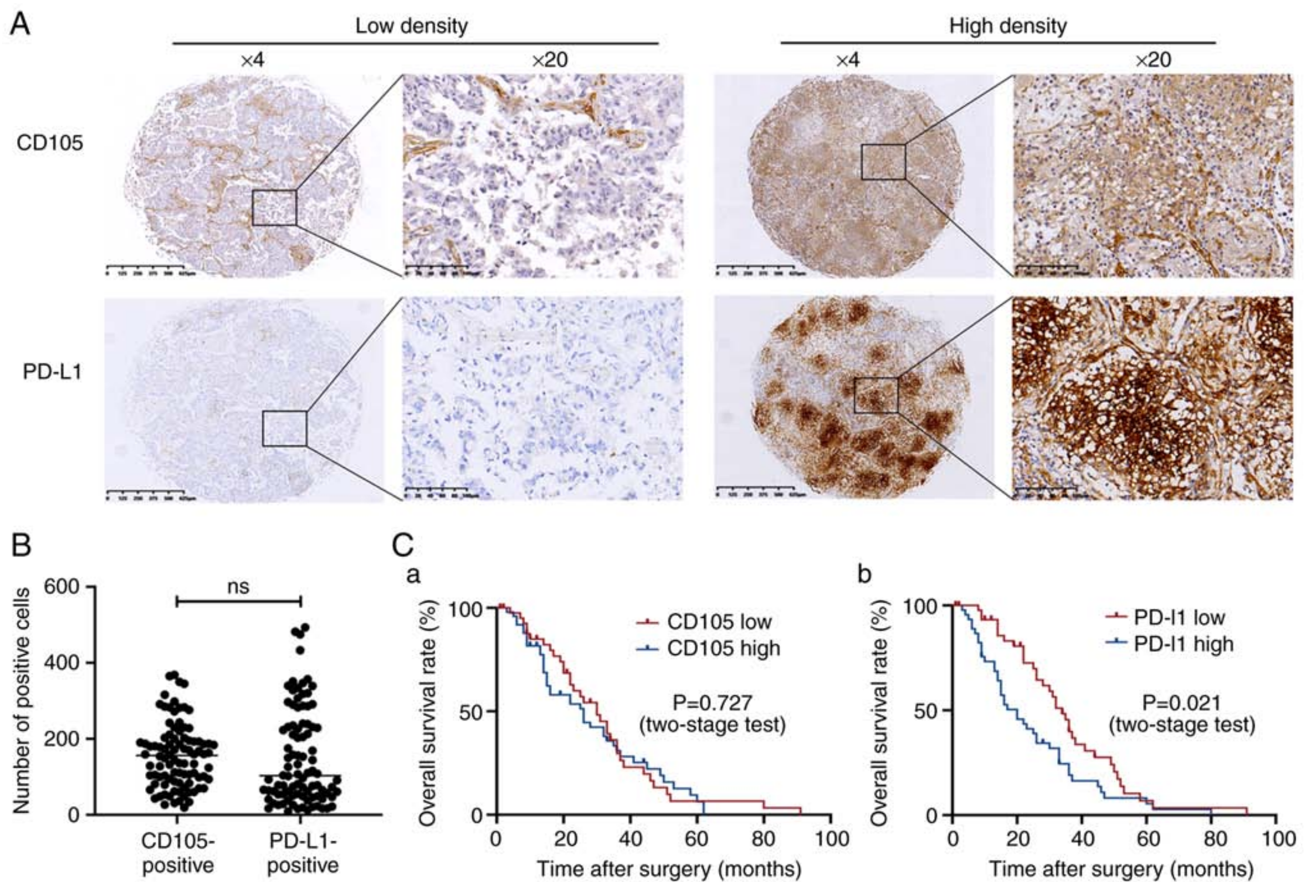

Figure 2. Expression of CD105 and PD-L1 in tissue microarrays and survival analyses. (A) Immunohistochemical staining for CD105 and PD-L1 expression in NSCLC tissues. (B) Quantitative data of CD105 positive and PD-L1 positive cells in tumor tissues (left panel: Magnification, x4; scale bar, 625 μm. Right panel: Magnification, x20; scale bar, 100 μm). (C) Cumulative overall survival of patients with NSCLC. (a) Tumor neo-vessels are not associated with the OS of patients with NSCLC. (b) Patients in the high PD-L1 expression groups have a higher risk of death. ns, not significant; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death-ligand 1.

