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Desmoplasia in non-small cell lung carcinomas is associated with low programmed death-ligand 1 expression and the absence of tumor-infiltrating lymphocytes

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Programmed death-ligand 1 (PD-L1) is the most widely utilized predictive marker used to identify non-small cell lung carcinoma (NSCLC) patients most suitable for immunotherapy approaches. The relationship between PD-L1 expression, the presence of CD8+ T cells, and other clinicopathological characteristics of NSCLC patients has not been elucidated yet. In this retrospective study, we immunohistochemically determined PD-L1 expression (using clone 22C3) and $CD8^+$ T cell count (using clone c8/144B) in surgical resection specimens from 698 advanced NSCLC patients. Results of PD-L1 expression and $CD8^+$ T cell count were correlated to various clinicopathological characteristics, including the presence of desmoplasia in NSCLC. Regarding the immunological attributes of the tumor microenvironment, we identified major differences between desmoplastic and non-desmoplastic areas in NSCLC. Tumor areas without desmoplasia were significantly more often PD-L1 positive than tumor cell clusters encased in a dense collagenous stroma ($p=0.004$). Furthermore, the desmoplastic stroma contained significantly less often an immune cell infiltrate rich in $CD8^+$ T cells ($p<0.001$). Also, the positivity of PD-L1 significantly correlated with advanced N-stage ($p<0.001$) and poor differentiation in adenocarcinomas ($p=0.032$) but not with other clinicopathological characteristics. In conclusion, to our knowledge, this is the first study that points to major differences in terms of immunological attributes between desmoplastic and non-desmoplastic areas in NSCLC. The desmoplastic component, therefore, may represent an immunologically distinct tumor area in which PD-L1 immunohistochemistry and $CD8^+$ T cell count should be evaluated separately.

Key words: non-small cell lung carcinoma; desmoplasia; tumor infiltrating lymphocytes; programmed death-ligand 1

Immunotherapeutic approaches have introduced a new era in the treatment of various types of human malignancies including advanced non-small cell lung carcinoma (NSCLC) [1, 2]. Immune checkpoint inhibitors (ICI) block the interaction between programmed cell death 1 (PD-1) and programmed death-ligand 1 (PD-L1), which leads the tumor to increased cytotoxicity of effector $CD8^+$ T cells [3]. Immunotherapeutic approaches using ICI have been shown to be superior to mainstay chemotherapy, yielding higher overall survival and less toxic side effects in NSCLC patients [4–7]. Immunohistochemical determination of the presence of PD-L1 on the surface of tumor cells [8] has proven to be a valuable tool in predicting response to ICI in numerous NSCLC patients [9–11]. On the other hand, over the last several years, PD-L1 expression has gained the status of an imperfect biomarker in some patients, which may be attributed to its complex nature [12]. PD-L1 lacks the straightforward dichotomy of other predictive factors in the treatment of NSCLC, like the *epidermal growth factor receptor* mutational status in which a wild-type genetic signature basically excludes the possibility of tyrosine kinase efficacy [13]. Regulation of PD-L1 expression is much more complex and is intricately connected with numerous signaling pathways and cellular constituents present within the NSCLC tumor microenvironment (TME) [14–16]. Many aspects of TME with presumable impact on the quality and quantity of immune responses have not yet been investigated in detail and are therefore not sufficiently understood [17]. Some

authors believe that desmoplasia, which refers to the presence of a dense collagenous stroma surrounding the tumor beds, can lead to the exclusion of effector cytotoxic $CD8^+$ T cells and other tumor-infiltrating lymphocytes (TIL) from TME, which may cause non-responsiveness to immunotherapy in some types of malignancies [18–20]. However, the impact of desmoplasia on antitumor activity in TME of NSCLC has not yet been investigated.

In this retrospective study, we aimed to determine the relationship between various clinicopathological characteristics, the expression of PD-L1, the presence of $CD8^+$ T cells and $CD3^+$ T cells in the TME of a large cohort of NSCLC patients diagnosed by surgical resection specimens. Furthermore, based on our hypothesis that desmoplasia may affect the immunological attributes within TME, a comparison between desmoplastic and non-desmoplastic areas in various types of NSCLC was performed.

Patients and methods

Sample characteristics and evaluated clinicopathological parameters. We evaluated 698 surgical resection specimens from patients with the diagnosis of NSCLC. The tumors were collected from various hospitals across Slovakia and all methodological steps, including the histopathological analysis, PD-L1 immunohistochemistry, $CD8^+$ T cells, and $CD3^+$ T cells evaluation were performed at the Department of Pathological Anatomy of the University Hospital in Martin. The conduction of this study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin (EK 43/2021).

