

M2 macrophage infiltration into tumor islets leads to poor prognosis in non-small-cell lung cancer

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Background: Lung cancer is the leading cause of cancer-related death worldwide. Although the macrophages can affect the development of tumor, the contribution of macrophages to the prognosis of non-small-cell lung cancer (NSCLC) is still controversial. Moreover, anti-PD-1 therapy can redirect macrophages from an M2 to an M1 phenotype, suggesting that tumor PD-L1 may affect the prognostic role of macrophages. Therefore, in this study, we aimed to display a macrophage landscape to clarify the function of macrophages, considering the localization and polarization of the macrophages, and evaluate the effect of M2 macrophages and tumor PD-L1 in combination on the prognosis of NSCLC.

Methods: We performed multiplex quantitative immunofluorescence staining of pan-cytokeratin (CK), CD68, CD163, PD-L1, and DAPI on one tissue specimen simultaneously from 137 NSCLC patients.

Results: M2 macrophages, involved marginM2 (M2 macrophages in tumor stroma), and centralM2 (M2 macrophages infiltrating into tumor islets) increased as the tumor stage increased. More macrophages were found in lung squamous cell carcinoma (LUSC) patients, patients with wild-type EGFR, and smokers than in patients with lung adenocarcinoma (LUAD), patients with EGFR mutations, and non-smokers. Infiltration of centralM2 was an independent prognostic factor of poor overall survival (OS) and disease-free survival (DFS) for NSCLC patients ($P < 0.05$), which was superior to total macrophages and total M2 macrophages. Moreover, patients with centralM2^{less}PD-L1⁻ tumors showed the best OS and DFS, while the patients with centralM2^{more}PD-L1⁺ tumors showed the worst OS and DFS, and the two groups with centralM2^{less}PD-L1⁺ and centralM2^{more}PD-L1⁻ were in the middle ($P = 0.002, 0.034$, respectively).

Conclusion: Tumor islet-infiltrating M2 macrophages influence the prognosis of NSCLC patients. The analysis of M2 macrophages and tumor PD-L1 in combination may enhance the accuracy of prognostic prediction. This study provides a new understanding of macrophages in the development of NSCLC through the analysis of macrophage landscape.

Keywords: macrophage, microenvironment, polarization, non-small-cell lung cancer, prognosis, PD-L1

Introduction

Lung cancer is the most common type of cancer and remains the leading cause of cancer-related death worldwide.^{1,2} Smoking, gene mutation, and reprogramming of the immune microenvironment are critical factors that may affect malignancy in lung cancer. The vital role of the tumor immune microenvironment (TIME) has attracted increasing attention as progress with immunotherapy in lung cancer is made.³⁻⁵

Among the various immune cells present in the TIME, macrophages are the predominant population of tumor-infiltrating immune cells.⁶ Macrophages may differentiate into either pro-inflammatory (M1) or anti-inflammatory (M2)

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macrophages, and each type plays different roles in the development of cancer. M1 macrophages mainly function in killing pathogens and tumor cells, whereas M2 macrophages function in promoting tumor development.^{7–15} Although the role of macrophages in tumor progression is well known, the effect of macrophages on tumor prognosis is still contradictory. Previous studies have reported that macrophage infiltration is associated with poor prognosis in lung cancer,^{16,17} breast cancer,¹⁸ and prostate cancer,¹⁹ whereas it is associated with good prognosis in prostate²⁰ and colon cancer.²¹ In addition, some other studies reported that no relationship was found between macrophages and prognosis of lung cancer.^{22,23} Those contradictory results may be due to the opposing roles of M1 and M2 macrophages,^{24–27} as well as the differing roles of macrophages in distinct tumor locations.^{28–30} In addition, the traditional immunohistochemistry regularly used in previous studies could not simultaneously exhibit the polarization states and distribution of macrophages objectively and accurately, which may also be an important reason for the contradictory results. Therefore, in this study, we aimed to clarify the function of macrophages with regard to both their localization and polarization in patients with NSCLC using multiplex quantitative immunofluorescence staining.

It is known that programmed death receptor 1 (PD-1), an inhibitory receptor mainly expressed on T, B, and NK cells that inhibits antitumor immunity, is also expressed on tumor-associated macrophages (TAMs).³¹ Recently, it was reported that anti-PD-1 therapy could redirect macrophages from an M2 to an M1 phenotype in osteosarcoma,³² suggesting that PD-1/PD-L1 signaling may affect macrophage reprogramming in the TIME. Moreover, although PD-L1 plays an important immunosuppressive role in tumor development, the prognostic effect of tumor PD-L1 has not been consistent in previous studies.^{33–41} One of the possible reasons was speculated that tumor PD-L1 may affect the prognosis by altering macrophage function. Therefore, PD-L1 and macrophages together may affect the prognosis of cancer. However, no previous studies have examined the effect of macrophages and tumor PD-L1 in combination on tumor prognosis.

In this study, we investigated the effects of macrophage number, localization, and polarization on the development of non-small-cell lung cancer (NSCLC) using multiple quantitative fluorescence staining. Additionally, we also examined the combined effects of tumor PD-L1 expression and macrophage infiltration on the prognosis of NSCLC.

These results provide a new understanding of the importance of macrophages in the development of NSCLC.

Materials and methods

Patients

One hundred and thirty-seven patients diagnosed with NSCLC between 2012 and 2014 were enrolled as the following criteria: NSCLC with stage I–III; no neoadjuvant therapy before surgery; received standard adjuvant therapy after operation. All the above patients were followed up at the First Hospital of China Medical University. The median follow-up period was 55.6 months (range, 8–75 months), 65 patients relapsed (47.44%), and 35 patients (25.55%) died during this period, with a 5-year overall survival (OS) probability of 34.31%. The study was approved by the Ethics Committee of China Medical University (No. 2017–236), and all procedures were conducted in accordance with ethical principles. Clinical information of all patients was retrieved from the Hospital Information System.

Multiple quantitative fluorescence staining

Multiple quantitative fluorescence staining was performed with the Opal 7-Color Manual IHC Kit (NEL811001KT, PerkinElmer Inc., Waltham, MA, USA) according to the protocol of the manufacturer, which allows for microwave treatment to remove primary and secondary antibodies while retaining an intact fluorescent signal. This process is repeated until all antigens have been stained with their respective fluorophores.

The antibodies were diluted as follows:

Anti-PD-L1 (#13,684, clone E1L3N, CST), 1:300,
Anti-CD68 (ZM-0060, zhongshanjinjiao, China), 1:200,
Anti-CD163 (ZM-0428, zhongshanjinjiao, China), 1:400,
Pan-cytokeratin (CK) (MAB-0671, Maixin Biotech, China), working fluid,
DAPI (FP1490A, PerkinElmer), working fluid.

