



# PD-L1 expression and immune cells infiltration in primary tracheobronchial neoplasm

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**Background:** Primary tracheobronchial neoplasm is rare yet poses a serious threat to life. Due to its low incidence, the immune microenvironment of such tumors remained unclear. This study aimed to clarify the expression of programmed death-ligand 1 (PD-L1) and infiltration of immune cells in primary tracheobronchial neoplasm, which might be useful for guiding treatment and evaluating clinical outcome.

**Methods:** We assessed retrospectively the expression of PD-L1 and infiltration in cells expressing CD8, CD16, CD68, CD163 and FOXP3 in 21 patients with primary tracheobronchial neoplasm who underwent surgery in Tangdu Hospital from January 2016 to July 2021. The expression of PD-L1 was assessed based on the tumor proportion score system. The density of immune cells was analyzed by automatic image analysis software.

**Results:** In this study, all of 16 participants with adenoid cystic carcinoma (ACC) had no expression of PD-L1, whereas 4/5 (80%) of those with squamous cell carcinomas (SCC) were positive for PD-L1 expression. Compared with ACC, the density of FOXP3<sup>+</sup> cells in both the intratumoral region and peritumoral region was higher in SCC (P<0.01). The density of FOXP3<sup>+</sup> cells was significantly higher than that of CD8<sup>+</sup>, CD16<sup>+</sup>, and CD163<sup>+</sup> cells in SCC in the intratumoral region (P<0.01). In contrast, the density of FOXP3<sup>+</sup> cells was significantly lower than that of CD8<sup>+</sup>, CD16<sup>+</sup>, and CD68<sup>+</sup> cells in ACC in both the intratumoral region and peritumoral regions. The density of CD68<sup>+</sup> cells was significantly higher than that of CD8<sup>+</sup> cells (P<0.05) and CD163<sup>+</sup> cells (P<0.01) in ACC in the intratumoral region. Furthermore, the tumors of patients with metastasis more commonly of immune-excluded status, in which the CD8<sup>+</sup> cells accumulated in peritumoral region.

**Conclusions:** This study demonstrated that the expression of PD-L1 in primary tracheobronchial neoplasm was mainly concentrated in patients with SCC. In the immune microenvironment of SCC, FOXP3<sup>+</sup> cells were the dominant immune cells, while in the immune microenvironment of ACC, CD68<sup>+</sup>

cells were the main immune cells. Therefore, the immune microenvironment was significantly different in primary tracheobronchial neoplasm according to histology.

**Keywords:** Primary tracheobronchial neoplasm; tumor immune microenvironment (TIME); programmed death-ligand 1 (PD-L1); immune cells

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## Introduction

The incidence of primary tracheobronchial neoplasm comprises only 0.2% of respiratory tract neoplasm (1,2), and within this minority, squamous cell carcinoma (SCC) is the predominant pathological pattern, followed by the adenoid cystic carcinoma (ACC). Together, SCC and ACC represent more than 2/3 of primary tracheobronchial neoplasms (3,4). Other histological subtypes, including mucoepidermoid carcinoma, sarcoma, plasmacytoma, lymphoepithelioma etc. are much rare (5). Surgical resection is the most effective treatment for primary tracheobronchial neoplasm (6). The 5-year overall survival (OS) for SCC and ACC has been shown to range from 20% to 39% and 52% to 85%, respectively, in resected patients (7-9). However, due to the limitation of tracheal length or other surgical contraindications, approximately 30% of patients with tracheobronchial neoplasm are unsuitable for surgery (7,10). The 5-year survival rate is 7.3–26% and 33–63.7% in unresectable patients with SCC and ACC, respectively (7-9). Conservative treatments, including endoscopic resection by various techniques, radiotherapy, and chemotherapy may be a favorable choice (11,12). However, the efficacy is still not very satisfactory. Therefore, finding new alternative treatments is of great significance for those patients with unresectable lesions or second relapse.

In recent years, immunotherapy has received increasing attention, which inhibits tumor progression by reactivating the immune system (13,14). Programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are key targets in immunotherapy (15). Currently, the application of PD-1 or PD-L1 inhibitors has been approved in multiple tumors (16), including non-small cell lung cancer (17), head and neck cancer (18), melanoma (19), and breast cancer (20). In recent years, several case reports have shown that patients with tracheobronchial neoplasm can benefit from PD-1 inhibitors, nivolumab and pembrolizumab (21-23). And SCC of primary tracheobronchial neoplasm was

found to have significant PD-L1 expression, even 95% of tumor cells (22). This indicated that immunotherapy may be a potential alternative treatment for patients with unresectable tracheobronchial neoplasms.

The immune cell infiltration of tumors is a critical component of the anti-tumor immune response. For example, compared with the absence of CD8<sup>+</sup> cells in the tumor immune microenvironment (TIME), a patient with a high level of CD8<sup>+</sup> cell infiltration is more likely to benefit from immunotherapy (24). Among several different types of immune cells, the infiltrations of CD8<sup>+</sup>, FOXP3<sup>+</sup>, CD16<sup>+</sup>, and CD68<sup>+</sup> cells have been seen as potential biomarkers of clinical response to immunotherapy (25). Further, immune cells in the TIME are also regarded as new prognostic factors in various cancers (26-30). Therefore, understanding the infiltration phenotype of immune cells in the TIME is important for treatment selection and prognosis assessment of patients. However, due to the low incidence of primary tracheobronchial neoplasms, the characterization of infiltration of immune cells phenotype of such tumors is still unclear. Moreover, the relationship between the expression of PD-L1 and immune cells requires further exploration.

In this study, we evaluated the density of immune cells expressing CD8 (a marker of cytotoxic T cells), FOXP3 [a marker of regulatory T cells (Treg)], CD16 (a marker of NK cells), CD68 (a marker of macrophages), and CD163 (a marker of M2 polarized macrophages), and expression of PD-L1 positive tumor cells in primary tracheobronchial neoplasms. The TIME was assessed based on the expression of PD-L1 positive tumor cells and the presence of tumor-infiltrating lymphocytes. The relationship between the immune cells and clinical features of primary tracheobronchial neoplasms was also examined to identify potential candidates who may benefit from immunotherapy. We present the following article in accordance with the REMARK reporting checklist (available at <https://dx.doi.org/10.21037/tlcr-21-958>).

## Methods

### Patients

From January 2016 to July 2021, a total of 28 patients underwent surgical resection of tracheobronchial neoplasm at the Tangdu Hospital of Air Force Military Medical University. Due to sample acquisition, 21 patients were included in this study. Baseline characteristics including age, gender, smoking status, size and location of lesions, histopathological types, margin status (R0: no residual tumor; R1: microscopic residual tumor), lymph node metastasis, extension of tumor (E0, tumor confined to the tracheobronchial area or spread outside, but not to adjacent structures; E1, neoplasm had invaded the adjacent structures) (8), postoperative adjuvant therapy, and complications were evaluated. Participants were reexamined every 3 months in the first 2 years after surgery and every subsequent 6 months. This study was conducted in accordance with the ethical guidelines of Declaration of Helsinki (as revised in 2013) and approved by the Ethics Committee of Tangdu Hospital (No. K202111-04). Individual consent for this retrospective analysis was waived.

### Immunohistochemistry and image analysis

Consecutive tissue sections (3  $\mu\text{m}$  thick) were obtained from formalin-fixed and paraffin-embedded primary tracheobronchial neoplasm samples stored in the pathology department. And the automated staining system (Ventana, Bench Mark XT, America) was used for immunohistochemical staining (IHC). Deparaffinization and rehydration were conducted under xylene and gradient ethanol, respectively. We used 3%  $\text{H}_2\text{O}_2$  to block endogenous peroxidases at room temperature, and heat treatment was performed for antigen retrieval. After being washed in phosphate-buffered saline (PBS), the tissue sections were incubated with CD8 (Abcam, Cambridge, UK; Cat# ab178089, RRID: AB\_2756374), CD16 [rabbit monoclonal antibody, Abcam (ab183354)], FOXP3 (Abcam Cat# ab215206, RRID: AB\_2860568), CD68 (Abcam Cat# ab213363, RRID: AB\_2801637), CD163 (Abcam Cat# ab182422, RRID: AB\_2753196) for 1.5 h at room temperature. The tissue sections were incubated with the peroxidase-conjugated secondary antibody for 18 min after washing in PBS for 15 min. Diaminobenzidine was used for chromogen and hematoxylin was used for counterstaining nuclei. Human placental tissue was used as positive controls for PD-L1.