Clinicopathological parameters analyzed in all NSCLC cases included sex, age, the specific histopathological diagnosis, and the presence or absence of desmoplasia. The NSCLC patients were diagnosed and classified in accordance with the 2021 World Health Organization Classification of Thoracic Tumors [21]. Desmoplasia was morphologically defined as a tumor area in which the proportion of a dense collagenous stroma to tumor tissue is greater than one. If such morphological characteristics comprised at least 10% of the tumor mass in a given NSCLC case, the tumor was designated as "NSCLC with desmoplastic areas".

Clinical data informing about the tumor size (T stage) and the lymph node status (N stage) was available in 157 NSCLC cases. All clinicopathological attributes were correlated with the results of PD-L1 immunohistochemistry.

"NSCLC with desmoplastic areas" group. NSCLC cases with desmoplasia comprising at least 10% of the tumor mass were allocated into the "NSCLC with desmoplastic areas" group in which a more complex histopathological analysis was performed. In this group, the histological growth pattern, the percentage of PD-L1 positive tumor cells, the $CD8^+$ T cell and $CD3^+$ T cell density of the tumor stroma was evaluated separately in the desmoplastic component (DC) and in the non-desmoplastic component (NDC). The results obtained from DC and NDC were quantified, and these two categories were compared.

"Adenocarcinoma with desmoplastic areas" and "squamous cell carcinoma with desmoplastic areas" subgroups. In the next steps of our histopathological analysis, the "NSCLC with desmoplastic areas" group was further subdivided into the "adenocarcinoma with desmoplastic areas" and the "squamous cell carcinoma with desmoplastic areas" subgroups based on the specific histological type of lung cancer. The histological growth pattern, the percentage of PD-L1 positive tumor cells, the $CD8^+$ T cell and $CD3^+$ T cell density of the tumor stroma were evaluated separately in DC and in NDC, which were recognized in each NSCLC case. The results obtained from DC and NDC were quantified, and these two categories were compared.

Evaluation and categories of PD-L1 expression. To perform the immunohistochemical analysis, sections were made of formalin-fixed and paraffin-embedded tissues. The tissue sections were revitalized in a pre-treatment link with a low pH solution at a temperature of 96°C for 20 min. Sections were then incubated with the mouse monoclonal anti-PD-L1 antibody clone 22C3 (Dako Agilent, Denmark, SK006). The immunohistochemical reactions were conducted on the platform Autostainer Link 48 using the PD-L1 IHC 22C3 PharmDx (RTU, Ready-to-use) and the detection reactions were performed using EnVision Flex (Dako Agilent, Denmark).

The level of PD-L1 expression, evaluated immunohistochemically in each histopathological slide, was reported as the overall percentage of tumor cells showing membranous staining. The immunohistochemical analysis was performed by certified thoracic pathologists (LP and AF). Based on the results of the immunohistochemical analysis of PD-L1 expression, NSCLC, DC, and NDC were allocated into one of five categories: no expression (positivity < 1%), low expression (positivity 1–9%), intermediate expression (positivity 10–49%), high expression (positivity ≥ 50%), or any expression (positivity ≥ 1%).

Evaluation and categories of $CD8^+$ T cell and $CD3^+$ T cell density. $CD8^+$ T cells and the $CD3^+$ T cells in the tumor stroma were detected immunohistochemically by using the antibody FLEX Monoclonal Mouse Anti-Human CD8, clone c8/144B (Dako Agilent, Denmark, IR623), RTU and the polyclonal anti CD3 antibody, clone 17617-1-AP (Dako Agilent, Denmark, 99-201). Revitalization was conducted on a pre-treatment link with a high pH (pH 9) solution and the immunohistochemical reactions were carried out using the Autostainer Link 48. Detection reactions were performed using the detection kit EnVision Flex, high pH (Link) K8000 (Dako Agilent, Denmark).

The density of $CD8^+$ T cells and $CD3^+$ T cells present in the tumor stroma of DC and NDC were evaluated quantitatively as the number of lymphocytes stained positive by the CD8 antibody and the $CD3^+$ antibody, respectively. The $CD8^+$ T cells and $CD3^+$ T cells were counted on high-power

fields evaluating the entire tumor stroma of DC and NDC and the reported result represented the average number of CD8 positive lymphocytes and. Two categories of $CD8^+$ T cell density were recognized: low density (less than ten $CD8^+$ T cells per one high power field) and high density (≥10 $CD8^+$ T cells per one high power field). Considering the $CD3^+$ T cell density, two categories were distinguished: low density (less than twenty $CD3^+$ T cells per one high power field) and high density (≥20 $CD3^+$ T cells per one high power field).