Fig. S1). Other major clinicopathological characteristics data available, such as smoking status, tumor size, histology and differentiation, are presented in Tables I and II.

IHC analysis, including distribution and quantitative measurement of $CD68^+$ TAMs, $CD206^+$ TAMs, CD105 and PD-L1, was performed on all TMAs. In certain cases, only few $CD68^+$ and $CD206^+$ TAMs were present compared with other cases with high expression ('expression' here and throughout means the density of cells with positive expression) (Fig. 1A and B). The quantitative data demonstrated that in tumor islets, the mean numbers of $CD68^+$ and $CD206^+$ TAMs were 146 (median, 131; range, 8-348) and 70 (median, 52; range, 2-220), respectively ($P<0.001$). In tumor stroma, the mean numbers of $CD68^+$ and $CD206^+$ TAMs were 186 (median, 169; range, 23-412) and 103 (median, 81; range, 7-358), respectively ($P<0.001$). Therefore, the quantity of $CD68^+$ TAMs was higher than that of $CD206^+$ TAMs in tumor islets and stroma. The $CD68^+$ and $CD206^+$ TAMs also had a higher distribution in tumor stroma than in tumor islets (both $P<0.0001$). In addition, significant correlations were found between the distributions of $CD68^+$ and $CD206^+$ TAMs in each area (tumor islets, $\rho=0.5179$; tumor stroma, $\rho=0.5081$; both $P<0.0001$; Fig. 1C). According to the median of $CD68^+$ and $CD206^+$ TAMs in the tumor islets and stroma as the cut-off, the patients were divided into the low-density or high-density group. The survival analysis demonstrated that patients with low expression of $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma had a favorable prognosis (Fig. 1D).

CD105 and PD-L1 staining were mainly located in the cytoplasm or on the cell membrane of the tumor stroma. Tumor neo-vessels and PD-L1 were also heterogeneously expressed in the TME, in certain cases with low expression and other cases with high expression. The quantitative density of $CD105^+$ cells in tumor tissues was 19-368 (median, 156). The quantitative density of PD-$L1^+$ cells in tumor tissues was 9-493 (median, 103) (Fig. 2A and B). According to the median density of cells with positive CD105 and PD-L1 expression in tumor tissues as the cut-off, patients were divided into the low-density or high-density group. The survival analysis demonstrated that the CD105 density had no association with OS time in patients with NSCLC, whereas patients with high PD-L1 expression had a higher risk of death (Fig. 2C).

Correlations between $CD68^+$ and $CD206^+$ TAMs, tumor neo-vessels, PD-L1 expression and clinicopathological features. The quantitative data of $CD68^+$ and $CD206^+$ TAM density are provided in Table I. For $CD68^+$ and $CD206^+$ TAMs in tumor islets and stroma, the cut-off to classify low and high subgroups. Subgroups were as follows: in the tumor islets, less than 131 is the low $CD68^+$TAM expression group, more than

Table III. Univariate and multivariate analyses of the clinicopathological factors for overall survival time of patients with non-small cell lung carcinoma.

