Spatial distribution of infiltrating T lymphocytes with Immunoscore® CR T Cells Exhaustion test helps stratification of NSCLC patients treated with PD1 / PDL1 inhibitors in the PIONeeR project

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Introduction

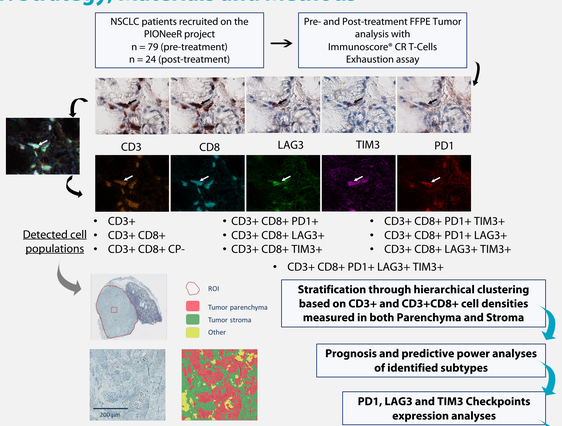
Immune checkpoint inhibitors (ICI), and particularly anti-PD1/L1, improved long-term outcome in ~20% of NSCLC patients, meaning that 80% present primary or secondary resistance and need to be identified at diagnostic to avoid inefficient therapy [1]. To date, neither PD-L1 tumor cell status nor TMB, both approved as companion diagnostics, can efficiently predict resistance.

Tumor-infiltrating lymphocytes (TILs) play a major role in the immune response against malignant cells by infiltrating and interacting with tumor cells to achieve their cytotoxic role.

TILs Immune Checkpoints' (CP) expression such as PD1, LAG3 or TIM3 may reflect their anti-tumor activity and be directly involved in response to ICIs through their regulation of T cell activity. Assessing their status within the tumor at diagnostic could help stratifying patients and refine population eligible to ICIs therapy.

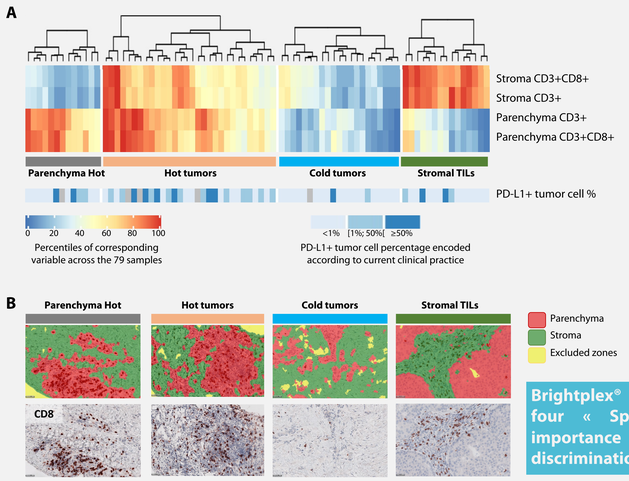
The PIONeeR project aims to predict the response/resistance to PD1/L1 ICIs in advanced NSCLC patients through a comprehensive agnostic multiparametric biomarkers assessment. Here, we aim to define clinicopathological implications of activated and exhausted TILs in a cohort of 79 patients through the multiplex immunohistochemistry (IHC) Brightplex® CR T-Cells Exhaustion assay. Among these patients, 24 were re-biopsied 6 weeks after anti-PD1/L1 treatment initiation, allowing comprehensive analysis of treatment action.

1. Strategy, Materials and Methods



4µm thick FFPE slides were sequencially stained with CD3, CD8, PD1, LAG3 and TIM3 primary antibodies on Leica Bond RX instrument and revealed with chromogenic substrate. Scanned images were analyzed according to Brightplex® Immunoscore® T-Cells Exhaustion protocol in HALO v3.0.311.314. Cell densities of populations of interest were reported for each sample and hierarchical clustering performed in Rstudio (R 3.6.3). The Kaplan Meier method was used to draw the survival curves, statistical significance of survival differences between groups was evaluated using the log-rank test. Univariate Cox model was used to estimate Hazard ratios of the survival groups, Fisher's test is used for distribution comparison and Wilcoxon test is used for means comparison between groups of interest. Figures were generated with the ggplot2, ggpubr and the rstatix packages.