Statistical analysis. In our study, we used the Exact binomial test of proportion for the statistical analysis of compared parameters. Results obtained from analysis of PD-L1, CD3, and CD8 immunohistochemistry were correlated with the various patients clinicopathological parameters. The test was two-sided and differences between the compared variables were considered statistically significant if the p-value was <0.05. All the statistical analyses were carried out using the software R V.3.5.0 of the DescTool library.

Results

PD-L1 expression in correlation with clinicopathological features. Immunohistochemical analysis of PD-L1 expression was performed in a sample of 698 tumors obtained from patients with NSCLC and the results were correlated with various clinicopathological variables. Most patients were men (64.8%) and the average age at the time of diagnosis was 66 years. No significant differences were found regarding the level of PD-L1 expression when comparing the various age groups and gender.

Considering the histological composition, most of the evaluated NSCLC cases (562/698; 80.5%) were categorized as adenocarcinomas (ADC). Acinar or papillary predominant (grade 2) ADC were the most common subtypes (306/562; 43.8%). Differences in the level of PD-L1 expression were noted when comparing different subtypes of ADC divided by the degree of differentiation: the proportion of PD-L1 positive cases in grade 3 ADC subtypes (136/207; 65.7%) was higher than in grade 2 (92/306; 30.0%) or grade 1 tumors (49/10; 20.4%) and these differences have been proven to be statistically significant (p=0.032). One hundred and thirty-six NSCLC cases (19.5%) were squamous cell carcinomas (SqCC). Most of them (107/136; 78.7%) belonged to the non-keratinizing subtype. The proportion of PD-L1 positive non-keratinizing SqCC cases (62/107; 58.0%) was similar to the proportion of PD-L1 positive cases present in the keratinizing SqCC category (17/29; 58.6%).

Desmoplastic areas that comprised at least 10% of the tumor mass were present in 78 NSCLC cases (11.2%). Out of these 78 NSCLC cases, 43 were ADC and 35 were SqCC. The proportion of PD-L1 positive NSCLCs with desmoplastic areas (32/78; 41.0%) was similar to those NSCLCs without desmoplasia (285/620; 46.0%).

Data regarding tumor size (T stage) and status of regional lymph node involvement (N stage) were available in 157 NSCLC patients. Approximately half of them (88/157; 56.0%) had T1 stage tumors. Forty-nine NSCLCs (31.2%) were categorized as T2 and twenty NSCLCs (12.7%) were defined as T3 or T4. No significant differences were observed between these three T stage categories with respect to the

Table 1. Clinicopathological characteristics of the sample with correlation with various categories of PD-L1 expression.