All tissue slides were digitized using Case Viewer 2.4 (3DHISTECH, Budapest, Hungary) after staining. Analysis was performed by 2 pathologists (Li Gong and Xue Wang) who were blind to the histopathological type and clinical dates. The expression of PD-L1 (22C3 clone) was evaluated according to the tumor proportion score (TPS) system based on the guidelines that have been established for non-small cell lung cancer (31). The expression of PD-L1  $>1\%$  was defined as positive. For CD8, FOXP3, CD16, CD68, and CD163 staining, the number of positive cells were counted automatically using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA), and among the positive cells labeled by Image-Pro Plus software, cells with false positive were manually removed. The intratumoral region (intra) was defined as tumor parenchyma and the peritumoral region (peri) was defined as the stroma surrounding nests of tumor cells (Figure S1) (32). We selected 5 high-powered representative fields ( $\times 400$ ) of each region for respective analysis. The density of cells was calculated based on the average number of cells in the 5 fields divided by the area ( $0.07 \text{ mm}^2/\text{field}$ ). The median density of immune cells positive for each maker was used as cut-off value, with a density of immune cells greater than the cut-off value defined as high level infiltration.

### Statistical analysis

The software SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical computation. Chi-square and Fisher's exact tests were used to compare groups on categorical variables. The Wilcoxon rank-sum test and Kruskal-Wallis test were used for evaluating the difference of the expression of immune markers among different groups. Kaplan-Meier method and log-rank test were used for survival analyzed. All statistics were 2-sided and P value less than 0.05 was considered statically significant.

## Results

### Clinical feature of 21 patients with primary tracheobronchial neoplasm

The baseline characteristics of participants are summarized in Table 1. In total, 21 patients diagnosed with primary tracheobronchial neoplasms who were treated with surgery were included in this cohort. The histopathology of 21 patients included ACC (16/21) and SCC (5/21). The median age of participants with ACC was 42 years

**Table 1** Baseline characteristics of patients with primary tracheobronchial neoplasm

Variable	ACC (n=16)	SCC (n=5)
Age, years, median [range]	42 [27–64]	56 [37–73]
Gender, n (%)		
Male	10 (62.50)	4 (80.00)
Female	6 (37.50)	1 (20.00)
Smoker, n (%)		
Yes	5 (31.25)	4 (80.00)
No	11 (68.75)	1 (20.00)
Presentations, n (%)		
Cough	10 (62.50)	3 (60.00)
Dyspnea	9 (56.25)	2 (40.00)
Hemoptysis	4 (25)	2 (40.00)
Tumor size, n (%)		
≤3 cm	9 (56.25)	4 (80.00)
>3 cm	7 (43.75)	1 (20.00)
Location, n (%)		
Trachea	12 (75.00)	2 (40.00)
Main bronchus	4 (25.00)	2 (40.00)
Carinal	0	1 (20.00)
Lymph nodes metastasis, n (%)		
Negative	14 (87.50)	4 (80.00)
Positive	2 (12.50)	1 (20.00)
Extension, n (%)		
E0	8 (50.00)	4 (80.00)
E1	8 (50.00)	1 (20.00)
Margin, n (%)		
R0	12 (75.00)	5 (100.00)
R1	4 (25.00)	0
Complications, n (%)		
Anastomotic fistula	4 (25.00)	0
Chylothorax	1 (6.25)	1 (20.00)
None	11 (68.75)	4 (80.00)
Adjuvant therapy, n (%)		
Radiotherapy	1 (6.25)	0
Chemotherapy	6 (37.50)	3 (60.00)
None	9 (56.25)	2 (40.00)
Distant metastasis, n (%)	2 (12.50)	2 (40.00)

ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma

(27–64 years), which was younger than that of patients with SCC. The gender ratio was about 2:1 (10 males *vs.* 6 females) in patients with ACC. Males comprised the majority of patients with SCC. Compared to those with ACC, most SCC participants were smokers, while the percentage of smokers in ACC was 31.25%. Cough was the most frequent symptom in these patients, following by dyspnea and hemoptysis. Among all 21 participants, none had a personal history of neoplasia, and only 1 participant with SCC had a family history of lung cancer (brother). Lymph node metastasis was observed in 3 participants (2 patients with ACC and 1 patient with SCC). The E1 extension was observed in 8 patients with ACC and 1 patient with SCC. Postoperative adjuvant therapy was performed in 10 patients.

All patients had recovered from surgery and were discharged without deaths. The median follow-up was 24.8 (1.6–45.6) months and 35.8 (2.6–51.9) months for SCC and ACC participants, respectively. At the time of analysis, 4 participants were recorded with distant metastasis, including 2 participants with ACC and 2 participants with SCC. In ACC, both of the metastases were in the lung, at 12.6 and 18.7 months, respectively. The metastasis in mediastinal lymph nodes and lung had occurred in SCC at 3.1 and 23.4 months postoperatively, respectively.

#### *PD-L1 expression and immune cell infiltration in primary tracheobronchial neoplasm*

The immunogenic features of ACC compared with SCC are showed in the *Table 2* and representative images of the stained immune cells and PD-L1 are shown in *Figure 1*. A significant difference in the expression of PD-L1 was observed between ACC and SCC ( $P=0.001$ ). All of 16 participants with ACC had no expression of PD-L1, whereas 4/5 (80%) of those with SCC were positive for PD-L1 expression, which was 6%, 10%, 20%, and 90%, respectively, based on the TPS. In the peritumoral region, the density of cells expressing CD16 was significantly higher in the SCC group than that in the ACC group ( $P<0.01$ ) (*Table 2*). In both intratumoral and peritumoral regions, the density of cells expressing FOXP3 were significantly higher in the SCC group ( $P<0.01$ ) (*Table 2*). In contrast, no significant difference was observed in the density of cells expressing CD8, CD68, and CD163 between SCC and ACC, neither in the intratumoral region nor peritumoral region (*Table 2*).

The comparison among different kinds of immune cells in primary tracheobronchial neoplasm is shown in

**Table 2** PD-L1 expression and immune cell infiltration in SCC and ACC

Immunomarker	ACC (n=16)	SCC (n=5)	P value
Expression of PD-L1, n (%)			0.001*
≥1%	0	4 (80.0)	
<1%, or no expression	16 (100.0)	1 (20.0)	
CD8 <sup>+</sup> cell density, /mm <sup>2</sup>			
Intra	168.5 (33–834)	429 (51–720)	0.368
Peri	251 (86–1,385)	897 (169–1,243)	0.056
CD16 <sup>+</sup> cell density, /mm <sup>2</sup>			
Intra	161.5 (54–1,382)	220 (89–991)	0.093
Peri	170 (54–1,100)	1134 (254–1,477)	0.003*
CD68 <sup>+</sup> cell density, /mm <sup>2</sup>			
Intra	403 (137–1,129)	469 (203–1,763)	0.368
Peri	351.5 (91–1,514)	1209 (206–2,583)	0.093
CD163 <sup>+</sup> cell density, /mm <sup>2</sup>			
Intra	187 (6–954)	317 (71–1,011)	0.263
Peri	180 (20–797)	460 (80–1,000)	0.181
FOXP3 <sup>+</sup> cell density, /mm <sup>2</sup>			
Intra	54 (0–1,094)	1,271 (871–2,763)	0.002*
Peri	80 (0–357)	477 (140–760)	0.005*

Cell density was showed as median (range). \*, the difference was significant. ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma; Intra, intratumoral region; Peri, peritumoral region.

*Figure 2.* In the ACC group, the density of CD68<sup>+</sup> cells were significantly higher than that of CD163<sup>+</sup> cells in the intratumoral region ( $P<0.01$ ) and peritumoral region ( $P<0.05$ ) and CD8<sup>+</sup> cells in the intratumoral region ( $P<0.05$ ) (*Figure 2A*). Similarly, the density of FOXP3<sup>+</sup> cells were significantly lower than that of CD8<sup>+</sup>, CD16<sup>+</sup>, and CD68<sup>+</sup> cells in the intratumoral region ( $P<0.01$ ) and peritumoral region ( $P<0.01$  and  $P<0.05$ ) (*Figure 2A*). However, no significant differences were observed among the distribution of each kind of immune cell in the intratumoral or peritumoral region (*Figure 2B*). In the intratumoral region of the SCC group, the density of FOXP3<sup>+</sup> cells were significantly higher than that of CD8<sup>+</sup> cells, CD16<sup>+</sup> cells, and CD163<sup>+</sup> cells ( $P<0.01$  and  $P<0.05$ ) (*Figure 2C*). Further, the density of FOXP3<sup>+</sup> cells located in the intratumoral region was higher than that in the peritumoral region ( $P<0.01$ ) (*Figure 2D*).