Image analysis

Multiple quantitative fluorescence staining data were collected by Mantra Quantitative Pathology Workstation (PerkinElmer, CLS140089) and analyzed by PerkinElmer inForm Analysis software. Each scanned image was visually examined by a pathologist, and regions/samples with staining artifacts and with large necrotic areas were excluded. Image processing comprised the steps of training session and image analysis session. The training

session included manual annotation of three distinct types of region (tumor, stroma, and blank areas) performed by a pathologist. A machine-learning algorithm built into the inForm software was used to create the tissue segmentation algorithm. Cell segmentation was performed on the basis of the nuclear DAPI staining.

We defined tumor PD-L1 positive as PD-L1-positive tumor cells accounting for more than 1% of the total cells and defined the macrophage as high infiltration if the macrophage density is greater than the median and the low infiltration is opposite.

Statistical analyses

Cell density among subgroups was analyzed using nonparametric test for continuous variables. Survival curves were determined using the Kaplan–Meier method and compared using the log-rank test. COX regression analysis with continuous variables was performed to prove the effect of centralM2 macrophages on prognosis, excluding the possibility of random fluctuations as shown in [Table S1](#). OS was calculated from the time of surgery till death or the last follow-up visit (June 30, 2018), and DFS was calculated from the time of surgery till the relapse of disease. Univariate and multivariate analyses were performed by Cox proportional hazards regression models to estimate HR and 95% CI. Statistical significance was considered at $P < 0.05$, and all statistical analyses were conducted using the SPSS statistical software package (version 16.0) and GraphPad Prism v7.0 for Windows.

Results

Patient characteristics and multiplex quantitative immunofluorescence staining of NSCLC patient specimens

To visualize the macrophage landscape in the microenvironment of NSCLC tumors, 137 patients with stage I-III NSCLC were enrolled. The clinicopathological features of the patients are summarized in [Table 1](#). The median age at diagnosis was 59 years (range, 34–75 years). Among the patients, 77 were male (56.2%) and 60 were female (43.8%), and 93 (67.9%) patients had adenocarcinoma and 44 (32.1%) had squamous cell carcinoma. Fifty-nine (43.1%) patients had EGFR mutations and 78 (56.9%) had wild-type EGFR, which is similar to the known rate of genetic mutations in NSCLC patients in Asia.⁴² Sixty-five (47.4%) patients had a history of smoking, and 81 (59.1%) patients had lymphatic invasion. According to the criteria

Table 1 Characteristics of the patients at baseline

Characteristic	All patients(N=137)
Age Median (range)	59 (34–75)
Gender Female Male	60 (43.8%) 77 (56.2%)
EGFR status EGFR mutant EGFR wild type	59 (43.1%) 78 (56.9%)
Smoking history Never Former or current	72 (52.6%) 65 (47.4%)
Stage I II III	46 (33.6%) 46 (33.6%) 45 (32.8%)
T 1–2 3–4	112 (81.8%) 25 (18.2%)
N 0 ≥1	56 (40.9%) 81 (59.1%)
Histologic diagnosis Adenocarcinoma Squamous-cell carcinoma	93 (67.9%) 44 (32.1%)

in the American Joint Committee on Cancer staging manual, 46 (33.6%) patients had stage I cancer, 46 (33.6%) had stage II, and 45 (32.8%) had stage III. Multiplex quantitative immunofluorescence staining of pan-CK, CD68, CD163, PD-L1, and DAPI was performed on the NSCLC tissues. DAPI labels nuclei, CK was used to label tumor cells, CD68 labels macrophages, and CD163 labels M2 macrophages ([Figure 1A](#)). Then, the total number of macrophages, macrophage subtypes, density, and the distribution of macrophages within the tumor was analyzed.

The correlation between macrophage distribution and tumor stages

First, the distribution of macrophages in the NSCLC microenvironment was analyzed. The results showed that the number of macrophages in the tumor tissues accounted for 15.84% of all cells in tumor tissue (27.8% of all stroma cells). Among the macrophages, 36.68% were M1 and 63.32% were M2 ([Figure 1B](#), [Table 2](#)); furthermore, 77.18% of macrophages