Clinicopathological variables			Attribute, number (%)	Percentage of PD-L1 positive tumor cells				
				<1%	1–9%	10–49%	≥50%	≥1%
All NSCLCs (n=698)	Sex		Female, 246 (35.2%)	131 (53.3%)	36 (53.3%)	40 (13.3%)	39 (15.9%)	115 (46.7%)
			Male, 452 (64.8%)	250 (55.4%)	51 (11.3%)	66 (14.6%)	85 (18.8%)	202 (44.6%)
	Age*		≤ average, 356 (51.0%)	193 (54.2%)	38 (10.6%)	52 (14.6%)	73 (20.5%)	163 (45.8%)
			> average, 342 (49.0%)	188 (55.0%)	49 (14.3%)	54 (15.8%)	51 (14.9%)	154 (45.0%)
	Histology		Total, 562 (80.5%)	324 (57.7%)	64 (11.4%)	75 (13.3%)	99 (17.6%)	238 (42.3%)
		ADC	Grade 1, 49 (7.0%)	39 (79.6%)	6 (12.2%)	3 (6.1%)	1 (2.1%)	10 (20.4%)
			Grade 2, 306 (43.8%)	214 (70.0%)	43 (14.0%)	32 (10.5%)	17 (5.5%)	92 (30.0%)
			Grade 3, 207 (29.7%)	71 (34.3%)	15 (7.2%)	40 (19.3%)	81 (39.1%)	136 (65.7%)
			Total, 136 (19.5%)	57 (41.9%)	23 (16.9%)	31 (22.8%)	25 (18.4%)	79 (58.1%)
		SqCC	Keratinizing, 29 (4.2%)	12 (41.4%)	4 (13.8%)	8 (27.6%)	5 (17.2%)	17 (58.6%)
			Non-keratinizing, 107 (15.3%)	45 (42.0%)	19 (17.8%)	23 (21.5%)	20 (18.7%)	62 (58.0%)
	Desmoplasia		Absent, 620 (88.8%)	335 (54.0%)	100 (16.1%)	163 (26.3%)	122 (19.7%)	285 (46.0%)
			Present, 78 (11.2%)	46 (59.0%)	24 (30.8%)	6 (7.7%)	2 (2.5%)	32 (41.0%)
NSCLCs with evaluation of T and N stage (n=157)	T stage		T1; 88 (56.0%)	47 (53.4%)	17 (19.3%)	9 (10.2%)	15 (17.0%)	41 (46.6%)
			T2; 49 (31.2%)	22 (44.9%)	7 (14.3%)	8 (16.3%)	12 (24.5%)	27 (55.1%)
			T3 and T4; 20 (12.7%)	12 (60.0%)	2 (10%)	3 (15.0%)	3 (15.0%)	8 (40.0%)
	N stage		N0; 104 (66.2%)	63 (60.6%)	11 (10.6%)	12 (11.5%)	18 (17.3%)	41 (39.4%)
			N1; 33 (21.0%)	16 (48.5%)	6 (18.2%)	5 (15.2%)	6 (18.2%)	17 (51.5%)
			N2; 20 (12.7%)	3 (15.0%)	8 (40.0%)	3 (15.0%)	6 (30.0%)	17 (85.0%)

Note: *age range from 26 to 96 years; average 66 years; Abbreviations: NSCLC-non-small cell lung carcinoma; ADC-adenocarcinoma; SqCC-squamous cell carcinoma; PD-L1-programmed death-ligand 1

overall positivity of PD-L1. Considering the status of the regional lymph nodes, most patients (104/157; 66.2%) had no local lymph node involvement. Thirty-three carcinomas (21.0%) were categorized as being N1 and twenty patients (12.7%) were defined as N2. A significant positive correlation was observed between the N stage and the level of PD-L1 expression: 34 out of 53 cases (64.2%) with a positive lymph node status (N1 and N2 stage) demonstrated PD-L1 expression in the primary tumor. Contrary to this, patients negative for lymph node involvement (N0 stage) had PD-L1 positive primaries in only 41 out of 104 (39.4%) cases (p<0.001). The results of PD-L1 expression in correlation with clinicopathological features are summarized in Table 1.

NSCLCs with desmoplastic areas – comparison between desmoplastic and non-desmoplastic components. Seventy-eight NSCLCs (43 of them were ADC and 35 cases were SqCC) in our sample were characterized by the presence of a desmoplastic component (DC), which comprised at least 10% of the tumor mass in a given case. The DC was compared with the non-desmoplastic component (NDC) present in the tumor. When quantifying and analyzing the results of the comparison of the DC with NDC, it was found that the positivity of PD-L1 expression was significantly more often seen in NDC compared to DC: the overall PD-L1 positivity in the NDC was 32 out of 78 cases (41.0%). On the other hand, only 12 of 78 tumor areas with the presence of desmoplasia (15.4%) had at least 1% PD-L1 positive tumor cells (p=0.004). PD-L1 positive DC were mostly in the low expression category, with no DC having ≥50% positive tumor cells. Compared to this, PD-L1 positivity was in the intermediate or high category in 15 (19.2%) and 13 (16.7%) NDC, respectively. Furthermore, the NDC category demonstrated a significantly higher proportion of cases showing high density of $CD8^+$ T cells and $CD3^+$ T cells in the tumor stroma compared to DC (42.3% vs. 10.2%, p<0.001). The $CD3^+$ T cell density correlated with the $CD8^+$ T cell density, i.e., cases that were characterized by a high $CD8^+$ T cell density had also a high $CD3^+$ T cell density. Considering the proportion, $CD8^+$ T cells made approximately one-third of the whole T cell count in each case. The results of the comparison between DC and NDC in 78 NSCLC cases with desmoplastic areas are summarized in Table 2. A representative picture that highlights differences between desmoplastic and non-desmoplastic areas in NSCLC is shown in Figure 1.