	Univariate analysis			Multivariate analysis		
Clinicopathological factor	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Sex (male vs. female)	0.980	0.572-1.678	0.941			
Age, years (>60 vs. ≤60)	1.269	0.804-2.005	0.306			
Smoking status (smoker vs. never-smoker)	1.162	0.698-1.932	0.564	0.505	0.160-1.593	0.244
Histological type (adenocarinoma vs. non-adenocarinoma)	0.734	0.462-1.168	0.192	0.444	0.167-1.183	0.104
Tumor size, cm (>5 vs. ≤5)	1.373	0.850-2.218	0.195	1.945	1.089-3.475	0.025
Differentiation			0.045			0.033
Low vs. moderate	2.118	1.087-4.127	0.028	0.595	0.252-1.406	0.237
Low vs. high	1.752	1.013-3.030	0.045	0.336	0.146-0.774	0.010
Lymph node metastasis (positive vs. negative)	0.909	0.694-1.190	0.486	0.810	0.384-1.710	0.581
Stage (III vs. I, II)	1.123	0.706-1.785	0.624	0.785	0.374-1.645	0.521
CD105 expression (high vs. low)	1.106	0.698-1.753	0.667	1.002	0.998-1.005	0.301
PD-L1 expression (high vs. low)	1.685	1.066-2.663	0.025	1.003	1.001-1.011	0.010
$CD68^+$ TAMs in tumor islets (high vs. low)	1.666	1.051-2.641	0.030	1.006	1.001-1.011	0.031
$CD206^+$ TAMs in tumor islets (high vs. low)	1.580	0.996-2.506	0.052	0.999	0.991-1.007	0.750
$CD68^+$ TAMs in tumor stroma (high vs. low)	1.916	1.202-3.055	0.006	1.000	0.995-1.005	0.941
$CD206^+$ TAMs in tumor stroma (high vs. low)	1.741	1.091-2.776	0.020	0.999	0.993-1.005	0.725

CI, confidence interval; HR, hazard ratio; PD-L1, programmed cell death-ligand 1; TAMs, tumor-associated macrophages.

or equal to 131 is the high $CD68^+$TAM expression group, less than 52 is the low $CD206^+$TAM expression group, more than or equal to 52 is the high $CD206^+$TAM expression group; in tumor stroma, less than 169 is the low $CD68^+$TAM expression group, more than or equal to 169 is the high $CD68^+$TAM expression group, less than 81 is the low $CD206^+$TAM expression group, more than or equal to 81 is the high $CD206^+$TAM expression group. For tumor neo-vessels and PD-L1 in tumor tissues, the cut-off to classify low and high subgroups was 156 and 103, respectively. In the low and high $CD68^+$ TAM subgroups, the high tumor neo-vessel density cases were 20 (43.5%) and 26 (56.5%), respectively. In the low and high PD-L1 expression subgroups, high densities of $CD68^+$ TAMs were observed in 18 (40.9%) and 26 cases (59.1%), respectively. In the low and high $CD206^+$ TAM subgroups, 21 (45.7%) and 25 (54.3%) cases had high tumor neo-vessel density, respectively. In the low and high PD-L1 expression subgroups, there were 15 (33.3%) and 30 (66.7%) cases with high $CD68^+$ TAM density, respectively. Of note, tumor neo-vessels, $CD68^+$ TAMs and PD-L1 expression were not significantly associated with any of the clinicopathological characteristics, which indicated that these key components of the TME were independent of clinical features, including tumor size, tumor histological type, degree of differentiation, lymph node metastasis and tumor staging (Tables I and II).

In addition, $CD68^+$ TAMs were mostly localized with tumor neo-vascularization, and the quantitative analysis demonstrated that $CD68^+$ TAMs and CD105 had similar trends in expression (ρ=0.2401; P=0.021; Fig. 3), while there was no obvious correlation between $CD206^+$ TAMs and CD105 (ρ=0.109; P>0.05; data not shown). Furthermore, the expression of $CD68^+$ TAMs was significantly correlated to the expression of PD-L1 in tumor tissues (ρ=0.332; P=0.030; data not shown) and there was a significant correlation between $CD206^+$ TAMs and PD-L1 (ρ=0.428; P=0.038; data not shown).