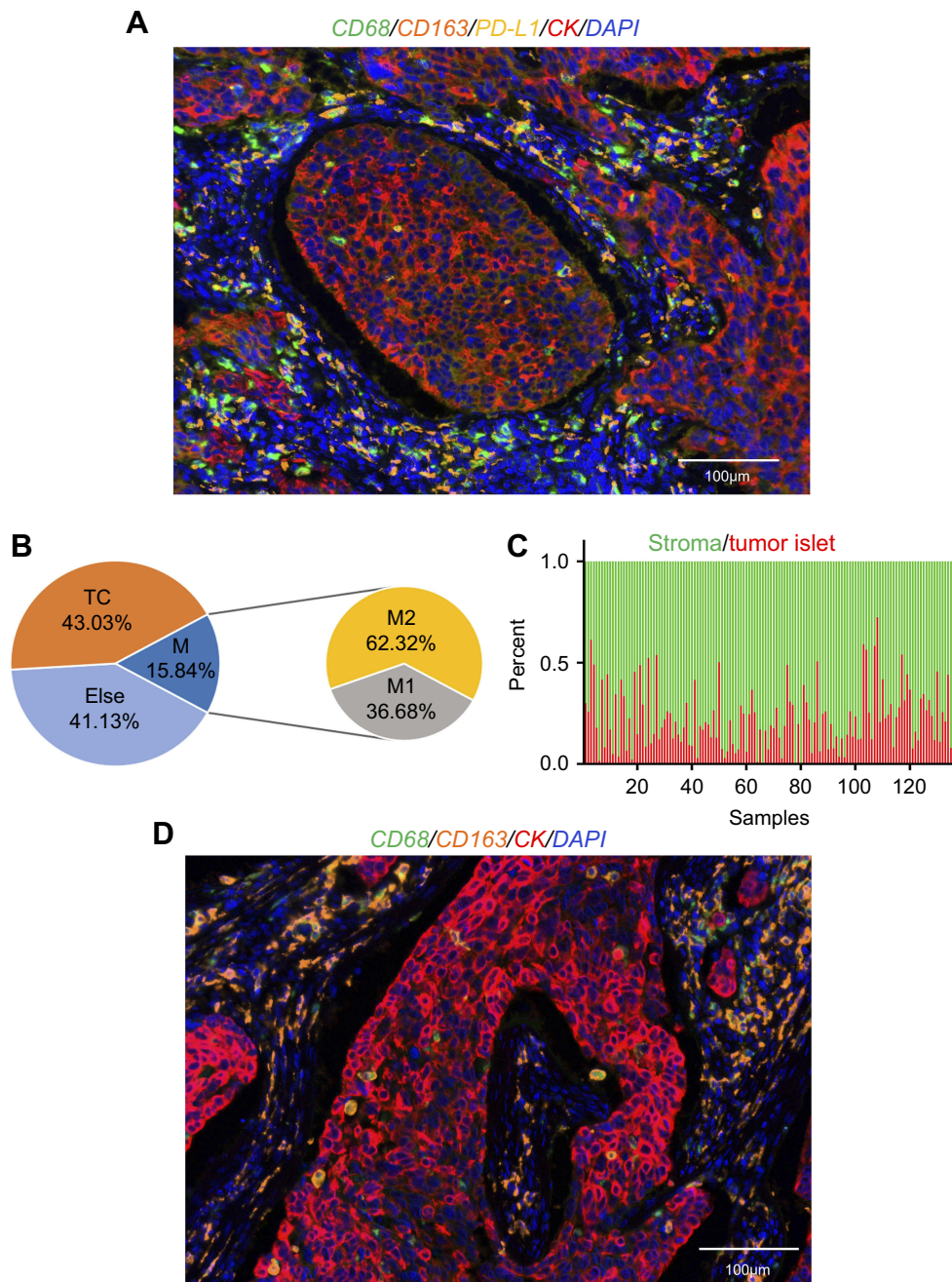


Figure 1 The macrophage landscape in the microenvironment of NSCLC. **(A)** A representative image using multiple quantitative fluorescence staining. Blue, DAPI; red, CK; green, CD68; orange, CD163; yellow, PD-L1. Scale bars =100 μ m. **(B)** Quantity of cell subpopulations normalized to the total cell numbers in TIME. **(C)** Proportion of macrophage distribution in stroma (green) and tumor islets (red) of 137 patients in NSCLC microenvironment. Each column represents a patient. **(D)** A representative image of macrophages distributed in the tumor microenvironment. Blue, DAPI; red, CK; green, CD68; orange, CD163. Scale bars =100 μ m.

Abbreviations: TC, tumor cells; M, total macrophages; M1, M1 macrophages; M2, M2 macrophages; NSCLC, non-small-cell lung cancer; TIME, tumor immune microenvironment.

were located around the tumor interstitial region (these macrophages are defined as marginM), whereas 22.82% of the macrophages infiltrated the tumor islets (defined as centralM) (Figure 1C, 1D, Table 2). These results indicated that macrophages, especially the M2 type, are abundant in the microenvironment of lung cancer and are mostly distributed in the interstitial region of the tumor margin.

Then, we analyzed the relationship between macrophage number, distribution, and the stage of NSCLC. Although there was no significant change in overall macrophage infiltration (Figure 2A, 2D, 2G), as shown in Figure 2B-C, the amount of total M2 macrophages and the M2 polarization status (M2/M) significantly increased from stage I to stage III ($P=0.005$, $P=0.02$, respectively). We also observed the sites

Table 2 Cell proportion of different subtypes

Cell type	Percent (%)
M/ALL	15.84
M1/ALL	5.81
M2/ALL	10.03
M1/M	36.68
M2/M	63.32
CentralM/M	24.56
MarginM/M	75.44
CenterM1/M1	37.80
MarginM1/M1	62.20
CenterM2/M2	16.89
MarginM2/M2	83.11

Abbreviations: M, total macrophages; M1, total M1 macrophages; M2, total M2 macrophages; centralM, M macrophages infiltrating into islets; marginM, M macrophages in stroma; centralM1, M1 macrophages infiltrating into islets; marginM1, M1 macrophages in stroma; centralM2, M2 macrophages infiltrating into islets; marginM2, M2 macrophages in stroma; ALL, total number of cells in tissue.

of macrophage infiltration in tumors of various stages. CentralM2 and marginM2 increased from state I to stage III (Figure 2E, 2H, $P=0.001$, $P=0.055$, respectively), and

the M2 polarization status (M2/M) significantly increased from stage I to stage III in both tumor islet and tumor margin (Figure 2F, 2I, $P=0.004$, $P=0.05$, respectively). CentralM1 (M1 macrophages infiltrating into tumor islets) rapidly increased while marginM1 (M1 macrophages infiltrating in stroma) decreased in stage II, and then central and marginM1 both decreased in stage III (Figure 2E, 2H). The amounts of total macrophages, especially M2 macrophages, marginM2, and centralM2, closely correlated to the NSCLC stage, suggesting that M2 macrophages may play an important role in the development of NSCLC.

The correlation between macrophages and pathological types, EGFR status, and smoking status

Next, to investigate whether different clinicopathological features of NSCLC affect macrophage status, the relationships between macrophages and pathological types, EGFR status, and smoking status were analyzed.