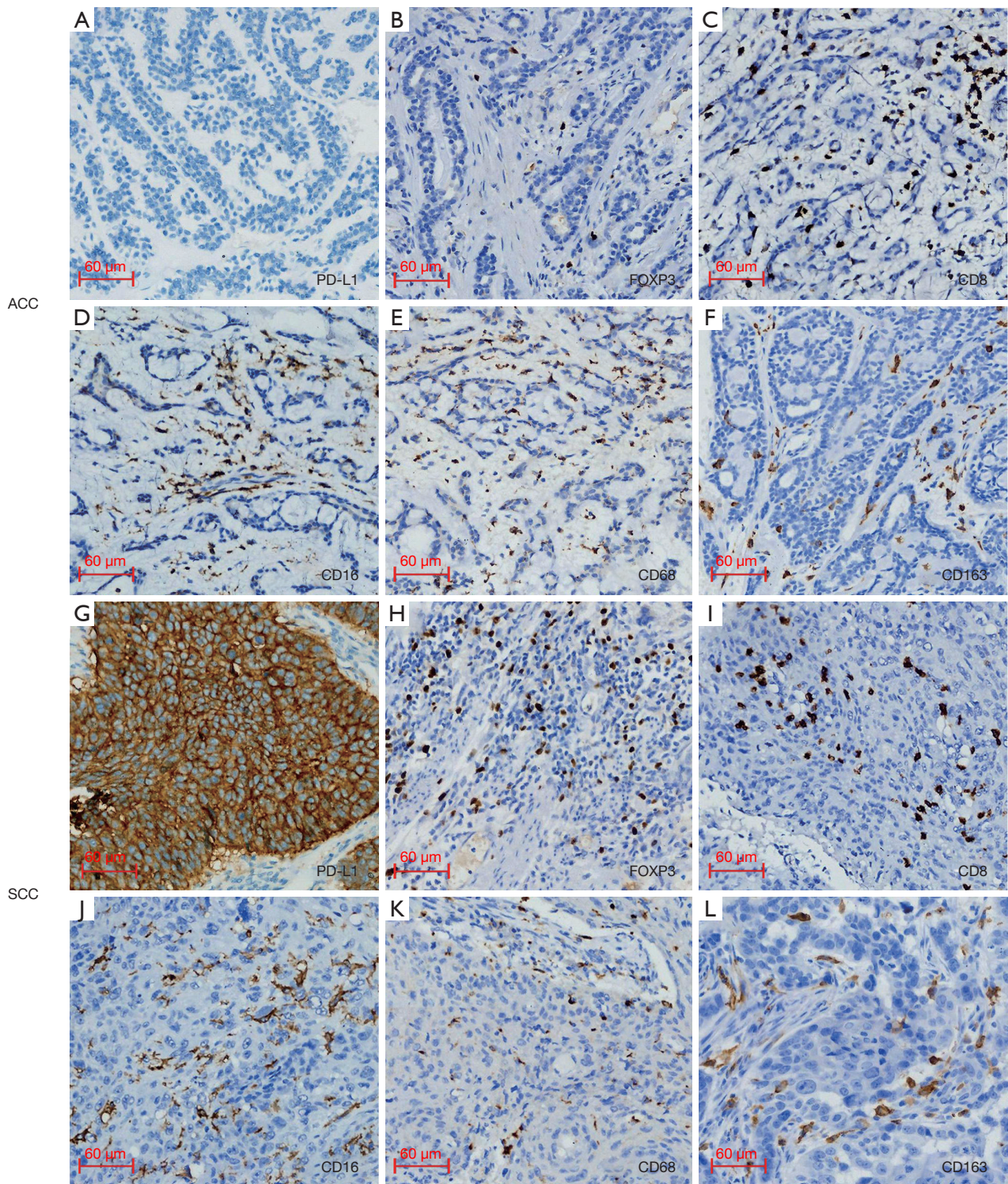
### *Relationship between the infiltration of immune cells and prognosis of tracheobronchial neoplasm*

As shown in *Figure 3A*, the densities of cells expressing CD16 in the intratumoral region was higher in participants without metastasis than in those with metastasis in the ACC group ( $P<0.05$ ). In contrast, no significant differences were observed between the density of CD16<sup>+</sup> cells and metastasis or extratracheal invasion in the peritumoral region (*Figure 3A,3B*). The differences between the density of cells expressing CD8, CD68, CD163, or FOXP3 and patients with or without metastasis were not significant ( $P>0.05$ ) in ACC (*Figure 3A*). As shown in *Figure 3B*, participants with or without extratracheal invasion were not associated with the infiltration of immune cells ( $P>0.05$ ). No statistical analysis was performed on the relationship between the density of immune cells and metastasis or extratracheal invasion, given the small sample size of SCC. The Kaplan–Meier curves showed that the degree of immune cell infiltration had no significant effects on the disease-free survival (DFS) and the OS of tracheobronchial neoplasm ( $P>0.05$ ) (*Figures S2–S5*).

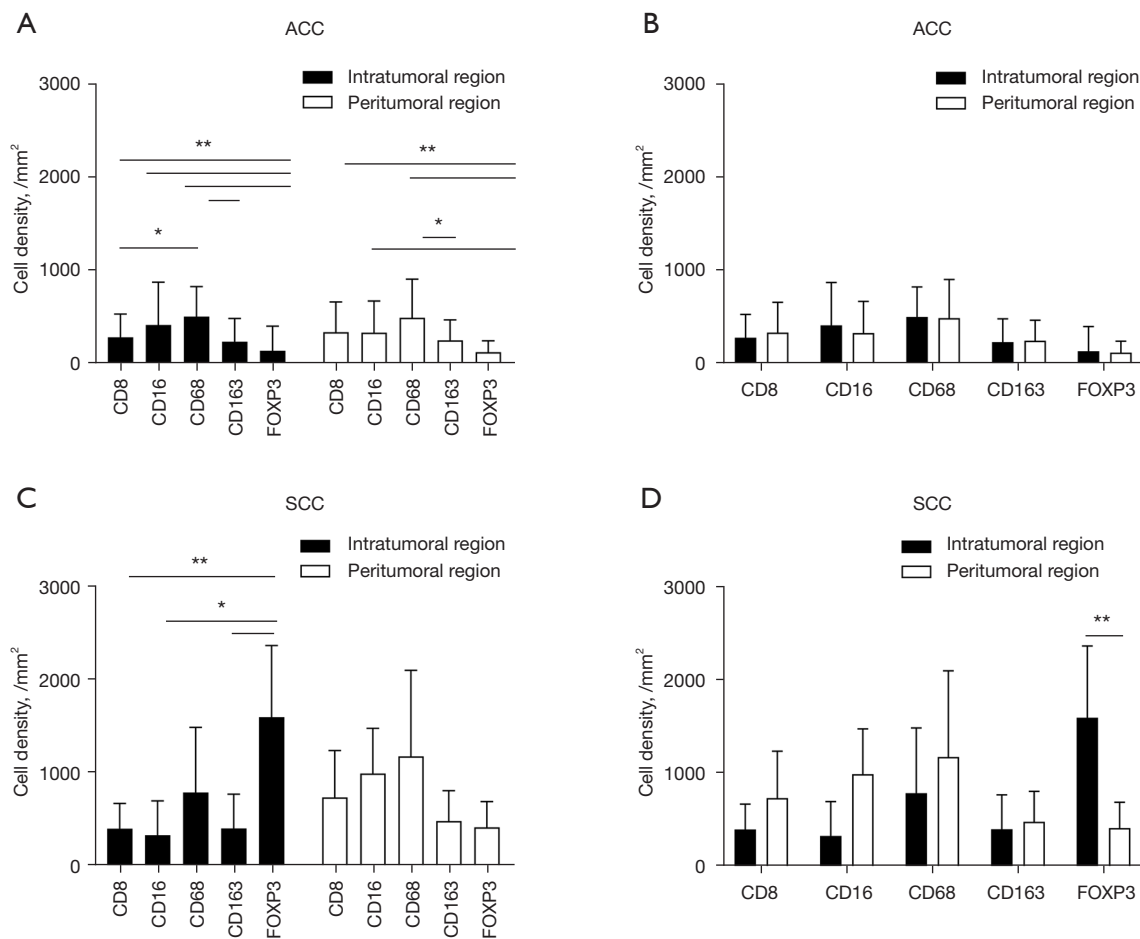
### *The immune status of primary tracheobronchial neoplasm*

According to the location of the infiltration of CD8<sup>+</sup> cells, (i.e., intratumoral or peritumoral region), the immune status was defined as immune inflamed, immune excluded, and immune desert (*Figure 4A*). The results demonstrated that there was no difference of immune status between ACC and SCC ( $P>0.05$ ) (*Figure 4B*). In the SCC group, 2 of 5 cases were in the immune excluded, 2 cases were in immune inflamed, and immune desert was observed in 1 case (*Figure 4B*). And the distribution of immune status might be associated with smoking history, although the difference was not significantly ( $P=0.082$ ). In the ACC group, the tumors under immune inflamed ( $n=6$ ) and immune desert were equal ( $n=6$ ), and 25% of participants ( $n=4$ ) with ACC were immune excluded (*Figure 4B*).