Prognostic significance of tumor stromal features in NSCLC. Univariate analyses demonstrated that clinical factors, such as tumor differentiation, were associated with OS time (P<0.05). PD-L1 and $CD68^+$ TAM densities in tumor islets and stroma were also negatively associated with DFS time (both P<0.05), but there was no statistically significant independent predictor of DFS time in NSCLC (Table SI). PD-L1 and $CD68^+$ TAM densities in tumor islets and stroma, as well as $CD206^+$ TAM density in tumor stroma, were negatively associated with OS time (P<0.05 for all). Furthermore, tumor size, differentiation degree, high density of $CD68^+$ TAMs in tumor islets and PD-L1 expression were statistically significant independent predictors of a poor prognosis in NSCLC (P<0.05 for all; Table III).

The aforementioned key components were explored collectively to reveal the association between TME and NSCLC prognosis. For the combined group, taking the median values for tumor neo-vessels, macrophages and PD-L1 as the cut-off, the patients could be divided into groups. The groups were assigned as follows: Group 1, all components were expressed at a low level; group 2, one or two of the components was expressed at a high level; group 3, all components were expressed at a high level. The combined analysis indicated that the OS rate of group 3 was worse than that of groups 1 and 2 (Fig. 4).

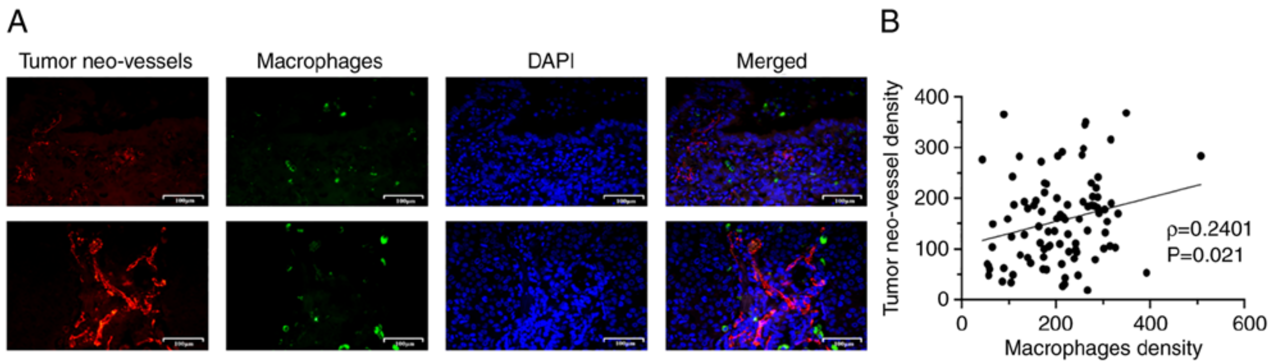

Figure 3. Co-expression of CD68+ TAMs and CD105 using multiplexed immunofluorescence. (A) Representative cases with low expression of both CD68+ TAMs and CD105 (above), and high expression of both CD68+ TAMs and CD105 (below). Staining is as follows: DAPI (nuclear DNA; blue), CD105 (tumor neo-vessel; red) and CD68 (macrophages; green). (B) Correlation between CD68+ TAMs and CD105 quantitative expression in microarrays comprising 92 non-small cell lung cancer tissues. TAMs, tumor-associated macrophages.