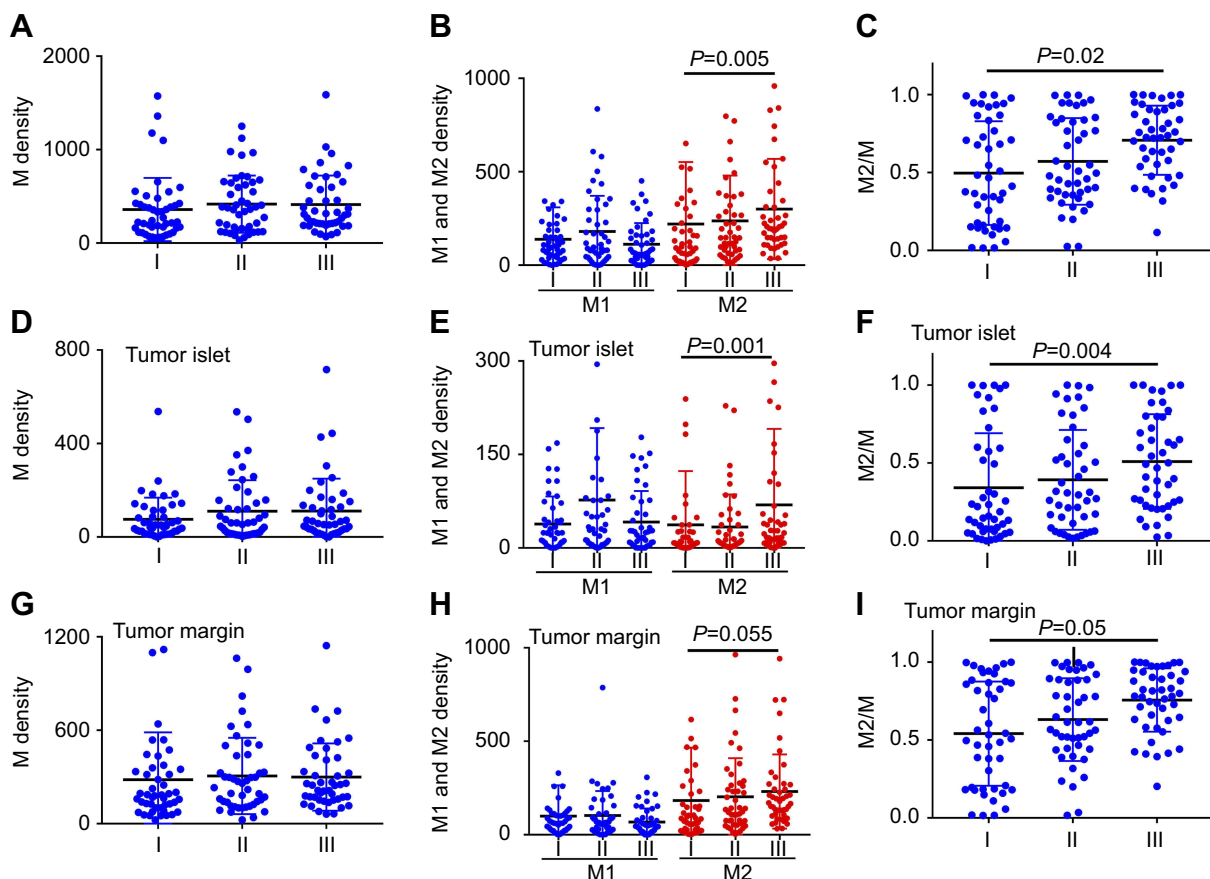


Figure 2 Distribution of macrophages in different tumor stages. (A-C) Represents the global distribution of macrophages. (D-F) Represents the distribution of macrophages in tumor islet. (G-I) Represents the distribution of macrophages in the stroma. Density = cell number/ 3.6×10^5 , m^2 . $P < 0.05$ indicates statistical significance. **Abbreviations:** M, total macrophages; M1, M1 macrophages; M2, M2 macrophages.

Compared to LUAD patients, patients with EGFR mutations, and non-smokers, higher densities of total macrophages were found in LUSC patients, patients with wild-type EGFR, and smokers, respectively ($P < 0.05$, Figure 3A, 3D, 3G). Similar results were seen when M1 and M2 macrophages were analyzed (Figure 3B-C, 3E-F, 3H-I). Additionally, M2 polarization (M2/M) levels were nearly identical between the subgroups (Figure 3J, 3K, 3L). These results indicate that different features of macrophages in patients with different pathological characteristics may lead to diverse conditions in the immune microenvironment.

Infiltration of macrophages and prognosis in NSCLC

To examine the effect of macrophage infiltration on NSCLC prognosis, we analyzed the OS and DFS of the patients. The results showed that the OS of patients with high infiltration of M2 macrophages ($M2^{\text{more}}$) was significantly shorter than that of patients with low infiltration of M2 macrophages ($M2^{\text{less}}$) ($P = 0.003$, Figure 4B), whereas the total numbers of macrophages and M1 macrophages did not affect the OS (Figure 4A, 4C). Compared to patients with high infiltration of centralM and centralM2, patients with low infiltration of centralM ($P = 0.003$,

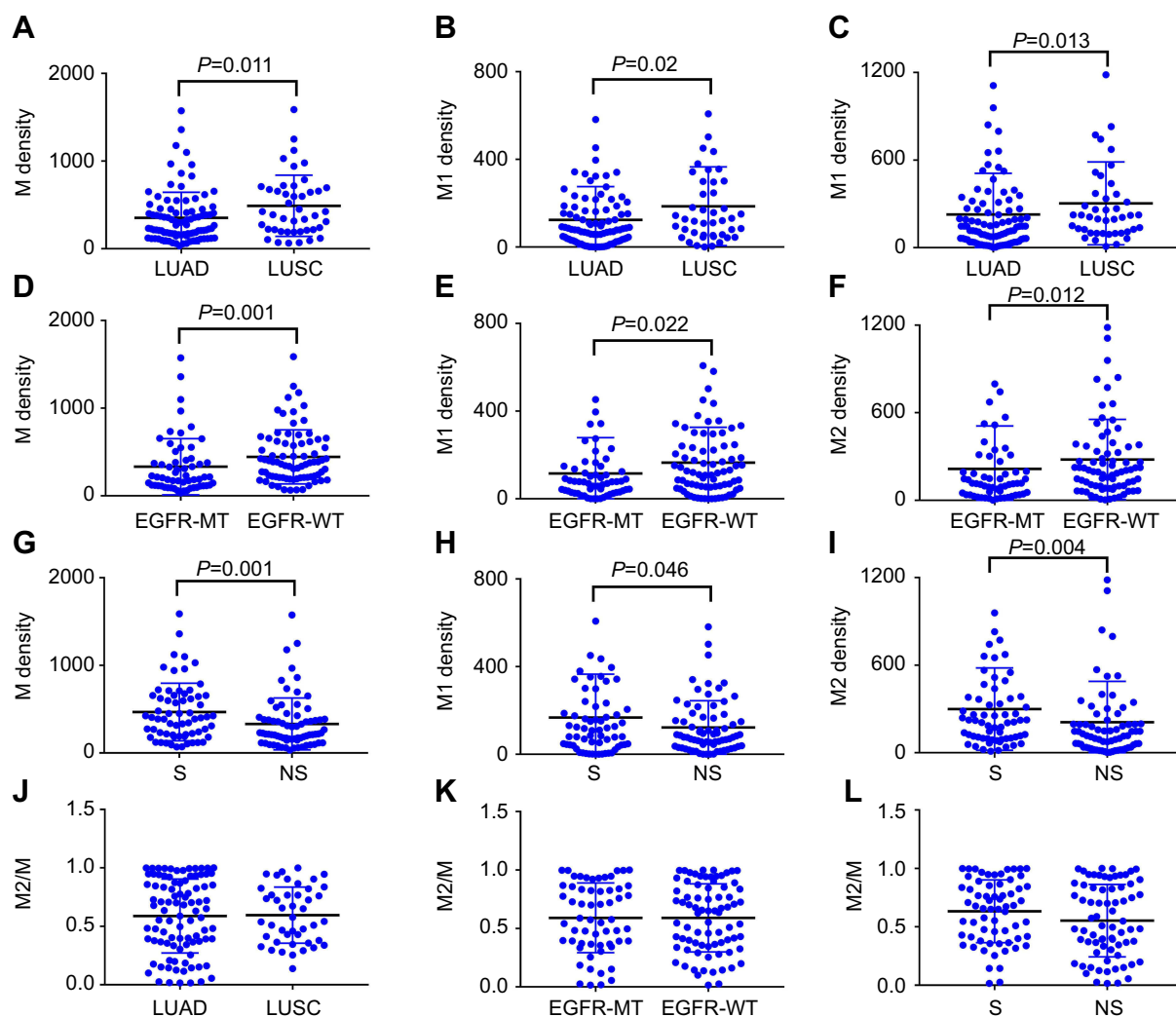


Figure 3 Quantity of macrophages in patients with different clinicopathological parameters. (A-C) Density of total macrophages (A), M1 macrophages (B), M2 macrophages (C) in patients with different pathological types. (D-F) Density of total macrophages (D), M1 macrophages (E), M2 macrophages (F) in patients with different EGFR status. (G-I) Density of total macrophages (G), M1 macrophages (H), M2 macrophages (I) in patients with different smoking history. (J-L) Proportion of M2 macrophages to total macrophages in patients with different pathological types (J), EGFR status (K) and smoking history (L). Density = cell number/ 3.6×10^5 , m^2 . $P < 0.05$ indicates statistical significance.

