Spatial resolution of the tumor and immune cell lineages in the hypoxic microenvironment of pancreatic ductal adenocarcinoma

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Introduction & Aim

Understanding how the tumor microenvironment (TME) evolves during tumorigenesis and therapeutic response is crucial to developing personalized treatments with the goal of improving cancer therapy. With robust and comprehensive multiplexed imaging technologies, immune biomarker antibodies can be used to interrogate immune cell lineages and structures. When combined with specific oncology biomarker antibodies, this approach can capture the immune response within the TME in a variety of neoplasms. The Cell DIVE™ Multiplex Imaging Solution allows probing and imaging of dozens of biomarkers on a whole single tissue section using an iterative staining and dye inactivation workflow. The broad portfolio of robust IHC-validated antibodies from Cell Signaling Technology (CST) enables the detection of key proteins in the TME, allowing immune cell detection and phenotyping in tissue.

Here, we demonstrate multiplexed Cell DIVE imaging using a novel CST panel to probe pancreatic ductal adenocarcinoma (PDAC). These biomarker antibodies define the immune cell landscape in the hypoxic tumor. Development of the antibody panel required minimal optimization, enabled the identification of complex cell types and revealed their cell-to-cell interactions within the tumor microenvironment. The availability of cell type specific biomarkers, combined with the ability to interrogate using multiplexed tissue imaging, provides unprecedented and novel insights and spatial resolution of immune cell populations with many cell types in the TME.

Results

Characterizing the tumor microenvironment can help to elucidate new mechanisms that contribute to poor patient outcomes. The tumor microenvironment is complex and commonly heterogenous both within a single sample and across patient samples. Iterative multiplexed staining and imaging enables TME interrogation across tissue samples even in cases where tissue availability is limited. In this study, we have examined the expression of 36 biomarker antibodies in PDAC (Table 1), with a focus on potential immunotherapy targets, predictive biomarkers and segmentation markers. Following 15 rounds of staining, there was negligable cell loss or movement, even in highly necrotic regions of the tumor (Fig. 1). Additionally, staining 6 months after initial staining resulted in similar levels of biomarker staining of fresh tissue in antibody validation studies (Fig. 1D-E, data not shown).

The markers used in this study help define immune cell types and subtypes in the tumor microenvironment (Fig. 2A). The hypoxia marker Glut1, Beta-Catenin and Pan-cytokeratin biomarkers enable identification of tumor regions with increased glucose uptake and hypoxia, a cancer hallmark, to be studied separately from normoxic tumor and normal pancreas (Fig. 2B). Following cell segmentation, these regions were classified using mean intensity of biomarker expression (Fig. 2C).

Cells were analyzed using cluster analysis and dimensional reduction (PaCMAP). Cluster analysis provides a non-biased approach to define the immune cell contribution within the aggressive tumor regions compared to less aggressive regions. PMN MDSC's inhibit immune responses, including those mediated by T cells, B cells and NK cells. Here, cluster resolution in the hypoxic tumor enables the identification of heterogeneous PMN MDSC's, with variable expression of CD79 and PD1 in regions of normoxic regions are less heterogeneous. These biomarkers have been previously defined in PMN MDSCs, where CD79a may be important for PMN MDSCs cell activation, and PD-1 may affect progenitor cell fate. Finally, these PMN MDSC's are spatially randomly distributed (Fig. 3C), possibly due to a general lack of tumor tissue architecture seen in the normoxic tumor region. Further study of these PMN MDSC's may lead to the development of novel approaches to improve immuno-therapeutic response in PDAC.

Methods & Materials

CST antibodies undergo a vigorous validation process to ensure antibody performance on FFPE tissue. All antibodies in this study were direct conjugates or off the shelf (OTS) commercial conjugates (Table 1). Following preliminary validation, conjugated antibody solutions with the optimum degree of labeling and concentration were randomly assigned to a round, without optimization and used for subsequent stainings of PDAC tissue. Tissue was obtained from a commercial source (Pantomics; Table 1). Slides were imaged on the Cell DIVE imager using four channels plus DAPI, with automatic AF removal, corrections and stitching. Imaging rounds were conducted over a 3 week period, and at round 14, slides were stored for 6 months and stained and imaged with additional recently validated antibodies. Fully stitched images were imported, fused, and analyzed using proprietary software developed by Leica Microsystems. Cells were first segmented, then classified into regions, gated for CD45 positivity then phenotyped and clustered accordingly.

Conclusions

- Iterative staining and imaging of PDAC with 36 CST biomarker antibodies using the Cell DIVE multiplexed imaging solution enabled immune cell type characterization within the TME.

- Cell DIVE multiplexing solution is tissue preserving, enabling further probing to define immune cell subtypes using additional CST antibodies on the same tissue sections months later. Data is automatically overlayed with all previous biomarkers from the study.

- In the more aggressive tumor regions, heterogenous PMN MDSCs express biomarkers that may indicate changes in cell activation and cell fate.

