Using a Multiplexed Immunofluorescence Assay to Detect Immunosuppressive Cells and their Mechanisms in the Pancreatic Tumor Microenvironment

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Background and Results

Background: Pancreatic ductal adenocarcinoma (PDAC) is characterized by an excessive amount of desmoplastic stroma seeded with inflammatory cells and it is one of the most aggressive forms of cancer with no current specific therapies. Myeloid cells are the primary recruited effector cells during inflammation. A subset of these, consisting primarily of tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs), accumulate in tumors where they establish an inflammatory tumor microenvironment (TME) that is favorable for tumor progression. TAMs can be described as classically activated M1 types with pro-inflammatory antitumor functions, versus alternatively activated M2 types with immunosuppressive pro-tumor functions. The immunosuppressive functions of M2 TAMs can be exerted through release of cytokines and growth factors as well as via direct recruitment of T regulatory cells (Tregs), a subset of lymphocytes responsible for immune tolerance of the system to the tumor. While the differentiation from M1 to M2 in PDAC has been shown to be associated with a worse prognosis, not much is known about PDAC TAM polarization and its potential correlation to Treg recruitment.

Methods: We have used MultiOmyx, a proprietary technology that enables visualization and characterization of multiple biomarkers **on a single 4µm tissue section**. MultiOmyx protein immunofluorescence (IF) assays utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Each round of staining is imaged and followed by novel dye inactivation chemistry, enabling repeated rounds of staining and deactivation for up to 60 protein biomarkers.

Results: Using a panel of 11 antibody markers to analyze 8 stage IIB PDAC FFPE tumor-samples, we were able to identify and quantify subsets of tumor-infiltrating lymphocytes and myeloid cell subsets. Using the pan macrophage marker CD68 in combination with either M1 marker HLA-DR or M2 marker CD163 we confirmed the presence of M1 (CD68+HLA-DR+) and M2 (CD68+CD163+) populations, the vast majority being of the M2 subtype. Moreover, we found a positive significant correlation (Pearson's correlation $p<0.05$) between the presence of M2 TAMs and Tregs (CD3+CD4+FoxP3+), but not between M1 TAMs and Tregs. Using our proprietary algorithms that takes into account the staining pattern for each specific biomarker, we also examined the spatial relationship between the M1/M2 subtypes of TAMs and Tregs and found M2 TAMs to be in closer proximity to Tregulatory cells than M1 TAMs.

Overview of Assay Workflow

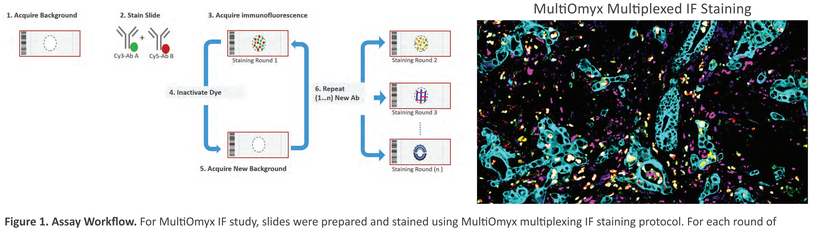

Figure 1. Assay Workflow. For MultiOmyx IF study, slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling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

Immune Marker Panel		
Round	**Cy3**	**Cy5**
1	**CD15**	**CD163**
2	**CD11b**	**CD68**
3	**CD14**	**FoxP3**
4	**PanCK**	PD-L1
5	**CD3**	PD-1
6	**CD4**	Arginase1
7	**CD8**	CD16
8	GranzymeB	Ki67
9	**HLA-DR**	CD33

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining. Markers used for this study are highlighted in bold.

Nomenclature	Tumor Tissue Phenotype
Myeloid cells	CD11b+
T helper cells	CD3+CD4+
T regulatory cells	CD3+CD4+FoxP3+
M1 TAMs	CD68+HLA-DR+
M2 TAMs	CD68+CD163+
M-MDSC	CD11b+CD14+CD15-HLA-DR-
G-MDSC	CD11b+CD15+CD14-HLA-DR-

Table 2. Phenotyping of human tumor-associated lymphocytes and myeloid cells. Cell surface markers associated with cell subsets analyzed in the PDAC samples. TAM: tumor-associated macrophage; MDSC: myeloid-derived suppressor cell; M-: monocytic; G-: granulocytic.