Abbreviations: M, total macrophages; M1, M1 macrophages; M2, M2 macrophages; M2/M, Proportion of M2 macrophages to total macrophages; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; EGFR-MT, EGFR mutation; EGFR-WT, EGFR wild-type; S, smoker; NS, nonsmokers.

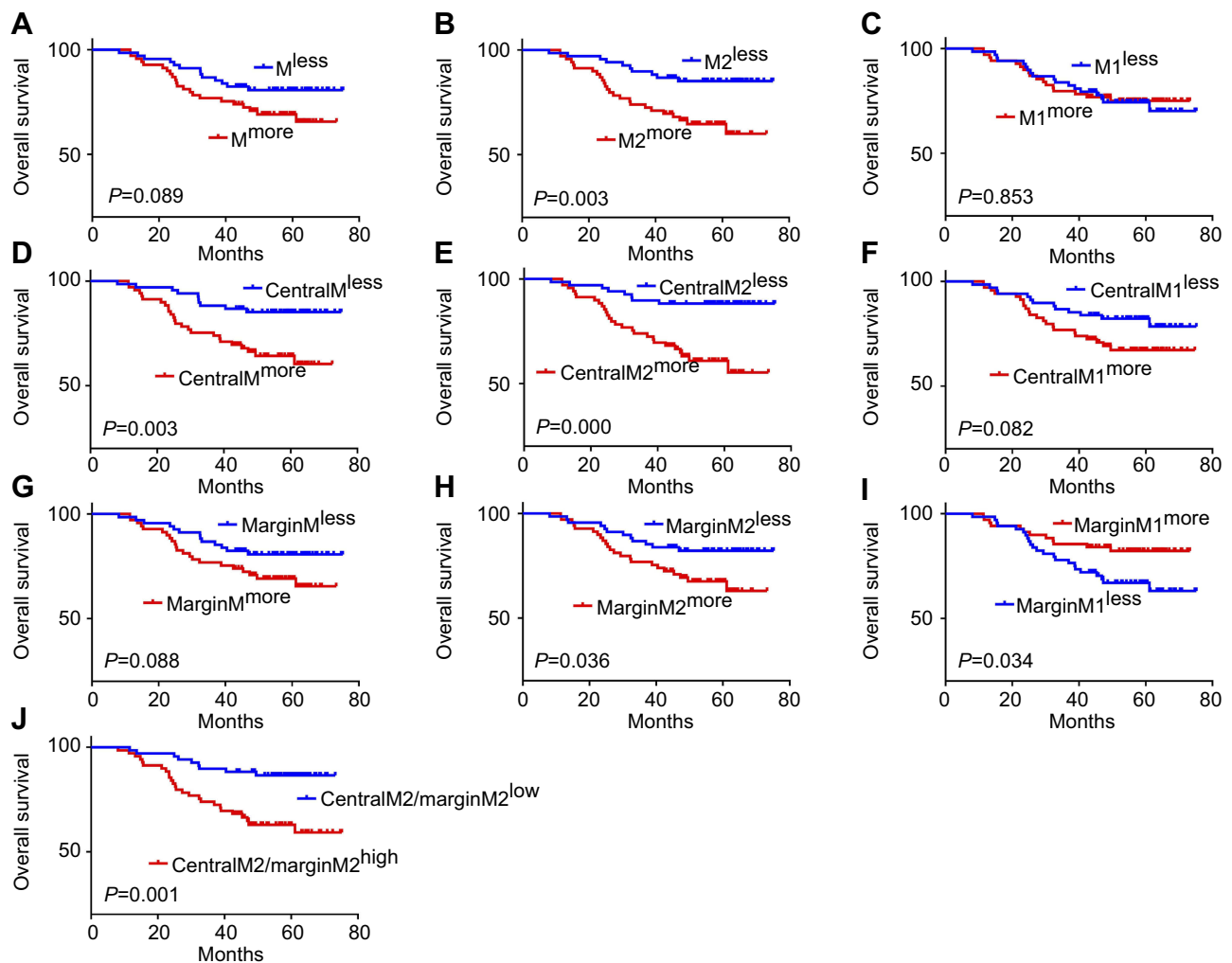


Figure 4 Overall survival (OS) analysis for macrophages of different polarization states and locations.

Notes: (A-C) Kaplan–Meier curve of the relationship between the total macrophages (M) (A), total M2 macrophages (M2) (B), total M1 macrophages (M1) (C) and the OS of NSCLC patients. (D-F) Kaplan–Meier curve of the relationship between the macrophages infiltrating in tumor islets (centralM) (D), M2 macrophages infiltrating in tumor islets (centralM2) (E), M1 macrophages infiltrating in tumor islets (centralM1) (F) and the OS of NSCLC patients. (G-I) Kaplan–Meier curve of the relationship between the macrophages in stroma (marginM) (G), M2 macrophages in stroma (marginM2) (H), M1 macrophages in stroma (marginM1) (I) and the OS of NSCLC patients. (J) Kaplan–Meier curve of the relationship between the ratio of M2 macrophages infiltrating in tumor islets to M2 macrophages infiltrating in stroma (centralM2/marginM2) and the OS of NSCLC patients. less: low infiltration; more: high infiltration.

Figure 4D), especially centralM2 ($P=0.000$, Figure 4E), had a better OS, but centralM1 did not affect the OS significantly ($P=0.082$, Figure 4F). MarginM1 did not affect the OS significantly ($P=0.088$, Figure 4G), while patients with high infiltration of marginM1 had a better OS than those with low infiltration of marginM1 ($P=0.034$, Figure 4I). In addition, high infiltration of marginM2 also decreased the OS ($P=0.036$, Figure 4H). To further clarify whether centralM2 or marginM2 has a more significant effect on the prognosis of NSCLC, we analyzed the correlation between the ratio of centralM2/marginM2 and the OS. Patients with centralM2/marginM2^{high} had a worse OS than those with centralM2/marginM2^{low} ($P=0.001$,

Figure 4J), suggesting that centralM2 have a greater impact on the OS of NSCLC patients than marginM2.