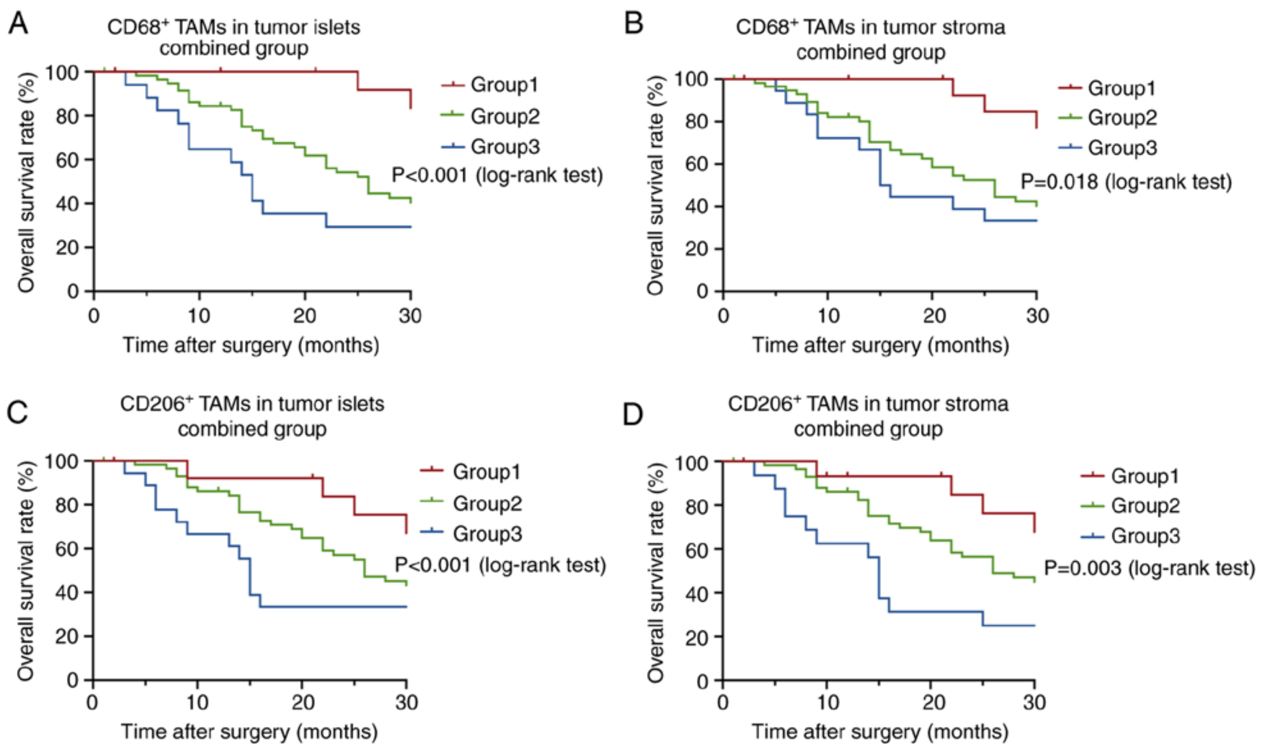

Figure 4. Cumulative OS of patients with NSCLC (within 30 months after surgery). (A) Combined CD68+ TAMs in tumor islets, CD105 and PD-L1 comprehensive analysis of OS of patients with NSCLC. (B) Combined CD68+ TAMs in the tumor stroma, CD105 and PD-L1 comprehensive analysis of OS of patients with NSCLC. (C) Combined CD206+ TAMs in tumor islets, CD105 and PD-L1 comprehensive analysis of OS of patients with NSCLC. (D) Combined CD206+ TAMs in the tumor stroma, CD105 and PD-L1 comprehensive analysis of OS of patients with NSCLC. In these combined groups, group 3 (the expressions of all components were at a high level) had a higher risk of death. NSCLC, non-small cell lung cancer; OS, overall survival; TAMs, tumor-associated macrophages; PD-L1, programmed cell death-ligand 1.

Discussion

A number of conflicting results have been reported regarding the prognostic significance of TAMs, tumor neo-vessels and PD-L1/PD-1 expression in NSCLC from clinical practice (31-35). The reasons for the inconsistent reports may be related to the choice of markers, as well as differences in statistical power and evaluation methods. In addition, these factors have not been observed to be adequately reliable as a single biomarker to evaluate the prognosis of patients with NSCLC (36,37). To the best of our knowledge, the present study was the first to compare TAM distribution detecting CD68+ and CD206+ in two intra-tumor areas, and to compare the distribution of TAMs, tumor neo-vessels and PD-L1 in NSCLC.