ADCs with desmoplastic areas – comparison between desmoplastic and non-desmoplastic components. Detailed morphological analysis of the 43 ADC cases with the presence of desmoplastic areas revealed further differences between DC and NDC: The prevalence of analyzed immunological attributes (PD-L1 expression, $CD8^+$ T cell and $CD3^+$ T cell density) was very low in the DC category: only 2 out of the 43 desmoplastic areas (4.7%) contained tumor cells showing PD-L1 expression and the positivity of PD-L1 was in the low range (1–9%) in both cases. Furthermore, a high density of $CD8^+$ T cells and $CD3^+$ T cells in the tumor stroma was present in only one DC (2.3%).

Compared to the DC, the proportion of PD-L1 positive cases in the NDC category was significantly higher (p=0.004). Also, the high density of $CD8^+$ T cells and $CD3^+$ T cells in the tumor stroma of NDC was demonstrated significantly more often (p<0.001). Furthermore, an association between PD-L1 positivity, high $CD8^+$ cell and $CD3^+$ T cell count, and poorly differentiated growth patterns was found in the NDC category: the proportion of PD-L1 positive, $CD8^+$ and $CD3^+$

Table 2. Comparison of histopathological characteristics, PD-L1 expression, $CD8^+$ T cell and $CD3^+$ T cell density between desmoplastic and non-desmoplastic components in 78 NSCLC cases.

Pathologic variables		Component		p-value
		Desmoplastic (n=78)	Non-desmoplastic (n=78)	
Histologic growth patterns present in the components	Lepidic	1 (1.3%)	7 (9.0%)	
	Papillary	0	5 (6.4%)	
	Acinar	41 (52.6%)	25 (32.1%)	
	Solid	1 (1.3%)	5 (6.4%)	
	Micropapillary	0	1 (1.3%)	
	Keratinizing	9 (11.5%)	9 (11.5%)	
	Non-keratinizing	26 (33.3%)	26 (33.3%)	
PD-L1 positive tumor cells in the component (%)	<1%	66 (84.6%)	46 (59.0%)	
	1–9%	9 (11.5%)	15 (19.2%)	
	10–49%	3 (3.8%)	13 (16.7%)	
	≥ 50%	0	4 (5.1%)	
	≥ 1%	12 (15.4%)	32 (41.0%)	p=0.004
$CD8^+$ T cell density of the component	Low	70 (89.8%)	45 (57.7%)	p<0.001
	High	8 (10.2%)	33 (42.3%)	
$CD3^+$ T cell density of the component	Low	69 (88.5%)	44 (56.4%)	p<0.001
	High	9 (11.5%)	34 (43.6%)	

Abbreviation: PD-L1-programmed death-ligand 1

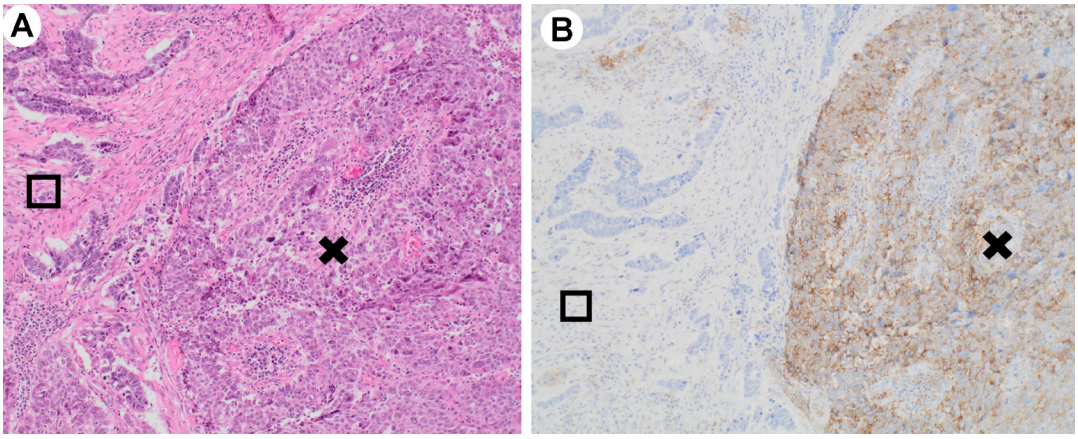

Figure 1. Comparison of PD-L1 expression between desmoplastic and non-desmoplastic areas in non-small cell lung carcinoma. A) A predominantly solid adenocarcinoma case is shown, in which tumor cell clusters are encased by a desmoplastic stroma on the left side of the picture (□). On the right side of the picture, tumor cell clusters are surrounded by minimum stroma (×). B) Differences in the level of PD-L1 positivity are shown between desmoplastic (□) and non-desmoplastic areas (×) in the example shown in (A). Original magnification x10; hematoxylin-eosin (A); PD-L1 immunohistochemistry (B)

TIL high cases was greater in NDC characterized by grade 3 morphology. The results of the comparison between DC and NDC in 43 ADC cases with desmoplastic areas are summarized in Table 3.