Furthermore, the relationship between the immune status and metastasis or extratracheal invasion were analyzed. All of the 5 participants (ACC,  $n=3$ ; SCC,  $n=2$ ) with metastasis (either lymph nodes metastasis or distant metastasis) were in the state of immune excluded or immune desert (*Figure 5A,5B*). Specifically, both participants with SCC and 2 of 3 participants with ACC were in the state of immune excluded, and the other 1 was in immune desert. When looking at extratracheal invasion, the results showed that the status of immune excluded accounted for 50% of participants with ACC (*Figure 5C*). In contrast, immune desert was the main



**Figure 1** Immunohistochemical staining for immune cell analysis in primary tracheobronchial neoplasm. Representative picture of cells expressing PD-L1 (A,G), FOXP3 (B,H), CD8 (C,I), CD16 (D,J), CD68 (E,K), and CD163 (F,L) in ACC and SCC. Scale bars =60  $\mu$ m ( $\times$ 200). ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma.



**Figure 2** Comparison of the average densities of immune cells in the intratumoral and peritumoral regions of ACC and SCC, respectively. The bar charts show the mean ± standard error of different types of immune cells densities in the intratumoral and peritumoral regions of ACC (A) and SCC (C); the comparison of the distribution of immune cells in different location of ACC (B) and SCC (D). \*, P<0.05; \*\*, P<0.01. ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma.

status of the immune microenvironment in patients with extratracheal invasion in SCC (Figure 5D).

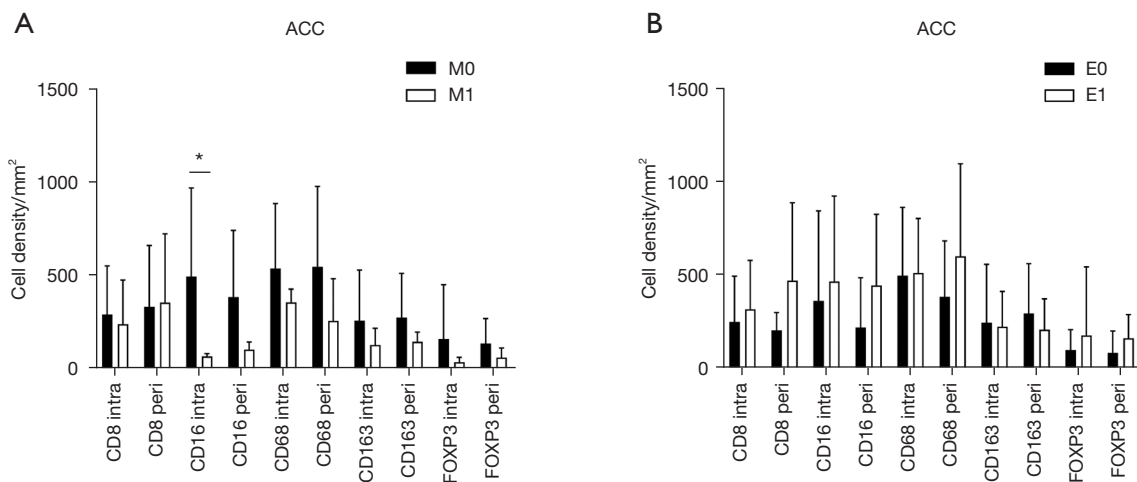
**Immunotherapy candidates of primary tracheobronchial neoplasm**

To develop a better immunotherapy strategy for patients with tracheobronchial neoplasm, the immune microenvironment was defined as 4 types based on the expression of PD-L1 and the CD8<sup>+</sup> cell density intratumorally. The representative images of PD-L1 and CD8<sup>+</sup> cell density in the 4 immunotypes are shown in Figure 6A. The expression of PD-L1 >1% and the median CD8<sup>+</sup> cell density was used as the cutoff. As shown in Figure 6B, most of the SCC cases were in the immunotype I (PD-L1<sup>+</sup>/CD8<sup>+</sup>, adaptive

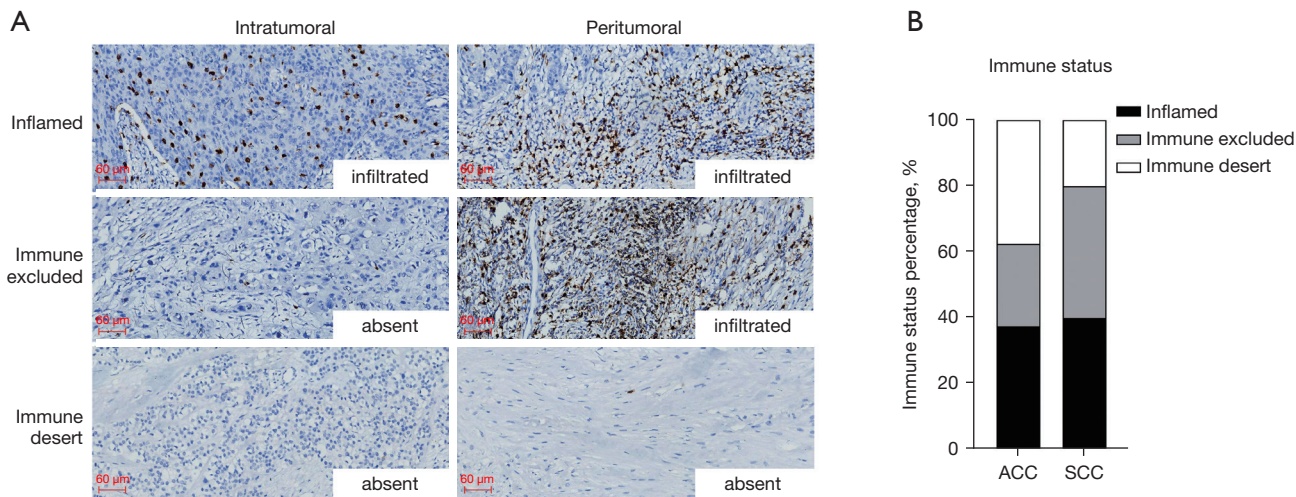
immune resistance), accounting for 60%. On the contrary, immunotype I was not observed in ACC cases; immunotype II (PD-L1<sup>-</sup>/CD8<sup>-</sup>, immunologic ignorance) or immunotype IV (PD-L1<sup>-</sup>/CD8<sup>+</sup>, immune tolerance) were the main immunotypes in ACC cases. Immunotype III (PD-L1<sup>+</sup>/CD8<sup>-</sup>, intrinsic induction) was only observed in SCC case.

**Discussion**

Immune microenvironment plays an important role in the development and progression of tumors, in which the immune cells have been regarded as treatment response markers and prognostic factors (33-37). In this study, based on current literature, several specific types of immune cells were analyzed, including CD8<sup>+</sup> T cells, CD16<sup>+</sup> cells, FOXP3<sup>+</sup>



**Figure 3** Comparison of the average densities of immune cells in the intratumoral and peritumoral regions among patients with metastasis and extratracheal invasion. (A) The relationship between the densities of immune cells and metastasis in ACC; (B) the relationship between the densities of immune cells and extratracheal invasion in ACC. \*, P<0.05. Metastasis was defined as patients with lymph node metastasis or distant metastasis. ACC, adenoid cystic carcinoma.