Table 1. Study Design: Antibodies and Tissues

CAT#	Antibody	Clone	Conjugate
*	SMA	D4K9N	AF_488
*	SMA	1A4	AF_488
*	SOX9	D8G8H	AF_488
*	NAKATPASE	D4Y7E	AF_488
*	CD79A	D1X5C	AF_555
*	CD3	D7A6E™	AF_555
*	CD20	E7B7T	AF_647
*	CD163	D6U1J	AF_647
*	KI-67	D2H10	AF_647
*	CD8	D8A8Y	AF_750
*	CD45	D9M8I	AF_750
*	PD-1	D4W2J	AF_750
*	VIM	D21H3	AF_750
*	FOXP3	D2W8E™	AF_647
*	CD11C	D3V1E	AF_647
*	CD4	D7D2Z	AF_555
*	CD56	E7X9M	AF_647
*	PDL-1	E1L3N	AF_647
*	CTLA-4	E2V1Z	AF_647
*	CD11B	D6X1N	AF_750
*	Granzyme B	D6E9W	AF_555
*	COL1A1	E8F4L	AF_488
*	GLUT1	E4S6I	AF_555
*	HLA-DRA	E9R2Q	AF_647
*	CD15	MC480	AF_750
*	PCK MIX	E6S1S/C11	AF_488
*	CD14	D7A2T	AF_555
*	BCAT	D10A8	AF_750
78226	TIM-3	D5D5R	AF_647
61255	CD31	89C2	AF_555
5108	EGFR	D38B1	AF_555
7497	pNDRG1	D98G11	AF_647
3906	GAPDH	14c10	AF_488
2866	SURVIVIN	71G4B7	AF_647
23308	CD68	D4B9C	AF_555

Slide	Tissue	Catalog
1333	Pancreatic ductal adenocarcinoma (PDAC)	PAC02

*Custom conjugations provided by CST

Figure 1.

Figure 1: Cell DIVE enables multiplexed imaging of CST biomarker panels over months of imaging and storage. Pancreatic ductal adeno-carcinoma was probed with biomarkers over 15 rounds of imaging conducted over an 8 month period. (A) Cell loss or movement was negligable (1B-C) when comparing DAPI images from round 1 and Round 15, even in necrotic tumor regions (1C). Slides were stored for 6 months between rounds 13 and 14, without antigen effects (1D-E), where CD45 positive cells from round 1 are easily pheno-typed as CD15 positive (Round 14) or CD14 positive (1D, Round 15).
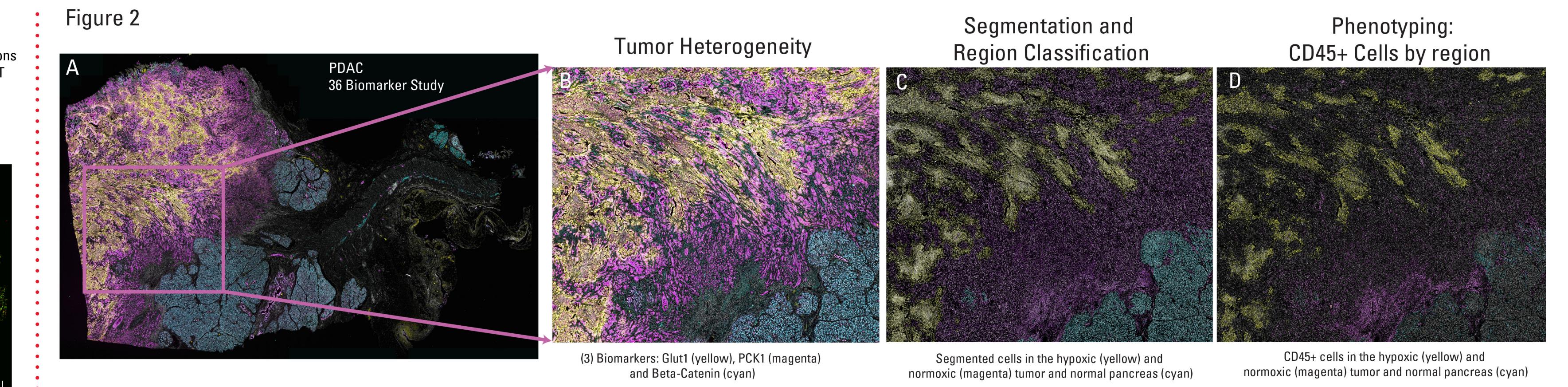
Figure 2