SqCCs with desmoplastic areas – comparison between desmoplastic and non-desmoplastic components. The morphological analysis of the 35 SqCC cases with the presence of desmoplastic areas did not reveal any differences in the sense of histological composition between DC and NDC. Overall, non-keratinizing morphology was the predominant histological pattern in DC as well as NDC (26/35; 74.3%). A tendency to be more often PD-L1 positive was noted in the NDC category. However, when compared with the subgroup of ADCs with desmoplastic areas, this trend did not achieve statistical significance (p=0.136). On the other hand, the proportion of NDC displaying high $CD8^+$ TIL as well as high $CD3^+$ TIL density was greater than in the DC category and achieved marginal statistical significance (p=0.043). The results of the comparison between DC and NDC in 43 SqCC cases with desmoplastic areas are summarized in Table 4.

Discussion

In this study, we have shown major differences regarding immunological characteristics of the TME in NSCLC in desmoplastic areas compared to the non-desmoplastic areas. Overall, the proportion of PD-L1 positive cases was significantly higher in NDC compared to NSCLC areas characterized as NDC (p=0.004). Furthermore, the density of $CD8^+$ T cells and $CD3^+$ T cells in NDC was significantly more often in the higher category compared to DC (p<0.001). These disparities in the level of PD-L1 expression can be explained by the lack of appropriate inducers of PD-L1 expression derived from immune cells in tumor areas with pronounced desmoplasia. In most cases, the expression of PD-L1 in tumor cells depends on stimuli coming from immune cells present in their proximity [22]. Interferon-γ elaborated by T cells in the TME of NSCLC is known to be the most effective extrinsic factor responsible for robust up-regulation of PD-L1 expression in NSCLC tumor cells [23].

The transforming growth factor beta (TGF-β) signaling pathway plays a pivotal role in regulating many aspects of the TME, including remodeling the tumor stroma [24–26]. The lack of TIL in the TME of desmoplastic areas in NSCLC can be attributed to the presence of a dense collagen fiber meshwork, which represents a mechanical barrier that effector $CD8^+$ T cells and $CD3^+$ T cells cannot cross [18]. This may have important clinical consequences because the response to various immunotherapeutic approaches has been linked to the presence of effector cytotoxic $CD8^+$ lymphocytes in the TME [27, 28]. Mariathasan *et al.* (2018) elucidated major determinants of responsiveness to immune checkpoints in a large cohort of metastatic urothelial carcinomas: response to atezolizumab (anti-PD-L1 antibody) correlated with high density of $CD8^+$ T cells in tumors. On the other hand, lack of efficacy was associated with signs of activation of the TGF-β signaling pathway in cancer-associated fibroblasts. Furthermore, tumors with a signature of TGF-β activation demonstrated exclusion of $CD8^+$ T cells from tumor cell clusters. Instead of infiltrating the tumor parenchyma, lymphocytes were "left behind and trapped" in the collagen and fibroblast-rich peritumoral stroma. In the same study, the authors demonstrated that administration of bifunctional antibodies blocking PD-L1 as well as the TGF-β axis resulted in the reversion of immune exclusion by $CD8^+$ T cells in analyzed tumors of mouse models. Reduction of TGF-β signaling facilitated infiltration of the tumor parenchyma by $CD8^+$ TIL, which was responsible for vigorous immune responses and tumor regression [18].

Table 3. Comparison of PD-L1 expression, CD8⁺ T cell and CD3⁺ T cell density between desmoplastic and non-desmoplastic components in the subtypes of 43 adenocarcinoma cases.