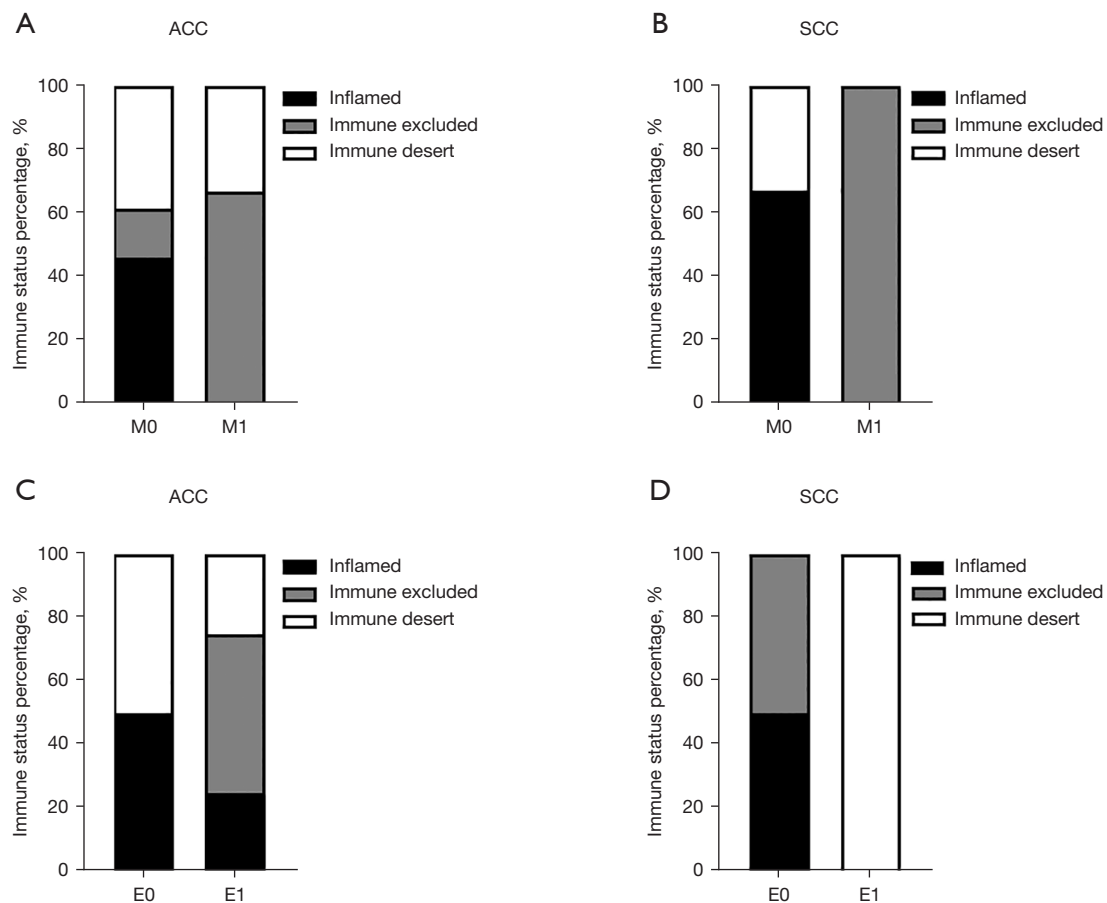
**Figure 4** The immune status in primary tracheobronchial neoplasm. (A) the representative IHC image for the immune status including inflamed, immune excluded, and immune desert; (B) the distribution of immune statuses in ACC and SCC. ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma; IHC, immunohistochemistry.

Treg cells, CD68<sup>+</sup> macrophages, and CD163<sup>+</sup>, indicating M2 macrophages. Currently, microsatellite instability (MSI), PD-L1 expression and tumor mutational burden (TMB) play important roles as predictive biomarkers for immunotherapy. However, the relationship between MSI, PD-L1 expression and TMB is different in different tumor types (38). Different tumor types should select different biomarkers (even combination) for evaluating immunotherapy response. In

NSCLC, the PD-L1 expression is approved as biomarker for determining usage of immunotherapy. Therefore, in this study, the PD-L1 expression in primary tracheobronchial was analyzed. To our knowledge, this is the first study to systemically describe the immune cells infiltration in primary tracheobronchial neoplasm.

The expression of PD-L1 varies widely in different solid tumors, ranging from 0% to 100% (39). A previous

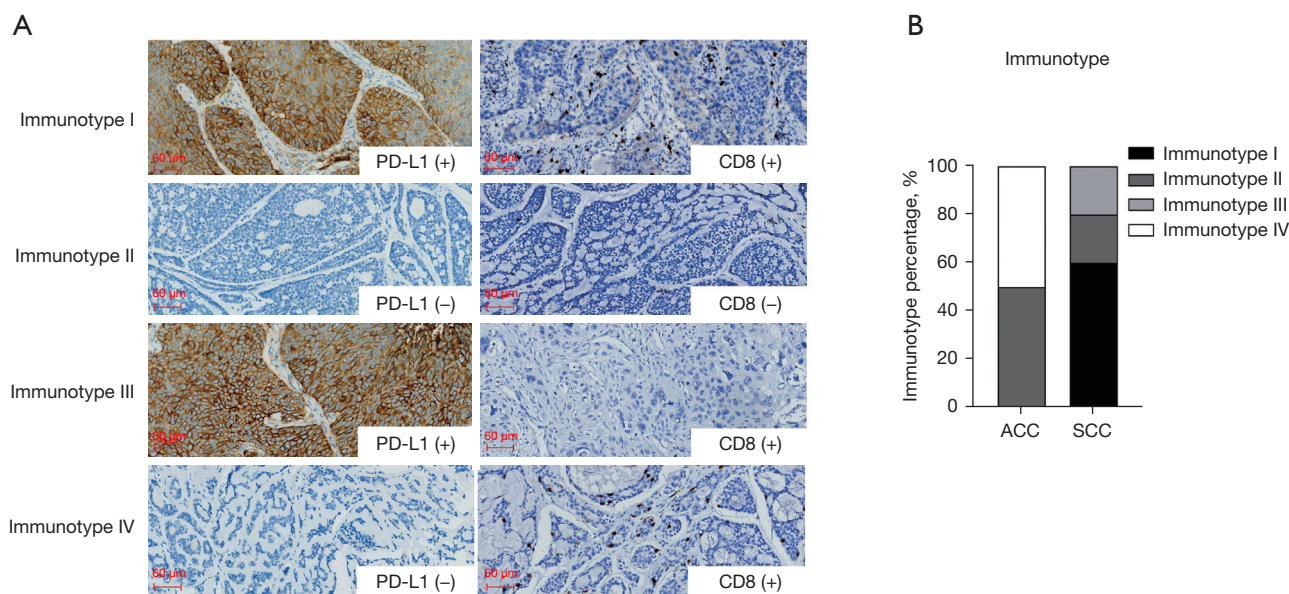




**Figure 5** The relationship between the immune statuses and metastasis and extratracheal invasion. The bar charts show the percentage of different immune statuses in patients with or without metastasis and extratracheal invasion. The relationship between the immune status and metastasis in patients with ACC (A) and SCC (B); the relationship between the immune status and extratracheal invasion in patients with ACC (C) and SCC (D). ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma.

study demonstrated that the expression of PD-L1 >1% in primary tracheal tumors with a predominant squamous cell component was 50%, and salivary-type tracheal tumors without any expression of PD-L1 (40). In this study, the expression of PD-L1 accounted for 80% of the SCC group, whereas ACC did not show any degree of PD-L1 expression. Overexpression of PD-L1 indicated poor clinical outcome in some studies (39,41,42). In this study, PD-L1 expression was positive in 2/2 SCC with distant metastases that the expression levels of PD-L1 was 10% and 90% and in 1/1 patient with lymph node metastasis that the expression levels of PD-L1 was 10%. Based on the staging system proposed by Bhattacharyya N (43), 3/3 of patients in stage I–II and 1/2 of patients in stage IV have high expression of PD-L1 (>1%). And in this study, no significant relationship is observed between the

expression of PD-L1 and the staging of SCC of primary tracheobronchial neoplasm. And PD-L1 overexpression also means that the patient may be sensitive to PD-L1 inhibitor. There have been 2 case reports showing the successful use of immune therapy in tracheal SCC with significant PD-L1 expression (21,22). Specifically, the patients were treated with nivolumab and pembrolizumab respectively, and both of them obtained completed tumor response after several months. These results indicated that the expression of PD-L1 should be examined in SCC. And due to the heterogeneity of tumor, the immune escape of tumor can occur through different immune checkpoints. Therefore, patients with low expression of PD-L1 can combine other immune checkpoint inhibitors. For example, in melanoma, combining anti-cytotoxic T lymphocyte associated antigen 4 (anti-CTLA) inhibitor and anti-PD-1 inhibitor showed



**Figure 6** Immunotypes of primary tracheobronchial neoplasm based on PD-L1 expression and median CD8<sup>+</sup> cell density intratumorally. (A) the representative IHC images of PD-L1 and CD8<sup>+</sup> cell density in the four immunotypes; (B) the distribution of four immunotypes in ACC and SCC. ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma; IHC, immunohistochemistry.

better therapeutic effects than that of single immune checkpoint inhibitor (44). Although more evidence is needed to support the actual effect of immune checkpoint inhibitor, patients with primary tracheobronchial squamous cell neoplasm who have failed to other therapies could try to use immune checkpoint inhibitor for treatment.