The results demonstrated that the number of CD68+ and CD206+ TAMs was higher in the tumor stroma but lower in tumor islets, and there was a correlation between the distribution of CD68+ and CD206+ TAMs in tumor islets and stroma. Furthermore, the mean numbers of CD68+ TAMs in each location of the tumor islets and stroma were significantly higher than those of CD206+ TAMs. Univariate analysis demonstrated

that a large number of CD68$^+$ and CD206$^+$ TAMs in the tumor stroma were associated with a shorter OS time, which is consistent with the results of Li *et al* (12) and Dai *et al* (11). The former study also demonstrated that the tumor stroma is the most suitable intra-tumor area for evaluating the relationship between TAMs and prognosis of NSCLC cases. The present study also found that CD206$^+$ stromal TAMs, CD105 and PD-L1 had a relationship with prognosis. When the number of positive cells in each section (tumor islets and tumor stroma) was summed, and CD68$^+$ or CD206$^+$ TAMs were combined with the other two key components, the high density of the three components may indicate a worse prognosis within 30 months of surgery.

New blood vessels support rapid tumor tissue growth, providing nutrients and oxygen to thriving tumor cells (38). However, in the present study, tumor neo-vessel density was not significantly correlated with the prognosis of patients with NSCLC, but there was a significant correlation between CD68$^+$ and CD105. These results suggested that macrophages have a significant role in tumor neo-vessels in the process of cancer invasion and metastasis. TAMs are considered to be 'angiogenesis switches' and a key factor leading to a proangiogenic environment (26,39).

In the present study, it was observed that PD-L1 expression was correlated with the prognosis of NSCLC and may be used as an independent prognostic factor for patients with NSCLC. PD-L1 mediates immunosuppressive signals and certain studies suggest that PD-L1 upregulation is associated with longer survival time in early NSCLC (40), breast carcinoma (41), gastric cancer (42) and colorectal cancer (43). However, another study has indicated that there is no association between PD-L1 expression and OS (44). A number of previous studies have reported that high PD-L1 expression is associated with poor prognosis in NSCLC (45-47). In these studies, the definition of PD-L1$^+$ or high density was different, leading to difficulties in concluding on the relationship between PD-L1 expression and NSCLC prognosis. Evidence suggests that PD-L1 upregulation is an adaptive mechanism and may be a response of tumor cells to host immune pressure (48). It is also understood that PD-L1 expression is related to the endogenous immune response, such as tumor-infiltrating lymphocytes in NSCLC and indoleamine 2,3-dioxygenase-1 expression by dendritic cells (49). It may therefore be suggested that any possible prognostic significance is not directly related to a single immune signal but to the overall balance between the host's antitumor immune response and tumor-mediated immunosuppression.

The present study demonstrated that the expression of PD-L1 in cancer cells was correlated with the density of CD68$^+$ and CD206$^+$ TAMs. M2 type macrophages have a weak antigen-presenting capacity and suppress T-cell immune responses by releasing immunosuppressive factors, such as TGF-β and IL-10 (50). In the hypoxic TME, the expression of certain immunosuppressive factors, such as prostaglandin E2 (PGE-2) and IL-10, not only inhibits the activation of M1 macrophages, but also converts the generated M1 type to the M2 type (51).

In conclusion, the distribution of stromal macrophages and M2 type TAMs are important factors affecting other key components in the TME. The present study demonstrated that the different immunological molecular profiles of the TME were associated with the prognosis of patients with NSCLC. There are also certain limitations to the present study. The number of cases was small and treatments of patients after postoperative recurrent-metastasis is incomplete. Further studies with a larger sample size are required to be conducted to gain a deeper understanding and explanation of this mechanism. To the best of our knowledge, the present study was the first to provide a multi-component combined prognostic survival analysis of different types of macrophages in different regions with tumor neo-vessels and PD-L1, and the combined analysis of key components may improve the prediction of the prognosis.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to restrictions applied by The Cancer Hospital of the University of Chinese Academy (Zhejiang Cancer Hospital, Hangzhou, China) but are available from the corresponding author on reasonable request.

Authors' contributions

Conception and design: MF and XL. Administrative support: MF and XL. Provision of study materials or patients: MF and XL. Collection and assembly of data: MF, QH and HY. Data analysis and interpretation: MF, QH, HY, GC and SY. Manuscript writing: HY and QH. HY and MF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in the present study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics committee of Zhejiang Cancer Hospital (Hangzhou, China; approval no. IRB-2021-111). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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