Similarly, the analysis of DFS showed that patients with low infiltration of centralM ($P=0.023$, Figure 5D), especially centralM2 ($P=0.006$, Figure 5E), had better DFS than patients with high infiltration of centralM and centralM2, and the patients with centralM2/marginM2^{high} had worse DFS than those with centralM2/marginM2^{low} ($P=0.005$, Figure 5J). However, the total numbers of macrophages, M1 macrophages, M2 macrophages, the marginM, marginM1, marginM2, and centralM1 did not affect the DFS (Figure 5A-C, F-I). These results indicate that centralM2 has an important impact on the DFS of NSCLC patients.

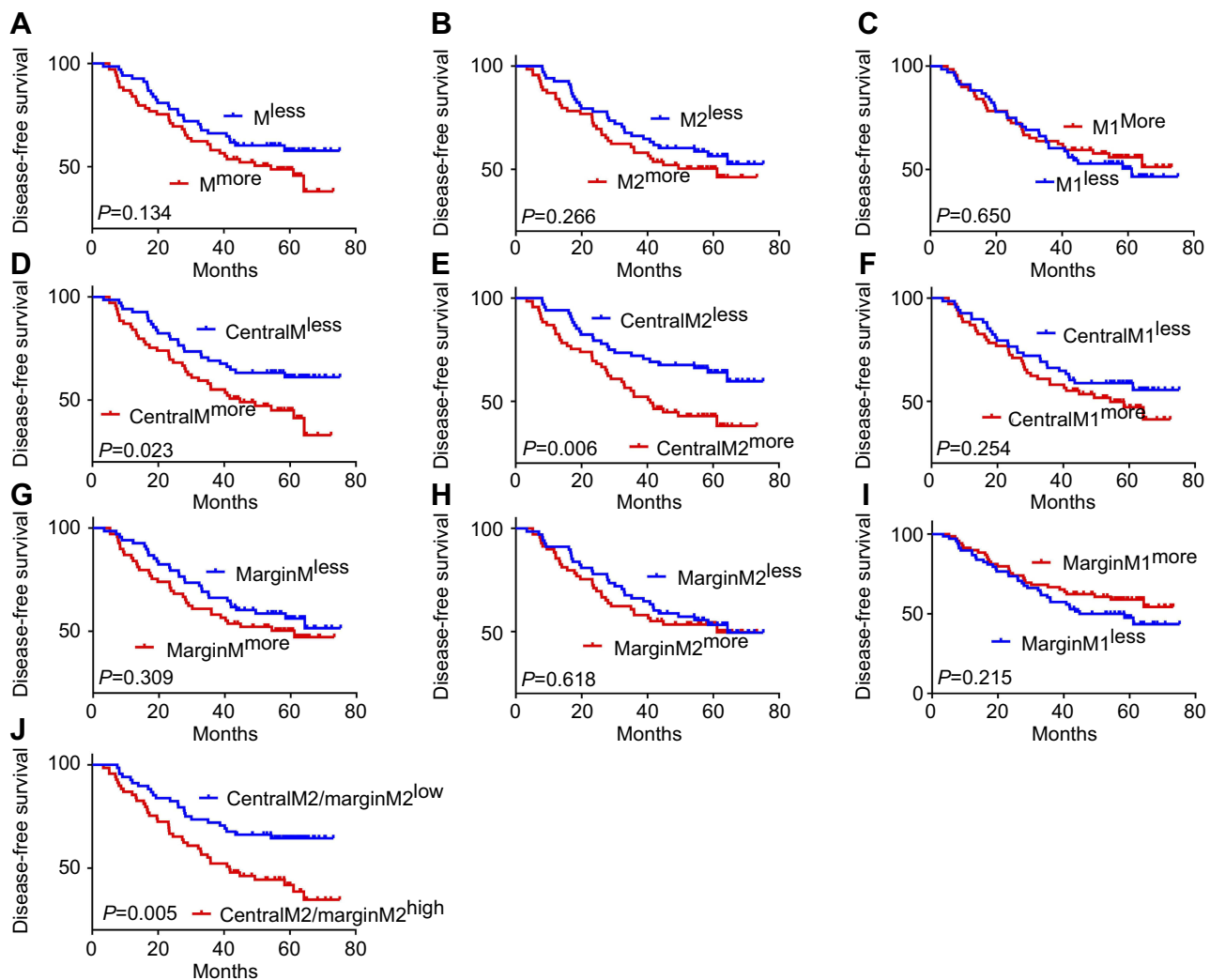


Figure 5 Disease-free survival analysis (DFS) for macrophages of different polarization states and locations.

Notes: (A–C) Kaplan–Meier curve of the relationship between the total macrophages (M) (A), total M2 macrophages (M2) (B), total M1 macrophages (M1) (C) and the DFS. (D–F) Kaplan–Meier curve of the relationship between the macrophages infiltrating in tumor islets (centralM) (D), M2 macrophages infiltrating in tumor islets (centralM2) (E), M1 macrophages infiltrating in tumor islets (centralM1) (F) and the DFS. (G–I) Kaplan–Meier curve of the relationship between the macrophages infiltrating in stroma (marginM) (G), M2 macrophages infiltrating in stroma (marginM2) (H), M1 macrophages infiltrating in stroma (marginM1) (I) and the DFS. (J) Kaplan–Meier curve of the relationship between the ratio of M2 macrophages infiltrating in tumor islets to M2 macrophages infiltrating in stroma (centralM2/marginM2) and the DFS. less: low infiltration; more: high infiltration.

Next, to determine whether the density of M1 and M2 macrophages is an independent factor of prognosis, univariable and multivariable analyses were carried out. Clinical characteristics and macrophage subtypes were subjected to univariable analysis, and the variables associated with OS or DFS at a significance level of $P < 0.05$ were then subjected to the multivariate analysis (Table 3, Table 4). The results revealed that the amount of centralM2 was an independent predictor of both OS and DFS (OS, HR = 2.859, 95% CI (1.125–7.262), $P = 0.027$; DFS, HR = 1.809, 95% CI (1.085–3.015), $P = 0.023$; Table 3, Table 4). These results suggest that infiltration

of M2 macrophages into the tumor islets has an important influence on the prognosis of NSCLC patients.

Combined prognostic analysis of M2 macrophages and tumor PD-L1

To investigate whether M2 macrophages and tumor PD-L1 expression have a combined effect on NSCLC prognosis, the patients were divided into 4 groups according to the density of M2 macrophages and tumor PD-L1 expression: $M2^{\text{less}}PD-L1^-$, $M2^{\text{less}}PD-L1^+$, $M2^{\text{more}}PD-L1^-$, and $M2^{\text{more}}PD-L1^+$. Patients with $M2^{\text{less}}PD-L1^-$ had the best OS, while patients with $M2^{\text{more}}PD-L1^+$ had the worst OS ($P = 0.027$, Figure 6A).