Component	Growth pattern, number (%)	Percentage of PD-L1+ tumor cells				Density of CD8⁺ TILs		Density of CD3⁺ TILs	
		<1%	1–9%	10–49%	≥1%	Low	High	Low	High
Desmoplastic (n=43)	Lepidic 1 (2.3%)	1 (100%)	0	0	0	1 (100%)	0	1 (100%)	0
	Acinar 40 (93.0%)	38 (95%)	2 (5%)	0	2 (5%)	39 (97.5%)	1 (2.5%)	39 (97.5%)	1 (2.5%)
	Solid 2 (4.7%)	2 (100%)	0	0	0	2 (100%)	0	2 (100%)	0
Non-desmoplastic (n=43)	Lepidic 6 (16.9%)	5 (83.3%)	1 (16.7%)	0	1 (16.7%)	5 (83.3%)	1 (16.7%)	5 (83.3%)	1 (16.7%)
	Acinar 25 (58.1%)	19 (76.0%)	4 (16.0%)	2 (8.0%)	6 (24.0%)	19 (76.0%)	6 (24.0%)	20 (80.0%)	5 (20.0%)
	Papillary 5 (11.6%)	3 (60.0%)	2 (40.0%)	0	2 (40.0%)	3 (50%)	3 (50%)	3 (50%)	3 (50%)
	Solid 5 (11.6%)	1 (20.0%)	1 (20.0%)	3 (60.0%)	4 (80.0%)	1 (20.0%)	4 (80.0%)	1 (20.0%)	4 (80.0%)
	Micropapillary 1 (2.3%)	0	0	1 (100.0%)	1 (100.0%)	0	1 (100.0%)	0	1 (100.0%)
p-value		p=0.004				p<0.001		p<0.001	

Abbreviation: PD-L1-programmed death-ligand 1

Table 4. Comparison of PD-L1 expression, CD8⁺ T cell and CD3⁺ T cell density between desmoplastic and non-desmoplastic components in the subtypes of 35 squamous cell carcinoma cases.

Component	Growth pattern number (%)	Percentage of PD-L1+ tumor cells					Density of CD8⁺ TILs		Density of CD3⁺ TILs	
		<1%	1–9%	10–49%	≥50%	≥1%	Low	High	Low	High
Desmoplastic (n=35)	Keratinizing 9 (25.7%)	8 (88.9%)	1 (11.1%)	0	0	1 (11.1%)	9 (100.0%)	0	9 (100.0%)	0
	Non-keratinizing 26 (74.3%)	17 (65.4%)	6 (23.1%)	3 (11.5%)	0	9 (34.6%)	19 (73.1%)	7 (26.9%)	18 (69.2%)	8 (30.8%)
Non-desmoplastic (n=35)	Keratinizing 9 (25.7%)	4 (44.4%)	3 (33.3%)	2 (22.2%)	0	5 (55.6%)	6 (66.7%)	3 (33.3%)	6 (66.7%)	3 (33.3%)
	Non-keratinizing 26 (74.3%)	12 (46.1%)	6 (23.0%)	4 (15.4%)	4 (15.4%)	14 (53.8%)	11 (42.3%)	15 (57.7%)	11 (42.3%)	15 (57.7%)
p-value		p=0.136					p=0.043		p=0.041	

Abbreviation: PD-L1-programmed death-ligand 1

Similar attributes suggestive of immune exclusion were demonstrated in our study. In most instances, we have shown a lack of CD8⁺ TIL and CD3⁺ T cells and an absence of PD-L1 expression in the DC of NSCLC. Because DC and NDC may represent tumor areas with different potential of responses to immunotherapy, a more complex approach to morphological evaluation is warranted in NSCLC with such complex histology. In these tumors, the response to immunotherapy may be hampered because of desmoplastic areas. We suggest that in NSCLC with desmoplastic areas comprising at least 10% of the tumor mass, PD-L1 immunohistochemistry should be evaluated in the DC and in the NDC separately. Combined therapy targeting the TGF-β signaling pathway as well as the PD-1 axis may act synergistically in amplifying the effect of immune checkpoints in NSCLC with DC. Clinical studies investigating this possibility are recommended in the future.

Our results have also shown that the most significant differences in the sense of immunological attributes were observed when comparing DC and NDC in tumors classified as ADC. Only 2 out of the 43 desmoplastic areas (4.7%) contained at least 1% of tumor cells showing PD-L1 expression and a high density of CD8⁺ and CD3⁺ TIL was present in only one DC (2.3%). Therefore, adenocarcinoma morphology with associated desmoplasia represents in most instances an immunologically poor component, pointing to a negative result of PD-L1 immunohistochemistry. These results suggest that especially in small biopsy samples, the presence of such histological features could be used as a surrogate for PD-L1 immunohistochemistry if tissue is short in supply or needs to be preserved for molecular testing.

