PD-1 and LAG-3 synergize to drive tumor-infiltration of T cytotoxic cells in NSCLC tumors

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Background and Results

Background: Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancer cases, and is characterized by a poor response to chemotherapy and a low survival rate. Treatment targeting the immune checkpoint inhibitor pathway PD-1/PD-L1 has been found to be effective against NSCLC with manageable side effects, but with only 20-25% of patients showing a positive response there is an urgent need for additional immunotherapy options for this group of patients. Lymphocyte Activation Gene-3 (LAG-3) is upregulated on activated T cells, and is often found co-expressed with PD-1 leading to immune exhaustion and tumor growth. Co-blockade of the LAG-3 and PD-1 pathways has been shown to synergize to improve T cytotoxic cell responses, and several LAG-3 modulating agents (BMS 986016, LAG525, MK-4280, REGN3767) have entered the clinic where they are currently being tested alone or in combination with PD-1/PD-L1 pathway inhibition.

Methods: In order to perform a comprehensive immunoprofiling of NSCLC tumors we used MultiOmyx™, an immunofluorescence (IF) multiplexing assay that utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Using a 16-marker panel we have analyzed the proportion of B cells, T cell subtypes, tumor-associated macrophages, as well as the expression of PD-1, LAG-3, and TIM-3 in 19 samples from patients with NSCLC.

Results: Both PD-1 and LAG-3 were found to be expressed mainly on T cells, with the proportion on T cytotoxic cells relative to T helper cells increasing inside the tumor area. When analyzing the proportion of T cytotoxic cells infiltrating into the tumor area, we observed an apparent synergy between LAG-3 and PD-1 co-expression driving this infiltration, suggesting a therapeutic benefit of dual checkpoint blockade of LAG-3 and PD-1 in NSCLC.

Multiplexing Setup - MultiOmyx™

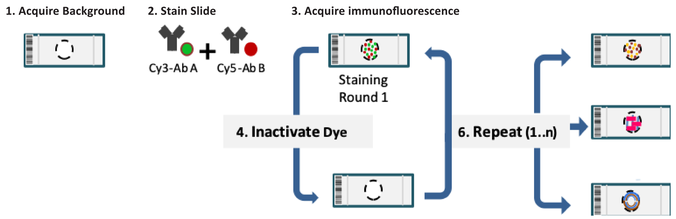

Figure 1. MultiOmyx Assay Workflow. Slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. A. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies.

16-Marker Panel		
Round	**Cy3**	**Cy5**
1	CD15	PD-L1
2	PanCK	ICOS
3	CD11b	LAG-3
4	CD3	OX40
5	CD8	TIM-3
6	CD4	FoxP3
7	CD20	CD163
8	CD68	PD-1

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining.

Nomenclature	Tumor Tissue Phenotype
T cells	CD3+
T helper cells	CD3+CD4+
T regulatory cells	CD3+CD4+FoxP3+
T cytotoxic cells	CD3+CD8+
B cells	CD20+
TAMs	CD68+
M2 TAMs	CD68+CD163+
Myeloid cells	CD11b+
Granulocytes	CD11b+CD15+
PanCK	Tumor cells

Table 2. Phenotyping of human tumor-associated lymphocytes and myeloid cells. Cell surface markers associated with cell subsets analyzed in the tumor samples. TAM: tumor-associated macrophage. PanCK: pan cytokeratin.

MultiOmyx™ Deep Learning Cell Classification

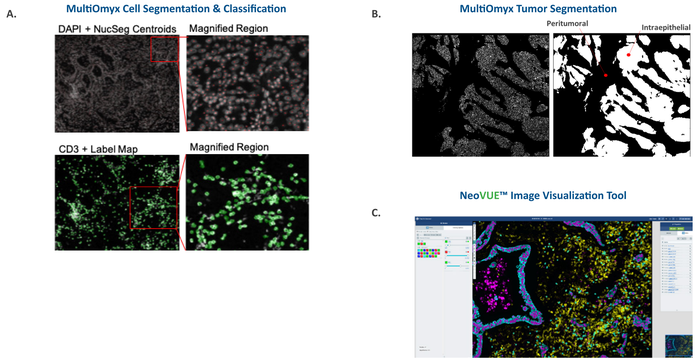

Figure 2. MultiOmyx Analytics Workflow. A. Proprietary cell segmentation algorithms generate unique IDs for every cell allowing them to be tracked through multiple rounds of staining. Deep learning based cell classification algorithms identify positive cells for each biomarker which are visualized via label masks shown here for CD3. B. A tumor marker such as pan-cytokeratin is used to generate a tumor mask in order to classify all cells as intraepithelial or peritumoral. C. The image and data visualization software NeoVUE was used to create all overlay images for this study.