Figure 2: PDAC Multiplexed imaging of CST panels enables an examination of immune cell components in pancreatic ductal adenocarcinoma (PDAC) tissue on a single slide. (Fig.2A). Slides were iteratively stained and imaged with multiple biomarkers (Table 1; Fig. 2A-B panels). Figure 2C. For segmentation: AI-DapI (nuclei) and markers (membrane) using a subset of the 36 biomarkers for membrane segmentationNAK, EGFR, PanCK-AE1, GLUT1, BCAT, VIM plus immune markers (CD45, CD31, CD11B, CD3E, CD8, CD11C, CD15, CD14). For classification: Machine learning and biomarker expression GAPDH, SMA, NAK, KI67 PanCK-AE1, PNDRG1, COL1A1, GLUT1, HLADRA, BCAT, VIM, enabled cell phenotyping by hypoxic and normoxic tumor and pancreas regions. Cells were gated based on CD45 expression (Fig. 2D).
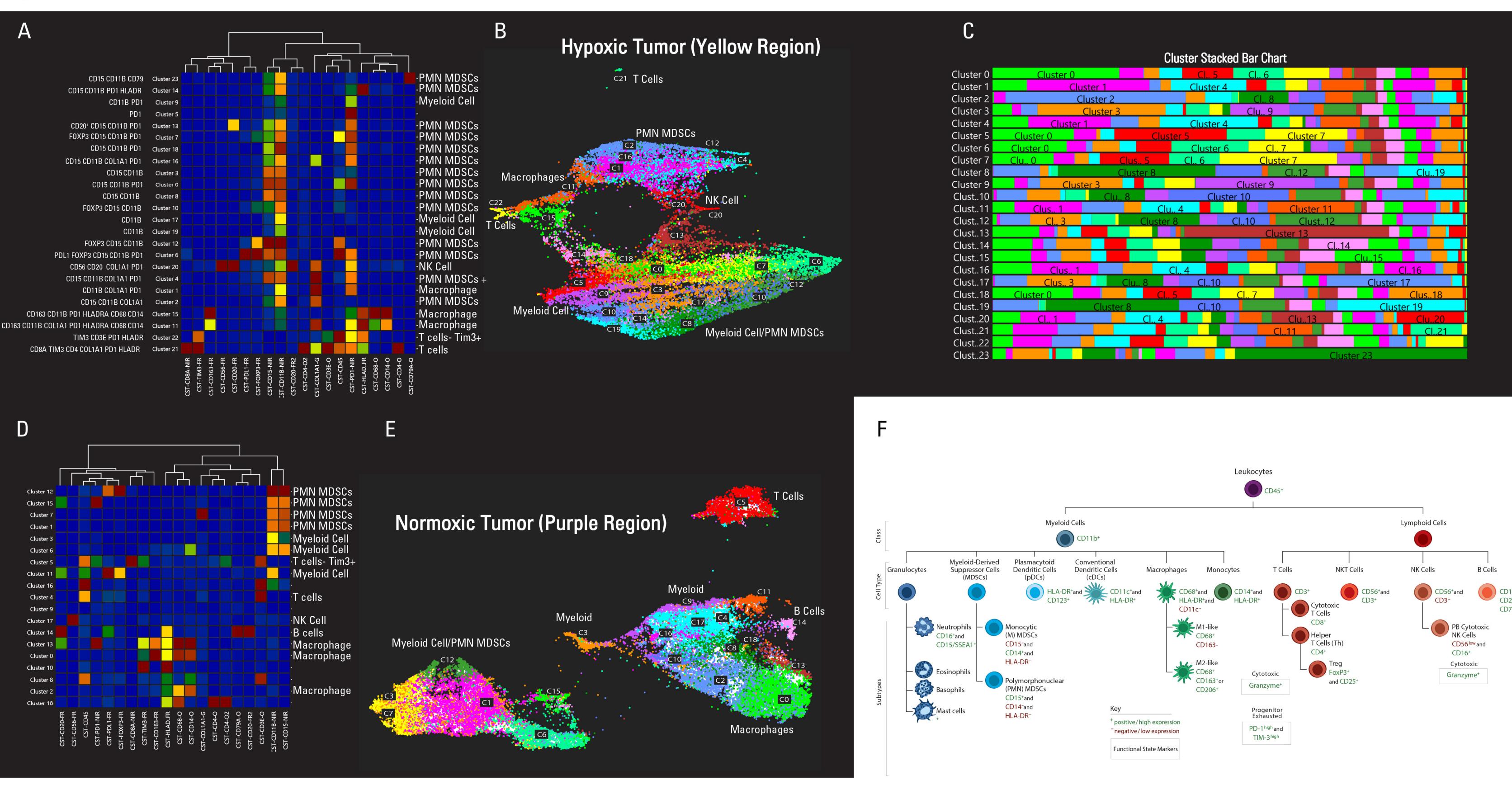
Figure 3.

Figure 3: Hypoxic highly metabolic regions of PDAC compared to normoxic tumor regions. CD45 gated cells were separately clustered by region. Hypoxic regions have abundant and heterogeneous PMN MDSCs (Fig. 3A-C), compared to normoxic tumor (Fig. 3D-E). Characterization of these cells in this aggressive region could illuminate potential targets for immunotherapy. Illustration (Fig. 3F) reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

Questions?
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