Our analysis also revealed further associations between the presence of immunological attributes and morphological characteristics of our samples. Although there was a tendency of NDC in the SqCC subgroup to be more often PD-L1 positive, this trend did not achieve statistical significance (p=0.136). It is possible that SqCC inherently need a higher level of PD-L1 expression because they represent genetically

more complex NSCLC compared to the ADC type [29]. A higher tumor mutational burden noted in this form of lung cancer leads to the formation of plenty of neoantigens, which leads to higher immunogenicity [30, 31]. To escape immune destruction by $CD8^+$ T cells, tumor cells of SqCC need to acquire mechanisms of immune disguise, especially in the form of PD-L1 expression [32].

Besides $CD8^+$ TIL, various subtypes of T cells may also have an impact on the characteristics of the TME in NSCLC. Our previous study showed that NSCLCs with the presence of a high amount of $CD3^+$ T cells are more likely to be PD-L1 positive [33]. In the present study, analysis of the $CD3^+$ T cell and the $CD8^+$ T cell count was performed in the 78 NSCLC cases with the presence of desmoplasia. Overall, the $CD8^+$ T cell count correlated with the $CD3^+$ T cell count, i.e., cases that were characterized by a high $CD8^+$ cell density had usually also the presence of a high $CD3^+$ T cell density. Considering the proportion of T cell subpopulations, $CD8^+$ T cells made up approximately one-third of the overall population of $CD3^+$ T cells. Many subtypes of T cells are known so far, including $CD3^+$ TIL, $CD4^+$ TIL, $CD8^+$ TIL, $CD20^+$ TIL, and $FoxP3^+$ TIL [34, 35]. The presence of a high density of $CD3^+$ TIL in tumor nests correlates with better overall survival (OS) and disease-specific survival (DSS) [35]. The presence of $CD8^+$ TIL in the tumor stroma as well as in tumor nests is associated with improved OS, DSS, progression-free survival (PFS), and disease-free survival (DFS) [36, 37]. Besides $CD8^+$ TIL, the complex subgroup of $CD4^+$ TIL has gained attention. Broadly, $CD4^+$ TIL can be divided into immune-promoting T helper cells (Th) and the highly immunosuppressive T regulatory cells (Treg) [35]. Because of the counteracting roles of the subsets of $CD4^+$ TIL, there are conflicting results regarding the prognostic value of $CD4^+$ TIL in the tumor nests and tumor stroma of NSCLC [36–39]. Similarly, inconsistent results exist regarding the impact of $FoxP3^+$ TIL on the survival of NSCLC patients. Some authors consider the presence of $FoxP3^+$ TIL in the TME of NSCLC as a good prognostic factor [40], while others envisage that $FoxP3^+$ TIL has a negative prognostic impact [41, 42]. The role of $CD20^+$ TIL is not fully elucidated yet. Chen and colleagues observed that a high density of $CD20^+$ TIL is associated with improved OS and suggest that humoral immunity is as important as cellular immunity in the cancer-eliminating immune response in NSCLC [35]. The overall proportion of subtypes of T cells may vary and their impact on PD-L1 expression as well as other immunological characteristics of the TME can differ. Because of this, further studies are suggested to determine the role of various subtypes of T cells in DC and NDC of NSCLC.

Although most compared categories of clinicopathological attributes in our NSCLC sample did not show an association with PD-L1 expression, the regional lymph node status represented an important exception. The level of PD-L1 expression was significantly higher in NSCLC with metastatic involvement of regional lymph nodes compared to NSCLC cases with benign lymph nodes ($p<0.001$). Poorly differentiated NSCLC, especially of the ADC type, have been shown to display a higher proportion of PD-L1 positive cases [43, 44]. Poorly differentiated tumors have a generally higher metastatic potential [45], which may explain the higher overall PD-L1 positivity in N1 or N2 stage tumors.

Lastly, because the focus of this study was the analysis of $CD8^+$ T cells, which made up only approximately one-third of the $CD3^+$ T cell count in our sample, a weakness of this study is the missing knowledge regarding the detailed phenotype of the rest of $CD3^+$ T cells.

In conclusion, the present study points to major differences in terms of immunological attributes between desmoplastic and non-desmoplastic areas in pulmonary adenocarcinomas. Tumor areas without desmoplasia were significantly more often PD-L1 positive compared to tumor areas encased in a dense collagenous stroma. Furthermore, the desmoplastic stroma was significantly less often with the presence of a high density of $CD8^+$ T cells and $CD3^+$ T cells. Desmoplastic component, therefore, may represent an immunologically distinct tumor area, in which PD-L1 immunohistochemistry, $CD8^+$ T cells, and $CD3^+$ T cell count should be evaluated separately.

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