PD-1 & LAG-3 T Cell Expression – In Stroma vs Tumor Area

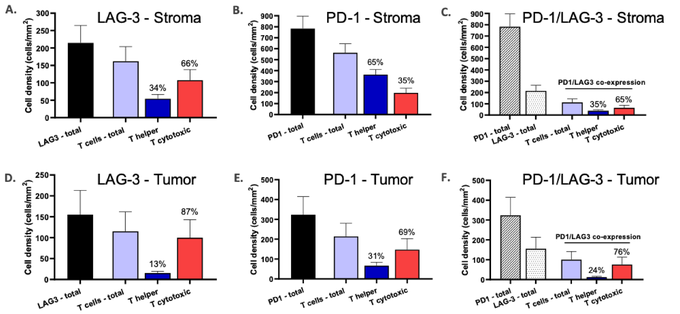

Figure 3. Immuno-profiling of FFPE NSCLC samples using MultiOmyx. Densities of all 16 markers were calculated following MultiOmyx multiplex staining protocol, using deep learning based cell classification algorithms. A+B+C. Bar graphs showing peritumoral/stromal densities of LAG-3 and PD-1. In the stromal region the density of PD-1 is approximately 4-fold higher than LAG-3. D+E+F. Bar graphs showing intraepithelial densities of LAG-3 and PD-1. Inside the tumor area the density of PD-1 is only 2-fold higher than that of LAG-3.

LAG-3 & PD-1 Immune Cell Expression

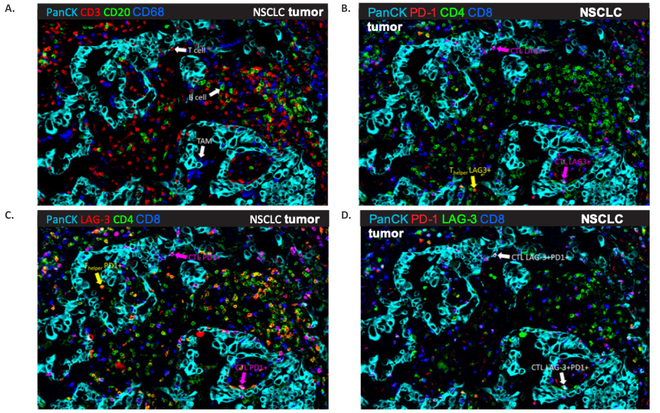

Figure 4. Multiplexed overlaid images of FFPE NSCLC samples of PanCK, CD3, CD20, CD68, CD4, CD8. **A. T cells** (CD3+) are red, **B cells** (CD20+) are green, and **TAMs** (CD68+) are blue. **B+C.** Yellow arrows indicate LAG-3 or PD-1 expressed on **T helper** cells, while magenta arrows indicate LAG-3 or PD-1 expressed on **CTLs**. **D.** CTLs co-expressing LAG-3 and PD-1 are indicated by white arrows.

LAG-3 Synergizes with PD-1 in Increasing Tumor-Infiltration of T Cytotoxic Cells

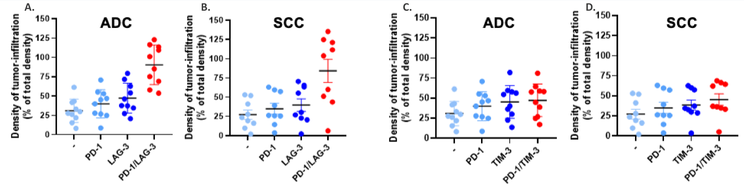

Figure 5. Tumor infiltration analysis using tumor masks generated as described in figure 2C, classifying all T cytotoxic cells as either peritumoral (non-infiltrating) or intra-epithelial (infiltrating). **A.** More T cytotoxic cells infiltrate the tumor are when co-expressing LAG-3 and PD-1, than cells expressing LAG-3 or PD-1 alone, in both adenocarcinoma (ADCD) samples, and in SCC samples. **B.** As a comparison, this apparent synergy is not observed for TIM-3 and PD-1 co-expression.

Conclusion

In this study, utilizing MultiOmyx™ technology, a platform offered exclusively by NeoGenomics Laboratories, protein expression in 19 NSCLC patients were analyzed for analysis of PD-1 and LAG-3 expression in the tumor microenvironment.

- Both PD-1 and LAG-3 were found primarily expressed on T cells. While LAG-3 was found mainly on CTLs in both the stroma and inside the tumor area, PD-1 was found mainly on T helper cells in the stroma but on CTLs inside the tumor area.
- When analyzing T cytotoxic tumor-infiltration we found that cells co-expressing LAG-3 and PD-1 infiltrated the tumor area more so than cells expressing LAG-3 or PD-1 alone. This apparent synergy suggests a potential therapeutic benefit of dual checkpoint blockade of LAG-3 and PD-1 in NSCLC.