However, the responses to the anti-PD-L1/PD-1 immune checkpoint inhibitors not only depended on the expression of PD-L1, but on a variety of factors, such as the infiltration of CD8<sup>+</sup> T cells. The TIME has been divided into 4 categories based on the expression of PD-L1 and the infiltration of CD8<sup>+</sup> T cells (24). The main immunotype in the SCC group was immunotype I (PD-L1<sup>+</sup>/CD8<sup>+</sup>), which was considered the group that was most likely to benefit from single-agent checkpoint blockade. The high level of pre-existing CD8<sup>+</sup> T cells intratumorally was the main contributor to response to anti-PD-L1/PD-1 therapy. Immunotype III (PD-L1<sup>+</sup>/CD8<sup>-</sup>) is another immune status with PD-L1 overexpression; however, different from immunotype I, a PD-L1/PD-1 inhibitor alone would not elicit response to treatment. In this situation, immunotherapy combined with stimulating CD8<sup>+</sup> cell infiltration is a good option. Radiation has been reported to enhance the number of CD8<sup>+</sup> cell infiltration in tissue (45). Therefore, anti-PD-1/PD-L1 combined with radiotherapy may be an optional treatment strategy

for patients with immunotype III. In this study, 25% of SCC group participants were in immunotype III, which means that evaluating the infiltration of CD8<sup>+</sup> T cells, rather than the expression of PD-L1, is necessary for primary tracheobronchial squamous cell neoplasm before conducting immunotherapy.

In the ACC group, the distribution of immunotypes was equal (immunotype II 50% *vs.* immunotype IV 50%). Besides anti-PD-1/PD-L1 or anti-CTLA4 therapy, inducing CD8<sup>+</sup> cell infiltration was a critical part in the therapeutic strategy for immunotype II (PD-L1<sup>-</sup>/CD8<sup>-</sup>). In contrast, instead of anti-PD1/PD-L1, patients in immunotype IV (PD-L1<sup>-</sup>/CD8<sup>+</sup>) should consider other immunosuppressive pathways, including vaccines and metabolites. Therefore, the treatment for primary tracheobronchial ACC may be focused on stimulating CD8<sup>+</sup> cells infiltration and some novel immunosuppressants. Based on the cancer immunity cycle, the immune cells was activated and migrated from immune organ to tumor bed through various factors, including stimulatory factors, such as IL-1, TNF- $\alpha$ , CD28/B7-1, OX40/OX40L, CX3CL1, CXCL9, LFA1/ICAM1, etc., and inhibitory factors, such as IL-10, IL-4, CTLA4/B7-1, PD-L1/PD-1, LAG-3, IDO, TGF- $\beta$ , etc. (46). And CD8<sup>+</sup> cell can be recruited by the secretion of CXCL11, which may be a direction for stimulating CD8<sup>+</sup> cells infiltration (47).

Whether CD8<sup>+</sup> T cells can play an anti-tumor role

depends on whether they can effectively infiltrate into the tumor parenchyma. Therefore, based on spatial distribution of CD8<sup>+</sup> cells, the tumor microenvironment was defined as 3 immune statuses, including inflamed tumor, immune-excluded tumor, and immune-desert tumor (48). The result showed that 4 of 5 patients with metastasis were of immune excluded status, which means that CD8<sup>+</sup> cells had accumulated in stroma rather than infiltrating into tumor parenchyma. These results indicated that patients with immune excluded status were more like to experience metastasis. Therefore, both the density and location of CD8<sup>+</sup> cells infiltration should be considered when evaluating the TIME of primary tracheobronchial neoplasm.

The cluster of differentiation molecule CD16 (Fcγ receptor III), expressing on natural killer (NK) cells, macrophages, and neutrophils, plays an important role in antibody-dependent cell-mediated cytotoxicity (ADCC) (49). When the antibodies secreted by plasma cells bind specifically to the target cells, NK cells can bind to the Fc segment of the antibody through CD16, and then release perforin to kill the target cells. Furtherly, the migratory pattern of NK cell is associated with a downregulation of CX3C chemokine receptor 1 (CX3CR1) and sphingosine-1-phosphate receptor 1 (S1PR1) and overexpression of C-X-C chemokine receptor type 6 (CXCR6) and C-X-C chemokine receptor type 5 (CXCR5) (50). And a study showed that the depletion of regulatory T cells mediated by ADCC has been regarded a possible mechanism of action of anti-CTLA-4 antibody therapy (51). Further, CD16<sup>+</sup> cells have been significantly associated with response to ipilimumab treatment in patients with metastatic melanoma (25). In this study, in the intratumoral region, the density of CD16<sup>+</sup> cell was significantly lower in participants with metastasis than those without metastasis ( $P < 0.05$ ). These results demonstrated that CD16<sup>+</sup> cell infiltration represented a favorable prognostic factor. Furthermore, high level of CD16<sup>+</sup> cell infiltration correlates with CD8<sup>+</sup> cell infiltration in colorectal cancer, indicating that CD16<sup>+</sup> cells may be able to trigger T-cell response (52). Therefore, stimulating the infiltration of CD16<sup>+</sup> cells could be an important treatment for improving the curative effect and prognosis of primary tracheobronchial neoplasm.

Tumor-associated macrophages (TAM) are important components of the TIME, among which CD68 is a characteristic generic macrophage maker and CD163 is the marker for M2 macrophages (53). And colony-stimulating factor-1 receptor (CSF1R)-mediated signaling is critical for the survival and differentiation of TAM (54).

Some studies have reported that higher density of TAM infiltration is associated with poor prognosis. For example, in laryngeal SCC, weak CD68<sup>+</sup> and CD163<sup>+</sup> cell infiltration presented with early tumor stage compared with advance tumor stage (55). Furthermore, strong CD68<sup>+</sup> cell infiltration was observed in the majority of patients who relapsed (55). However, in contrast, ACC patients with metastasis exhibited lower-level density of CD68<sup>+</sup> cells and CD163<sup>+</sup> cells infiltration, although the difference was not significant. This was similar to the results of a study of duodenal cancers, which showed that CD68<sup>+</sup> TAMs were a positive prognostic factor (56). The positive prognostic function of macrophages has also been observed in human colorectal cancer (57). Therefore, larger participant cohorts are necessary to further explore the function of TAM in primary tracheobronchial neoplasm.

Overexpression of FOXP3<sup>+</sup> cells has been found to indicate an immunosuppressive microenvironment, and FOXP3 is regarded as the most accurate maker of Tregs (41). And FOXP3<sup>+</sup> Treg cell will be recruited by the high expression of chemokine CCL22 and CCL17, and TGF-β activation can further promote Treg differentiation and inhibit T cell activation (58). Toss *et al.* (41) and Li *et al.* (59) showed that higher infiltration of FOXP3<sup>+</sup> cells was significantly associated with decreased recurrence-free time and OS in breast cancer. Previous studies have also demonstrated that Tregs are associated with worse OS in uveal melanoma (60). In this study, the density of FOXP3<sup>+</sup> cells in ACC were significantly lower than that of FOXP3<sup>+</sup> cells in SCC. Furthermore, the density of cells expressing FOXP3 was significantly lower than that of cells expressing CD8, CD16, and CD68 in ACC. However, the density of cells expressing FOXP3 was significantly higher than that of cells expressing CD8, CD16, and CD163 in SCC. These results indicated that the TIME between ACC and SCC of primary tracheobronchial neoplasm was significantly different.

It should be noted that there were several limitations to this study. Firstly, due to the low incidence of tracheobronchial neoplasm, the sample size was small. Larger cohorts are needed to further confirm our results. Secondly, only ACC and SCC of primary tracheobronchial neoplasm were included in this study. Recently, a study showed that patients with primary tracheal lymphoepithelioma-like neoplasm and primary tracheal large-cell neuroendocrine neoplasm can also benefit from immunotherapy (23). Therefore, other than ACC and SCC, more pathological types of primary tracheobronchial neoplasm should be included in subsequent studies.

## Conclusions

In primary tracheobronchial neoplasm, the expression of PD-L1 was mainly concentrated among patients with SCC, whereas patients with ACC did not show any expression of PD-L1. The infiltrations of immune cells in primary tracheobronchial neoplasm were significantly different between ACC and SCC. Compared with other immune cells, the density of FOXP3<sup>+</sup> cells were the highest in SCC, while the infiltration degree of FOPX3<sup>+</sup> cells were the lowest in ACC. The CD68<sup>+</sup> cells were the main immune cells in the TIME of ACC. Notably, the location of CD8<sup>+</sup> cells infiltration is an important factor in tumor progression, in which immune excluded and immune desert were more common in primary tracheobronchial neoplasm with metastasis. Therefore, immunotherapy should be realized as a potential component of treatment strategies for patients with primary tracheobronchial neoplasm. The infiltration of immune cells should be considered for immunotherapy and prognosis.