2. Spatial distribution of TILs stratifies NSCLC patients into 4 subtypes



A – Hierarchical clustering of 79 NSCLC patients based on CD3+ and CD3+CD8+ cell densities respectively measured in the parenchyma and in the stroma of each tumor highlights four patient subtypes:

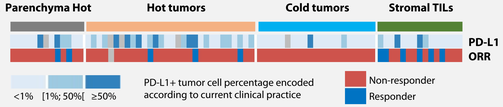
- « **Parenchyma Hot** », with important CD3+CD8+ cell densities in the Parenchyma only
- « **Hot** », with important CD3+CD8+ cell densities in the whole tumor
- « **Cold** » with negligible CD3+CD8+ cell densities in the whole tumor
- « **Stroma infiltrated** » with important CD3+CD8+ cell densities in the Stroma compartment only.

Distribution of PD-L1+ Tumor cell percentage status according to pathologist interpretation is represented below each tumor profile.

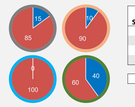
B – Representative tumors of each tumor subtype based on CD8 IHC staining. Detected Stroma (green), Parenchyma (red) and excluded areas (yellow) are represented on the upper images. CD8 IHC staining of corresponding fields is shown. Positive cells are brown, hematoxylin counterstaining is blue.

Brightplex® T-Cell Exhaustion assay highlights four « Spatial TILs subtypes » revealing importance of Parenchyma and Stroma discrimination when analyzing TILs.

3. Spatial TILs stratification enriches anti-PD1/L1 immunotherapy responders' group



A – Association between Spatial TILs subtypes and PD-L1 tumor status shows significant enrichment of PD-L1 positive tumors in both Parenchyma Hot and Hot tumors, whereas Cold tumors and Stromal TILs are depleted in PD-L1+ tumors (p=0.012). However, each subtype displays samples of all PD-L1 status.
Overall Response Rate (ORR) according to RECIST 1.1 criteria is displayed for each tumor.



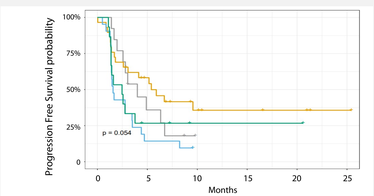
Spatial TILs subtype	Response status NR	R
Parenchyma Hot	11 (85%)	2 (15%)
Hot	26 (90%)	3 (10%)
Cold	21 (100%)	0 (0%)
Stromal TILs	9 (60%)	6 (40%)

Fisher's Test: p = 6.7e-3

PDL1 Tumor status	Response status NR	R
<1%	43 (91%)	4 (9%)
[1%;50%[	12 (75%)	4 (25%)
≥50%	8 (73%*	3 (27%)

Fisher's test : NS

B – ORR to anti-PD1/L1 therapy according to RECIST 1.1 criteria is significantly associated to subtypes ($p=6.7e^{-3}$), whereas it is not to PD-L1 tumor status, suggesting Spatial TILs subtypes are stronger predictor than PD-L1. Particularly, no responder is classified into Cold subtype, whereas Stromal TILs subtype is enriched in responders.



C – Log-rank test shows a trend but not significant association between Spatial TILs subtypes and Progression Free Survival (PFS) (p=0.054). Kaplan Meier curves show that **Hot tumors** have the best long term PFS, and **Parenchyma Hot** tumors have intermediate prognosis.
All **Cold Tumor** patients progress before 10 months of treatment (the majority before 5 months). **Stromal TILs** tumors PFS seems as poor as Cold tumors' even though half of the responders (n=6) are classified in this group. This suggests that TILs infiltration in the stroma could be equivalent to No infiltration in terms of PFS, and that ORR is not associated to survival at least in this subtype.

Spatial TILs subtypes may improve NSCLC patients' stratification regarding anti-PD1/L1 therapy response and progression-free survival.

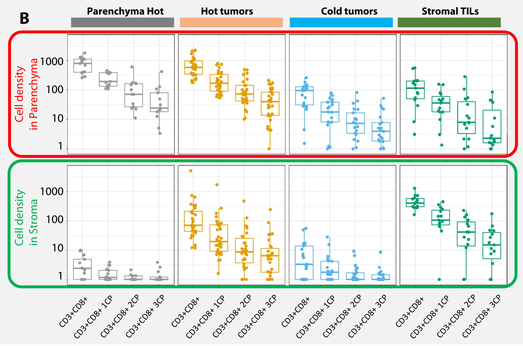
4. Checkpoints expression across progression status and Spatial TILs Subtypes

A

Parenchyma TILs populations	No progression n=39	Progression n=39	p-value
CD3+CD8+TIM3+	57	10	0.00084***
CD3+CD8+PD1+ or LAG3+	74	33	0.002**
CD3+CD8+ Activated (1CP)	132	52	0.002**
CD3+CD8+PD1+ TIM3+	32	5	0.002**
CD3+CD8+ 2 CP	68	12	0.002**
CD3+CD8+ CP-	158	67	0.005**
CD3+CD8+PD1+	54	14	0.008**
CD3+CD8+LAG3+ TIM3+	7	1	0.027*
CD3+CD8+ Exhausted	21	5	0.035*

A – Across 78 patients, only parenchyma compartment shows significant TILs differences regarding progression: TILs are enriched in non-progressive patients.
PD1 expression on TILs seems important to discriminate progressive vs non-progressive patients, as well as TIM3 and LAG3 suggesting the activation status is important.

