Using Multiplexed Immunofluorescence to Quantitatively Analyze MDSCs in Relation to Tertiary Lymphoid Structures (TLS) in Bladder Cancer

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Background and Results

Background: An intriguing phenomenon in many solid tumors is the de novo lymphoid neogenesis of tertiary lymphoid structures (TLS). However, it remains unclear whether spontaneous TLS restricts or promotes tumor progression and more studies are required to define the role of TLS. One interesting question is the pathological and functional connection of myeloid-derived suppressor cells (MDSCs), known inhibitors of T cell-mediated anti-tumor immunity, and TLS.

Methods: To perform a spatial analysis of MDSCs and other immune cell phenotypes in the bladder tumor microenvironment we used MultiOmyx™, a multiplexed immunofluorescence (IF) assay utilizing a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Using a 14-marker panel and proprietary cell segmentation and classification algorithms developed at NeoGenomics, we have analyzed the presence of TLS (positive for CD20, CD3, and PNAd), followed by a spatial analysis of MDSCs, T cell subtypes, and M1/M2-type tumor-associated macrophages (TAMs) in relation to the TLS in 25 FFPE samples from patients with bladder cancer.

Results: As expected we found a significantly higher density of B cells and T cells present inside the TLS compared to either near or far from the TLS, while the density of tumor associated macrophages (TAMs), which are not associated with TLS, was the same in all regions. To test our initial hypothesis that MDSCs repel TLS in bladder cancer, we quantitated the presence of MDSCs and found a linear decrease related to their proximity to the TLS, with a 33% lower density in the region near the TLS, and a 48% decrease in the regions far from the TLS. This pattern was consistent for both M-MDSCs and G-MDSCs, as well as for MDSCs expressing CXCR2, a chemokine receptor which has been shown to regulate the migration of MDSCs.

Conclusion: The finding of the co-abundance of MDSCs and T/B cells following the distance from TLS suggests that the TLS may attract MDSCs through cell-cell interaction, a finding with potential important implications for bladder cancer immunotherapy.

TLS and Immune Cell Proximity Workflow – Step 1

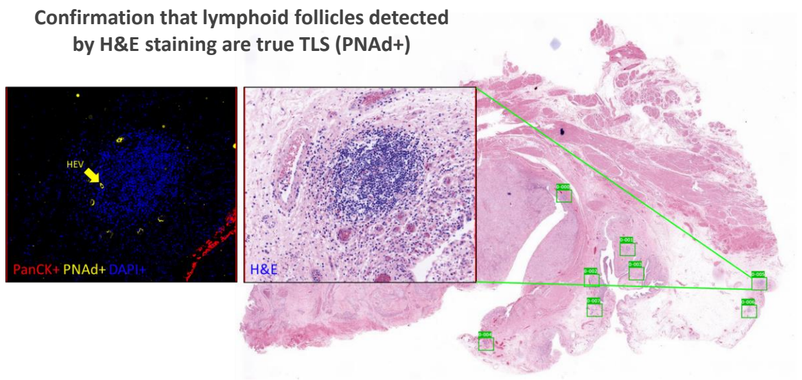


Figure 1. Assay Workflow Step 1. Potential TLS were selected as regions of interest (ROIs) from an H&E stain. An adjacent FFPE section was stained with a multiplex panel consisting of DAPI, PanCK, and PNAd for confirmation that the lymphoid follicles are positive for the high endothelial venules marker peripheral node addressin (PNAd).

TLS and Immune Cell Proximity Workflow – Step 2

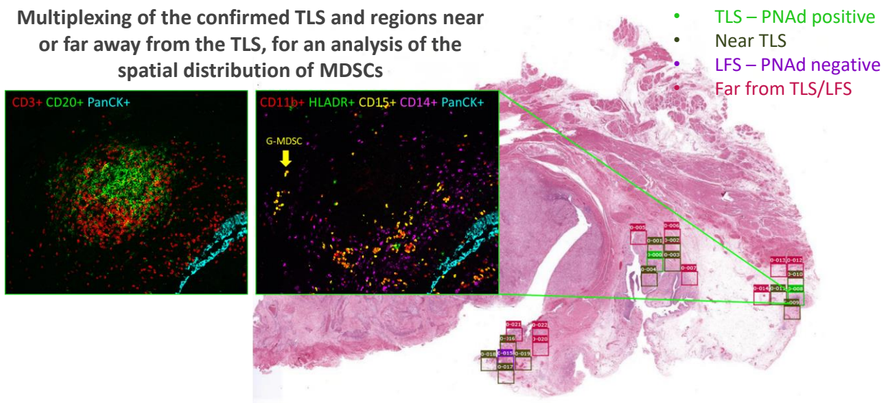
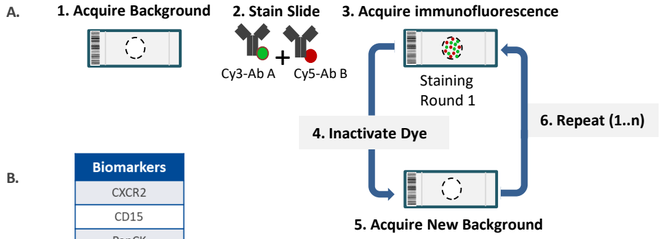


Figure 2. Assay Workflow Step 2. ROIs were selected to cover TLS identified in step 1, as well as ROIs near and far from the TLS on an adjacent FFPE section. These regions were then multiplexed with the full 14-marker panel for a detailed immunophenotyping of the TLS and the tumor microenvironment surrounding it.

MultiOmyx Multiplexing and Biomarker Panel



B.