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## Footnote

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the ethical guidelines of Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Tangdu Hospital (No. K202111-04). Individual consent for this retrospective analysis was waived.

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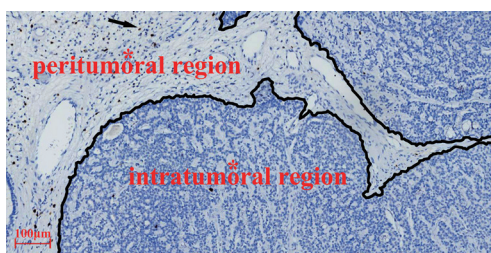
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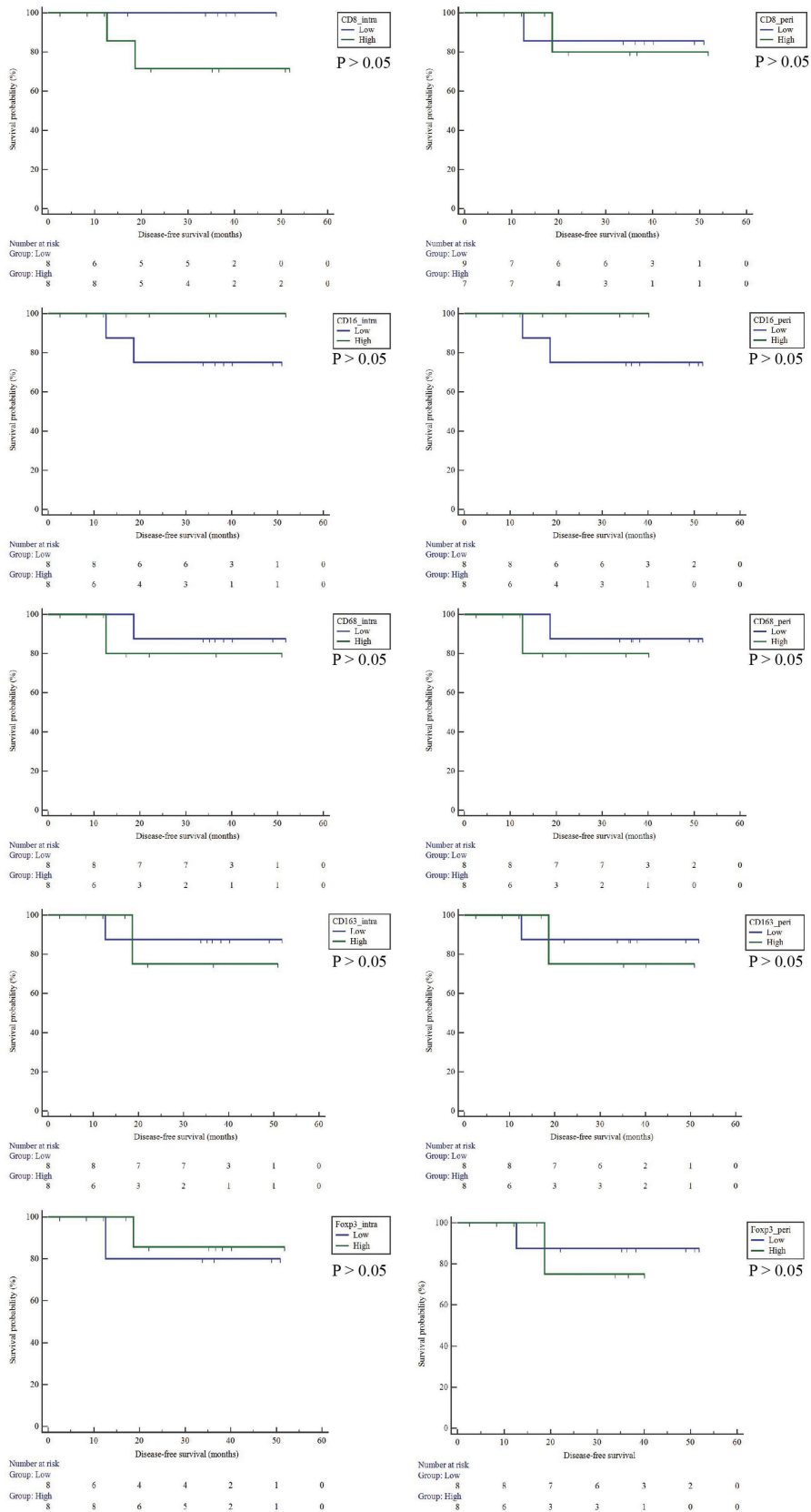
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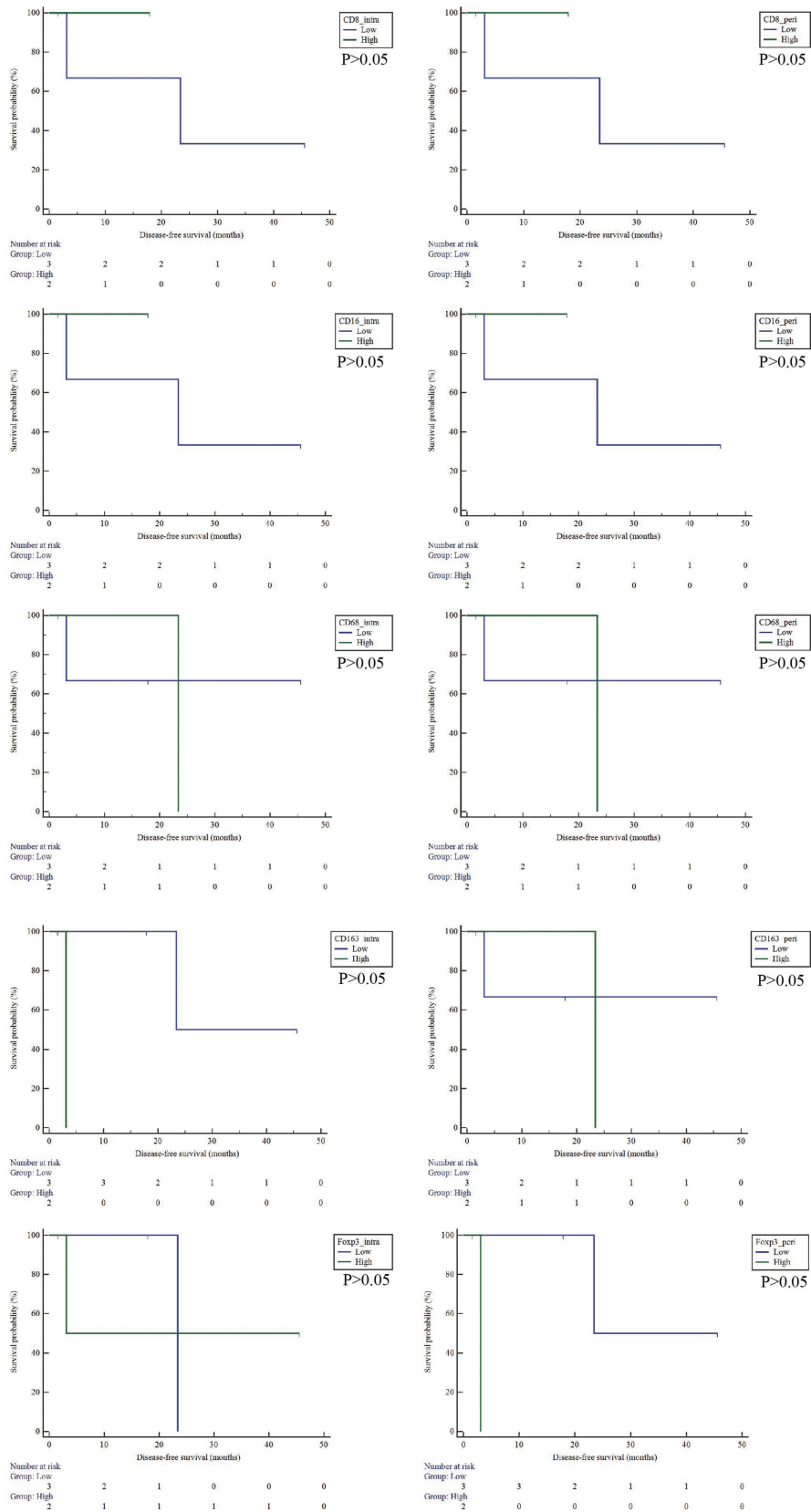


**Figure S1** Representative picture shows intratumoral region and peritumoral region in primary tracheobronchial neoplasm. (Immunohistochemical staining for CD8+ cells; Black line: the boundary between intratumoral region and peritumoral region; Black arrow: CD8+ cell).