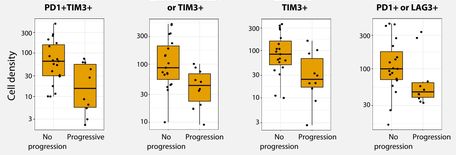
B – Distribution of TILs activation status across subtypes: boxplot representation of CD3+CD8+ cells expressing 1CP (activated enriched), 2CP (intermediate) and 3CP (exhausted) in parenchyma (red) and in stroma (green).
No significant difference between subtypes is observed in terms of TILs activation landscape across Spatial TILs subtypes.



5. Progression in Hot subtype is associated to lower checkpoints' expression

In Hot subtype (n=29), Wilcoxon test shows that activated TILs (1CP or 2CP), are enriched in parenchyma of patients that do not progress before 3 months. The four significant TILs populations are detailed in A and B.

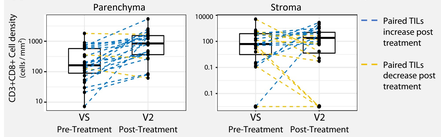
Hot Tumors Parenchyma TILs populations	No progression n=19 Median	Progression n=10 Median	p-value
CD3+CD8+ PD1+TIM3+	65	18	0.012*
CD3+CD8+ LAG3+ or TIM3+ (Activated)	87	43	0.018*
CD3+CD8+ TIM3+ (Activated)	84	25	0.024*
CD3+CD8+ PD1+ or LAG3+ (Activated)	100	47	0.05*



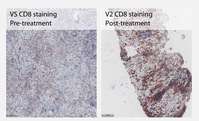
PD1, LAG3 & TIM3 Checkpoints expression assessment in Parenchyma may improve patients' stratification to further enrich eligible patients population to anti-PD1/L1 therapy.

6. Post-treatment induction of stromal infiltration can predict PFS

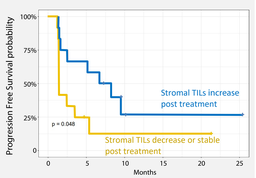
A CD3+CD8+ cell density evolution before and 6 weeks after anti-PD1/L1 therapy initiation



A – Boxplots representation of CD3+CD8+ cell densities in 24 pre-treatment (V1) and paired 6-weeks post-treatment (V2) tumor samples reveal significant enrichment of TILs in Parenchyma (left boxplot, p<0.0001) but not in Stroma (right boxplot, NS). CD8 IHC staining shows TILs enrichment in both Parenchyma and Stroma.



B – Kaplan Meier curves and log-rank test show that Stromal TILs increase after treatment (n=12) is associated to better PFS than Stromal TILs decrease (n=12), suggesting that TILs recruitment in the stroma, associated to TILs recruitment in Parenchyma in this cohort, involves efficient T-cells.



Anti-PD1/L1 therapy induces TILs recruitment in the parenchyma. Post-treatment recruitment in Stroma is associated to longer PFS.

Conclusion

Brightplex® T-Cells Exhaustion assay could enrich NSCLC patients' population eligible to anti-PD1/L1 therapy through the stratification into four Spatial TILs subtypes:

- Cold, 100% of patients progress within 10 months despite anti-PD1/L1 treatment
- Stroma-infiltrated, enriched in ORR but with short time to progression
- Parenchyma Hot subtype, with intermediate time to progression
- Hot subtype, enriched in ORR, with long time to progression for more than 40% of patients (>10 months), whatever the PDL1 status

In the Hot subtype, activated T-cell densities are higher in tumors of patients with longer time to progression, suggesting stratification could be even more accurate integrating checkpoints expression such as PD1, LAG3 and TIM3.

Finally, whatever the subtype, anti-PD1/L1 therapy seems to recruit T-cells in the parenchyma, but not as systematically in the Stroma. Tumors with such a Stromal T-cell recruitment present longer time to progression.

These preliminary results based on a first set of patients are under validation on the next 100 patients.

Reference

1 - Nabet et al., 2020, Cell 183, 363–37

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