Biomarkers
CXCR2
CD15
PanCK
CD3
CD8
CD20
CD14
CD4
PNAd
iNOS
CD11b
CD68
FoxP3
HLA-DR

Co-expression	Phenotypes
T helper cells	CD3+CD4+
T regulatory cells	CD3+CD4+FoxP3+
T cytotoxic cells	CD3+CD8+
B cells	CD3-CD20+
Antigen-presenting myeloid cells	CD11b+HLADR+
MDSC	CD11b+HLADR-
MDSC CXCR2 pos.	CD11b+HLADR-CXCR2+
MDSC iNOS pos.	CD11b+HLADR-iNOS+
M-MDSC	CD11b+CD14+CD15-HLADR-
G-MDSC	CD11b+CD15+CD14-HLADR-
TAM	CD68+
M1 TAM	CD68+HLADR+
M1 TAM	CD68+iNOS+
PanCK iNOS pos.	PanCK+iNOS+

Figure 3. A, MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to a 4 µm section, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. Proprietary cell segmentation algorithms generate unique IDs for every cell allowing them to be tracked through multiple rounds of staining. **B**, **Table 1.** 14-marker antibody panel. **Table 2**. Phenotyping of human immune and tumor cells. TAM: tumor-associated macrophage. PanCK: pan cytokeratin.

Immune Cell Spatial Proximity Analysis in Bladder Cancer TLS

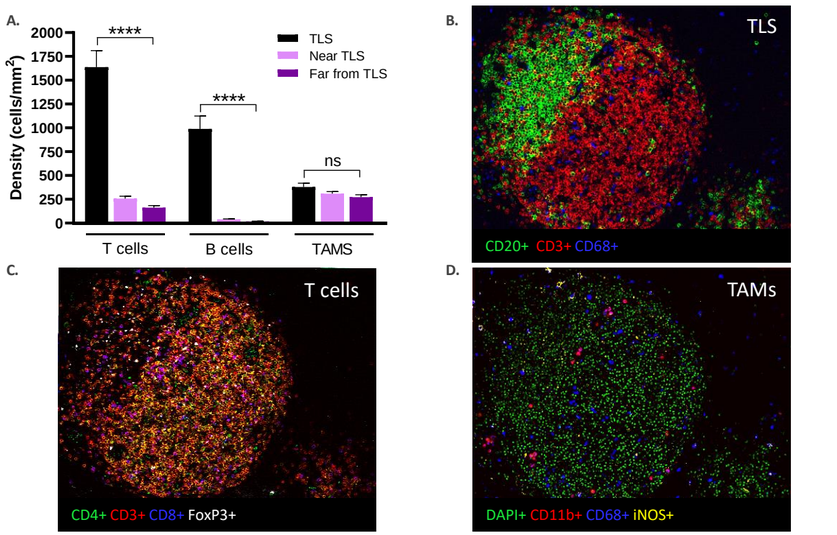


Figure 4. Spatial Analysis of TLS regions in Bladder Cancer Tumors and Multiplexed Images. **A**, Bar graph displaying densities of T cells (CD3+), B cells (CD20+) and TAMs (CD68+) in TLS, regions near to the TLS, and far from the TLS. There is a high statistically significant difference (P value<0.0001, one-way ANOVA test) in T and B cell densities between the three regions, but no difference in TAM density is observed. **B-D**, Multiplexed overlaid images of a TLS. **B**, Overlay image of CD20 (B cells), CD3 (T cells), and CD68 (TAMs). **C**, Overlay image of CD3, CD4 (T helper cells), CD8 (T cytotoxic cells), and FoxP3 (Tregs/white). **D**, Overlay image of DAPI (nuclei), CD11b (myeloid cells), CD68 (TAMs), and iNOS (M1 TAM marker).

MDSC Spatial Proximity Analysis in Bladder Cancer TLS

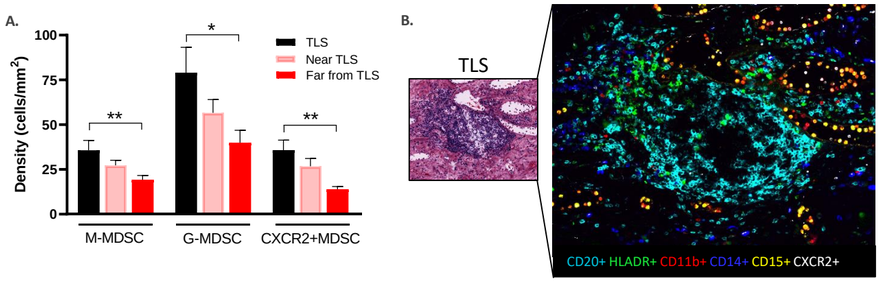


Figure 5. Spatial Analysis of TLS regions in Bladder Cancer Tumors and Multiplexed Images. **A**, Bar graph displaying densities of M-MDSCs (CD11b+HLADR-CD14+CD15-), G-MDSCs (CD11b+HLADR-CD14-CD15+), and CXCR2+ MDSCs (CD11b+HLADR-CXCR2+) in TLS, regions near to the TLS, and far from the TLS. There is a statistically significant difference (one-way ANOVA test) in all groups of MDSC densities between the three regions. **B**, Overlay image of CD20 (B cells), HLA-DR, CD11b, CD14, CD15, and CXCR2 (white) for a detection of M-MDSCs and G-MDSCs. MDSCs expressing CXCR2 can be seen in white.

Key Findings

- We found a significantly higher density of B cells and T cells present inside the TLS compared to either near or far from the TLS, but no difference in TAM density.
- When quantitating MDSCs we found a linear decrease related to their proximity to the TLS, with a 33% lower density in the region near the TLS, and a 48% decrease in the regions far from the TLS.
- This study demonstrates that blocking MDSCs may promote T cell-mediated anti-tumor immunity within the TLS, potentially leading to increased T cell proliferation and tumor-infiltration.

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