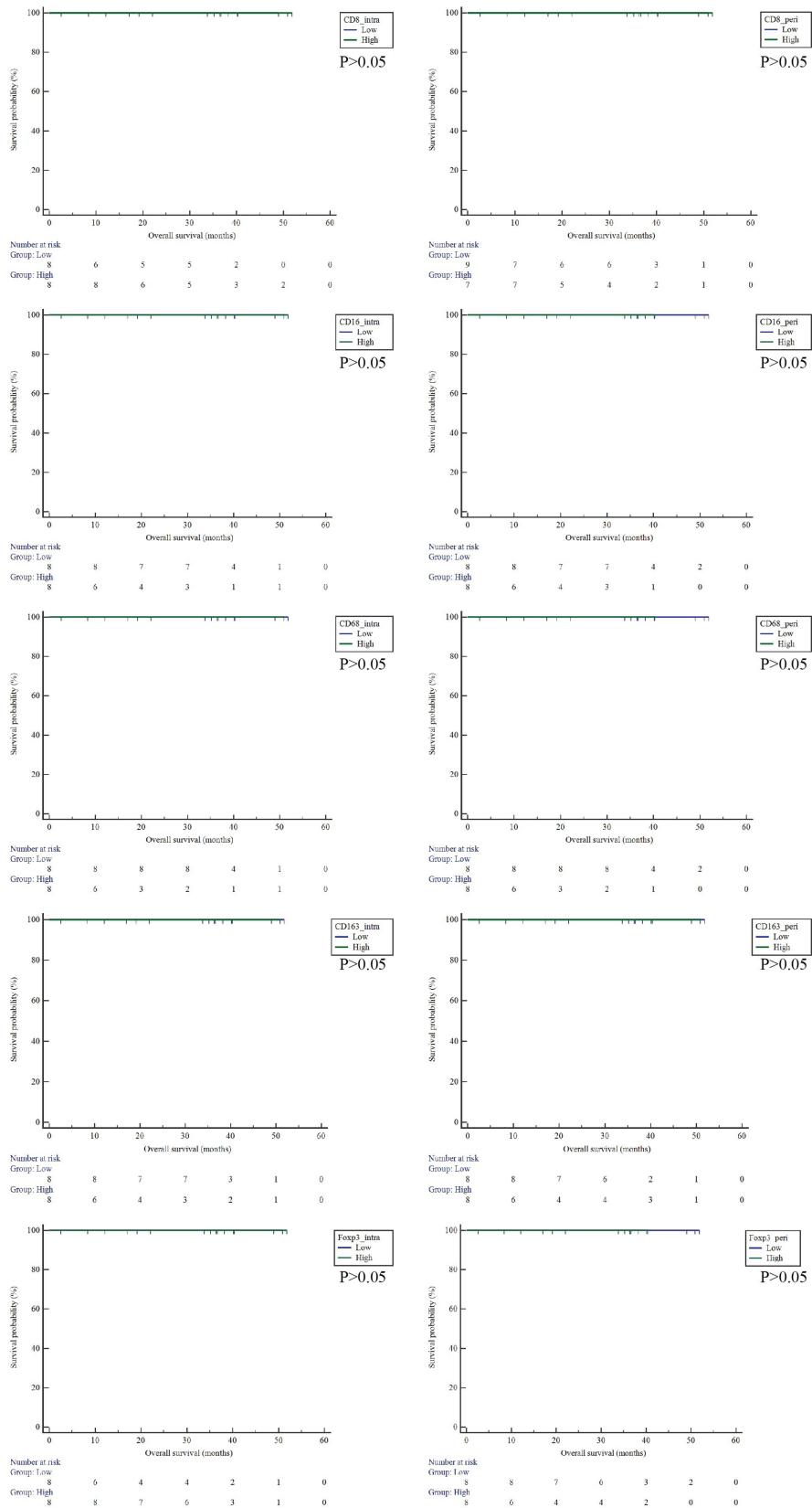


**Figure S2** The relationship between the infiltration of immune cells and disease-free survival in ACC. ACC, adenoid cystic carcinoma.

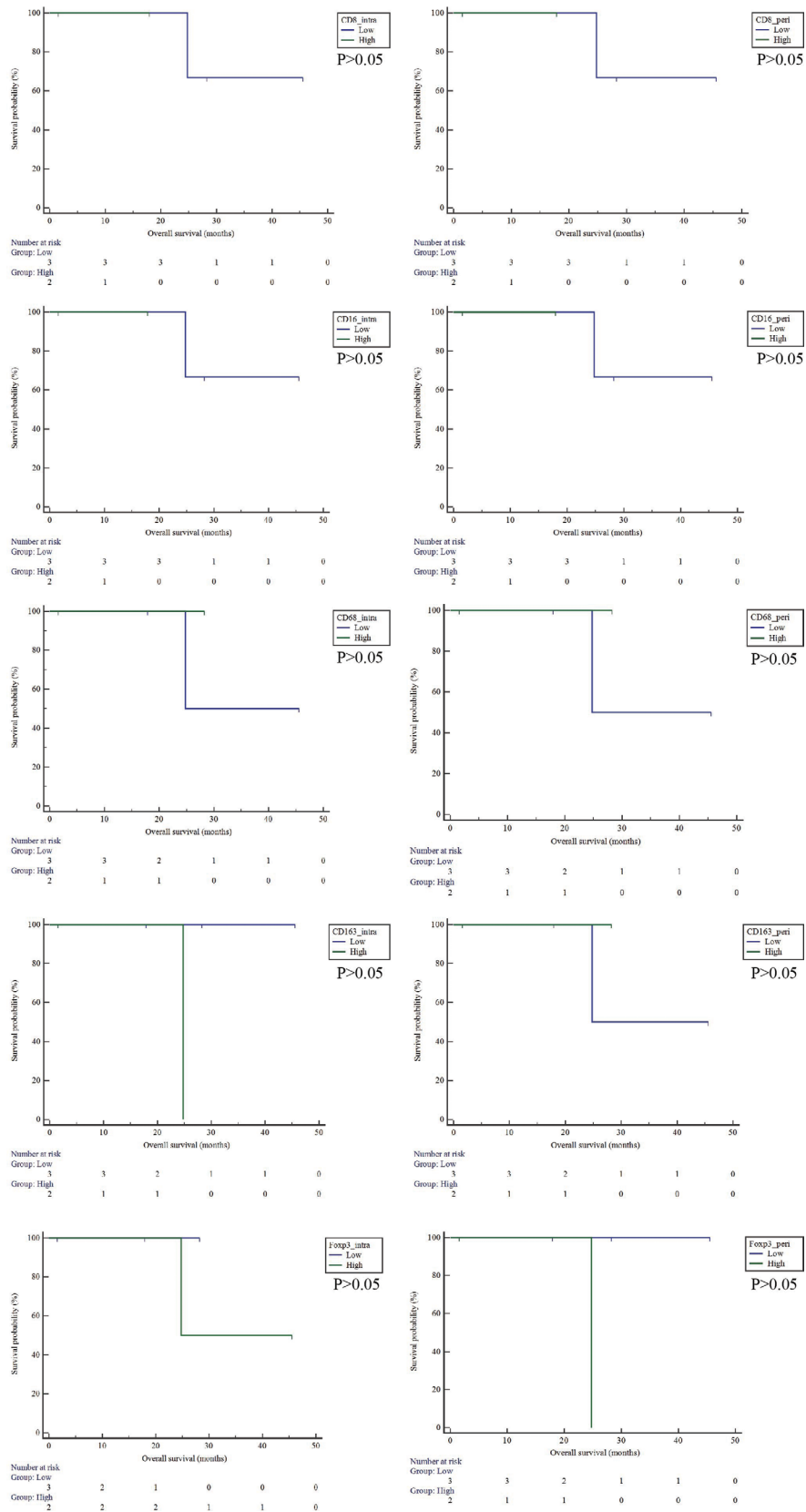




**Figure S3** The relationship between the infiltration of immune cells and disease-free survival in SCC. SCC, squamous cell carcinoma.



**Figure S4** The relationship between the infiltration of immune cells and overall survival in ACC. ACC, adenoid cystic carcinoma.



**Figure S5** The relationship between the infiltration of immune cells and overall survival in SCC. SCC, squamous cell carcinoma.