Table 3 Univariate and multivariate analysis for overall survival in 137 NSCLC patients

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Gender	1.254 (0.637–2.467)	0.512		
Age	1.152 (0.593–0.593)	0.677		
Pathology	0.858 (0.412–1.787)	0.682		
EGFR	0.837 (0.426–1.647)	0.607		
Smoke history	1.309 (0.672–2.55)	0.428		
T	2.654 (1.319–5.338)	0.006	1.673 (0.793–3.529)	0.176
N	5.102 (1.976–13.177)	0.001	4.053 (1.555–10.563)	0.004
CentralM1	1.820 (0.917–3.615)	0.087		
MarginM1	0.494 (0.245–0.993)	0.048	0.711 (0.34–1.484)	0.363
CentralM2	4.194 (1.897–9.27)	0.000	2.859 (1.125–7.262)	0.027
MarginM2	2.152 (1.069–4.329)	0.032	1.029 (0.465–2.276)	0.944

Notes: Bold values indicate statistical significance, $P < 0.05$.

Abbreviations: centralM1, M1 macrophages infiltrating into islets; marginM1, M1 macrophages in stroma; centralM2, M2 macrophages infiltrating into islets; marginM2, M2 macrophages in stroma.

Table 4 Univariate and multivariate analysis for disease-free survival in 137 NSCLC patients

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Gender	0.877 (0.539–1.428)	0.598		
Age	1.001 (0.614–1.631)	0.998		
Pathology	0.783 (0.454–1.349)	0.378		
EGFR	1.258 (0.773–2.048)	0.356		
Smoke history	0.852 (0.521–1.391)	0.521		
T	1.499 (0.842–2.67)	0.169		
N	2.591 (1.499–4.479)	0.001	2.325 (1.335–4.05)	0.003
centralM1	1.275 (0.783–2.076)	0.33		
marginM1	0.762 (0.467–1.244)	0.278		
centralM2	2.092 (1.264–3.463)	0.004	1.809 (1.085–3.015)	0.023
marginM2	1.183 (0.726–1.927)	0.499		

Notes: Bold values indicate statistical significance, $P < 0.05$.

Abbreviations: centralM1, M1 macrophages infiltrating into islets; marginM1, M1 macrophages in stroma; centralM2, M2 macrophages infiltrating into islets; marginM2, M2 macrophages in stroma.

However, no statistically significant differences in DFS were seen among these 4 groups (Figure 6B).

Next, analysis of correlation between the prognosis and the combination of centralM2 and tumor PD-L1 was performed. The OS and DFS in the centralM2^{less}PD-L1⁻ group were the best, while the centralM2^{more}PD-L1⁺ group had the worst OS and DFS ($P=0.002$, 0.034 , respectively, Figure 6C, 6D). Moreover, the prognosis of the centralM2^{less}PD-L1⁺ group was better than that of the centralM2^{more}PD-L1⁻ group, suggesting that the centralM2 macrophages may be more important than tumor PD-L1 expression in NSCLC prognosis. Taken together, these results suggest that the combined

analysis of macrophages and tumor PD-L1 could improve the accuracy of prognosis prediction.

Discussion

In this study, we found that M2 macrophages are important immune cells in TIME of NSCLC, that high infiltration of centralM2 was an independent predictor of poor OS and DFS, and that the analysis of macrophage subtype and tumor PD-L1 in combination could improve the accuracy of prognosis prediction in NSCLC. The multiplex quantitative fluorescence staining method used in this study could not only distinguish the tumor and stroma areas

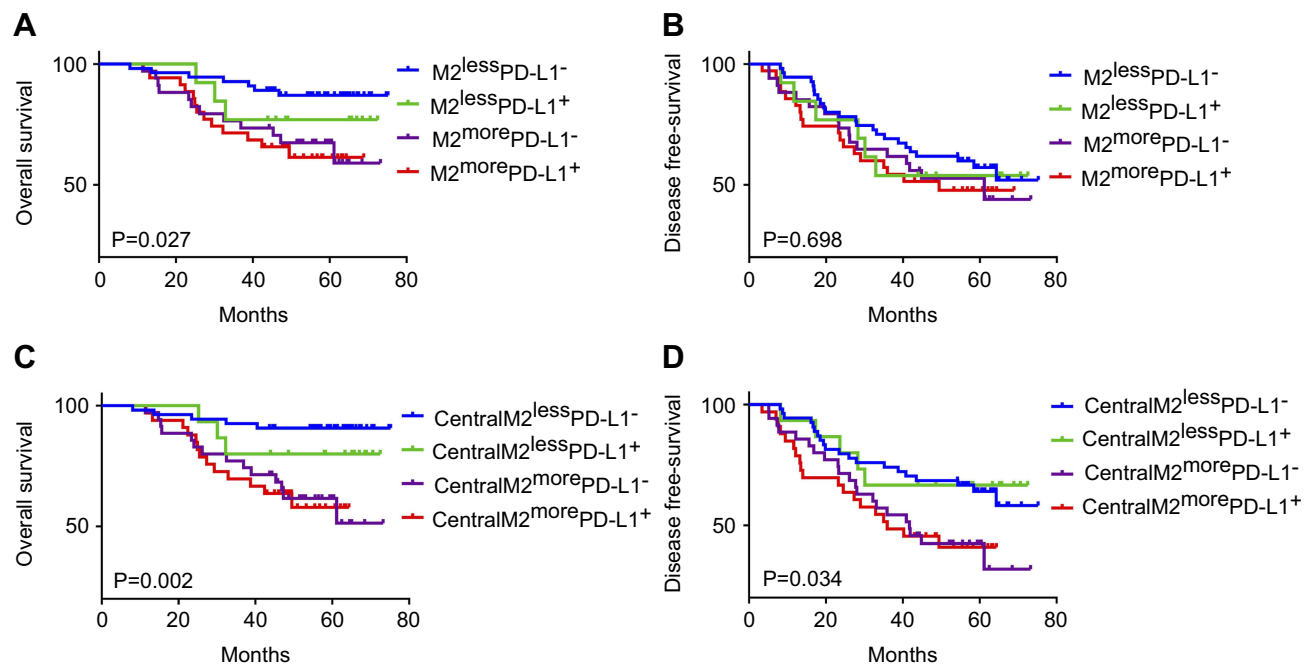


Figure 6 Prognostic analysis of M2 macrophages and tumor PD-L1 combination of NSCLC. **(A)** Kaplan–Meier curve of the relationship between the combination of M2 macrophages and tumor PD-L1, and the overall survival (OS). **(B)** Kaplan–Meier curve of the relationship between the combination of M2 macrophages and tumor PD-L1 and the disease-free survival (DFS). **(C)** Kaplan–Meier curve of the relationship between the combination of centralM2 macrophages and tumor PD-L1, and the OS. **(D)** Kaplan–Meier curve of the relationship between the combination of marginM2 macrophages and tumor PD-L1, and the DFS.