Localization of Immunosuppressive Cells in the Tumor Microenvironment

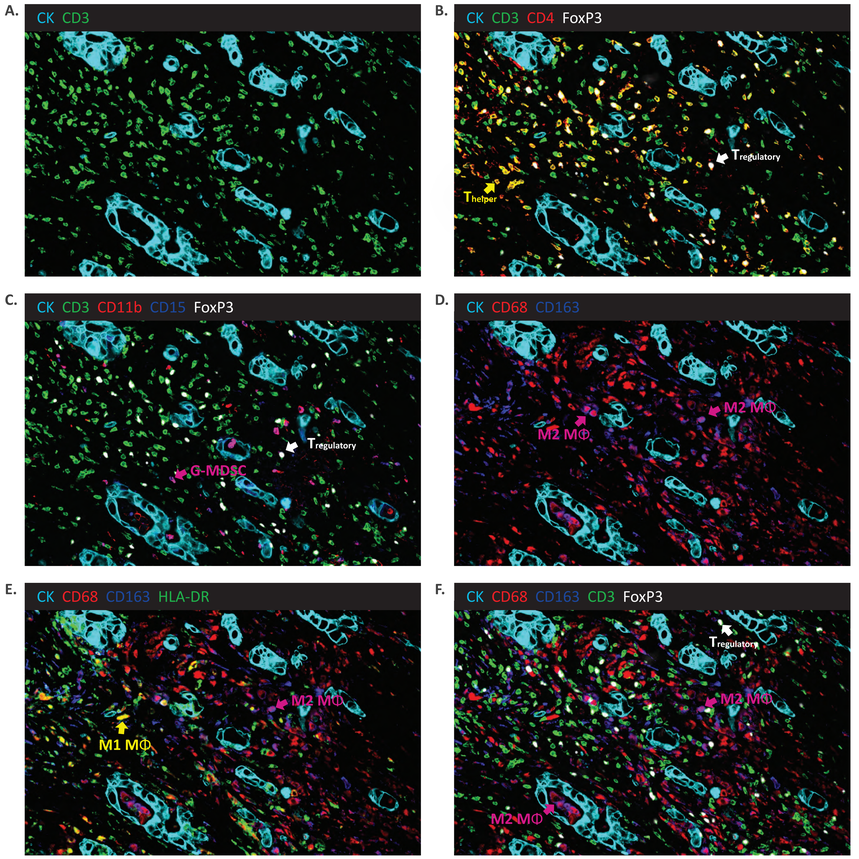

Figure 2. Immuno-profiling of an FFPE PDAC tumor using MultiOmyx multiplexed IF assay. Representative color overlaid images of PanCK, CD3, CD4, FoxP3, CD11b, CD14, CD15, CD68, HLA-DR, and CD163. **A,** **T cells** (CD3+) are green. **B,** **T helper cells** (CD3+CD4+) are yellow and T regulatory cells (CD3+CD4+FoxP3+) are white. **C,** Magenta cells are **G-MDSC** cells (CD11b+CD15+CD14-), and red cells are myeloid cells not expressing the granulocytic marker CD15. **D,** **Tumor-associated macrophages (TAMs)** not expressing the M2 marker CD163 are red, while **M2 type TAMs** (CD68+CD163+) are magenta. **E,** **M1 type TAMs** (CD68+HLA-DR+CD163-) are yellow, while **M2 type TAMs** CD68+CD163+HLA-DR- are magenta. F, T regulatory cells (CD3+CD4+FoxP3+) are white and **M2 type TAMs** (CD68+CD163+) are magenta. Green cells are non-regulatory T (CD3+FoxP3-), while red cells are TAMs that are negative for the M2 marker CD163.

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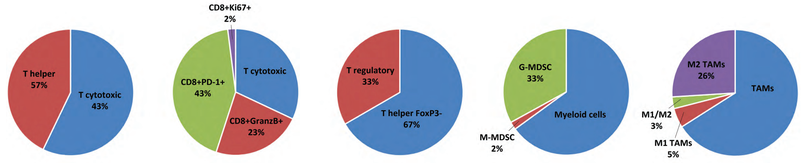
T Cell, MDSC, and TAM Distributions



Correlations to T Regulatory Cells

Patient #	Myeloid cells (CD11b)	G-MDSCs (CD11b+CD15+CD14-)	M1 TAMs (CD68+HLA-DR+)	M2 TAMs (CD68+CD163+)	T regs (CD3+CD4+FOXP3+)
1	187	11	31	36	20
2	<10	1	33	47	69
3	68	27	13	41	17
4	114	11	22	131	106
5	134	36	<10	<10	<10
6	35	3	<10	48	42
7	98	18	<10	51	46
8	288	31	<10	52	41
Pearson (R)	-0.21	-.58	0.41	**0.91**	
p value	0.612	0.136	0.313	**0.002**	

Table 3. Data shown are mean cell number per ROI. There is a significant positive correlation between the presence of M2 TAMs and the presence of T regulatory cells in the tumor microenvironment of these 8 PDAC patients.

MultiOmyx Spatial Analytics – Nearest Neighbor Analysis

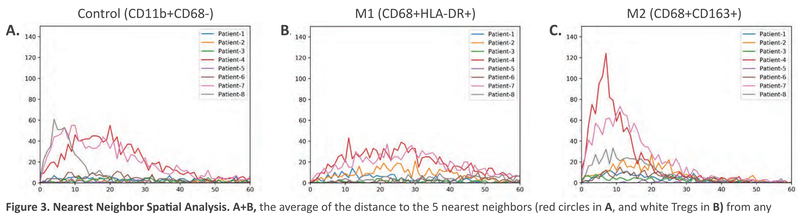


Figure 3. Nearest Neighbor Spatial Analysis. A+B, the average of the distance to the 5 nearest neighbors (red circles in **A**, and white Tregs in **B)** from any given phenotype is calculated. **C-F**, graph depicts the average distance against number of cells for non-TAM myeloid cells **(D)**, M1 type TAMs **(E)**, and M2 type TAMs **(F)** respectively. The M2 TAMs were found to be in closer proximity to Tregs compared to both the control cell population and M1 TAMs.

Conclusion

In this study, utilizing MultiOmyx technology, a platform offered exclusively by NeoGenomics Laboratories, protein expression in 8 patients with pancreatic ductal adenocarcinomas were analyzed for possible correlations between subtypes of myeloid cells and immune-suppressive T regulatory cells present in the tumor microenvironment.

- Utilizing a panel of 11 antibody markers, we quantified the number of myeloid cells, G-MDSCs, T regulatory cells and TAMs.
- We were able to distinguish between M1 subtypes of TAMs (CD68+CD163-) and M2 subtypes of TAMS (CD68+CD163+), the majority of which were found to be of the M2 subtype.
- We demonstrate a positive significant correlation between the presence of M2 TAMs and Tregs in the TME of PDAC, suggesting a possible pathway in which TAM polarization plays an immunosuppressive function by recruiting Tregs.
- When performing a MultiOmyx spatial "Nearest Neighbor Analysis", M2 TAMs were found to be in closer proximity to Tregs compared to both the control cell population and M1 TAMs.