Notes: M2^{less}PD-L1⁻: low infiltration of M2 macrophages and tumor PD-L1 negative; M2^{less}PD-L1⁺: low infiltration of M2 macrophages and tumor PD-L1 positive; M2^{more}PD-L1⁻: high infiltration of M2 macrophages and tumor PD-L1 negative; M2^{more}PD-L1⁺: high infiltration of M2 macrophages and tumor PD-L1 positive. centralM2^{less}PD-L1⁻: low infiltration of centralM2 macrophages and tumor PD-L1 negative; centralM2^{less}PD-L1⁺: low infiltration of centralM2 macrophages and tumor PD-L1 positive; centralM2^{more}PD-L1⁻: high infiltration of centralM2 macrophages and tumor PD-L1 negative; centralM2^{more}PD-L1⁺: high infiltration of centralM2 macrophages and tumor PD-L1 positive.

clearly, but also allowed us to identify and count macrophages accurately by simultaneously labeling multiple markers on the same tissue slice. Thus, this method is extremely advantageous for TIME research and allows for more detailed studies than classical immunohistochemistry methods.

Our results provide important new information about macrophages in the TIME. First, macrophages, especially the M2 type, were abundant in the microenvironment of NSCLC and were mostly distributed in the interstitial region of the tumor margin. This is consistent with previous studies. Second, the polarization of macrophages from M1 to M2 during the development of NSCLC is a dynamic process. We found that macrophage number, polarization, and infiltration sites were different in various tumor stages, which may illustrate the development of macrophages from tumor suppression to tumor promotion in cancer development. In the early stages of tumor development, M1 macrophages increasingly infiltrate to the tumor islets to suppress tumor progression. However, as the tumor develops, the M1 macrophages gradually transform into M2 macrophages. In the late stages, most of the

macrophages are polarized to M2 and promote tumor progression. Third, high infiltration of M2 macrophages, especially centralM2, was associated with poor prognosis in NSCLC. As mentioned above, the results from previous studies examining the prognostic role of macrophages were controversial. Several recent studies have shown that the polarization status and localization of macrophages, but not the total amount of macrophage infiltration, were related to the prognosis of lung cancer.^{22,23} Jurgita et al reported that tumor islet-infiltrating M1 macrophages and the number of total M2 macrophages were independent predictors of NSCLC patient survival,³⁰ while Li et al found that a high number of M2 macrophages in tumor stroma, but not in tumor islets or alveolar space, was a significant prognostic factor for DFS in NSCLC patients.²⁸ We considered that limitations of traditional immunohistochemistry might be one of the reasons for the inconsistent conclusions, which is dependent on the manual judgment for the identification of cell types, dividing tissue regions and counting cells. Therefore, to perform a more precise and objective analysis, we used multiplex quantitative immunofluorescence staining, of

which the counting process is quantitative and automatic, to examine the macrophage landscape in the TIME. We demonstrated that high M2 infiltration led to poor prognosis, and that the effect of M2 macrophages infiltrating into the tumor islets was stronger than that of M2 macrophages in the tumor margin. This suggests that the closer the M2 cells are to the tumor cells, the stronger the effect they have on tumor development. In general, our results suggest that centralM2 plays an important role in the prognosis of NSCLC, a result that is different from those of previous studies.

M2 macrophages are known to promote tumor development through direct and indirect mechanisms. M2 can directly promote angiogenesis, tumor matrix remodeling, and invasion by secreting multiple proangiogenic mediators,^{7–12,18} growth factors, and proteolytic enzymes.^{13,14} M2 macrophages can also inhibit T-cell proliferation and activation by secreting immunosuppressive factors such as CCL22, IL-10, and TGF- β , and by recruiting regulatory T cells (Treg) to tumor tissues. Therefore, M2 can indirectly promote tumor development by dampening the antitumor immune response.¹⁵ In this study, centralM2 had a stronger effect on tumor development than marginM2. However, the reasons for this are unclear. One of the possible mechanisms is that the cytokines and inhibitory inflammatory factors secreted by the centralM2 may be closer to the tumor cells, which allows for higher concentrations of these factors to interact with the tumor cells for longer durations. Moreover, direct interaction between molecules on the surface of the centralM2 and tumor cells may also contribute to malignant biological behavior in tumor cells. Further studies are needed to define these mechanisms.

We also found that patients with different infiltration levels of M2 macrophages and PD-L1 expression had different prognoses. The OS and DFS in centralM2^{less}PD-L1⁻ group were the best, while the centralM2^{more}PD-L1⁺ group had the worst OS and DFS. Therefore, the analysis of M2 macrophage infiltration and PD-L1 expression in combination could further increase the accuracy of prognostic prediction for NSCLC. Macrophages and tumor PD-L1 may affect each other, reflecting the complexity of the TIME.

While analyzing the association of macrophages and various clinical subgroups, it was found that the infiltration of macrophages in patients with LUSC, wild-type EGFR, and a history of smoking was higher than in patients with LUAD, EGFR mutations, and non-smokers. These results indicate strong correlations between the amount of macrophages and clinical subgroups, EGFR status, and smoking status of NSCLC patients. Several studies have shown that

NSCLC patients who are smokers and have wild-type EGFR were able to benefit the most from anti-PD-1 therapy.^{43–47} Therefore, anti-PD-1 therapy may be the most beneficial for patients with abundant macrophage infiltration. Our findings provide a new potential strategy for priority population selection for anti-PD-1/PD-L1 therapy.

Conclusion

Tumor islet-infiltrating M2 macrophages influence the prognosis of NSCLC patients, and the analysis of M2 macrophages and tumor PD-L1 in combination may enhance the accuracy of prognostic predictions. This study provides a new understanding of macrophages in the development of NSCLC and provides a means of prognostic prediction through the analysis of the macrophage landscape in NSCLC.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of China Medical University (No. 2017-236), and all patients have signed the informed consent.

Abbreviation list

NSCLC, non-small-cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; OS, overall survival; DFS, disease-free survival; TIME, tumor immune microenvironment; PD-1, programmed death receptor 1; PD-L1, programmed cell death-Ligand 1; EGFR, epithelial growth factor receptor; AJCC, the American Joint Committee on Cancer.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 COX analysis of continuous variables

	OS		DFS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
M2	1.088 (0.993-1.192)	0.071	1.016 (0.937-1.103)	0.696
CentralM2	1.571 (1.237-1.996)	0.000	1.438 (1.155-1.790)	0.001
MarginM2	1.061 (0.943-1.194)	0.327	0.973 (0.873-1.085)	0.628
cM2/mM2	1.92 (1.276-2.889)	0.002	1.971 (1.402-2.771)	0.000

Notes: Bold values indicate statistical significance, $P < 0.05$.

Abbreviations: centralM2, M2 macrophages infiltrating into islets; marginM2, M2 macrophages in stroma; cM2, M2 macrophages infiltrating into islets; mM2, M2 macrophages in stroma.

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