

Pro-Tumorigenic Mechanisms of M2 Tumor-Associated Macrophages in Triple-Negative Breast Cancer

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Background and Results

Background: Triple-negative breast cancers (TNBCs) are defined as tumors negative for both estrogen (ER), progesterone, and HER-2 receptors. It is a heterogeneous subtype of breast cancer with a high propensity for systemic metastases and poor survival with only chemotherapy available for treatment. Compared to other hormone-positive breast cancer subtypes, TNBC features a unique tumor microenvironment (TME) characterized by a large number of tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs). TAMs can exert their immunosuppressive functions through release of cytokines and growth factors as well as via promotion of T regulatory (Treg) cells and it has been reported that the presence of M2 TAMs positively correlates with TNBC, hormone receptor negativity, as well as higher tumor proliferation. However, the mechanisms by which TAMs interact with TNBC cells and their phenotypes in TNBC versus ER-positive patients is not well understood.

Methods: Using MultiOmyx™, a proprietary, immunofluorescence (IF) multiplexing assay that enables visualization and characterization of up to 60 biomarkers on a single FFPE section, we have characterized TIL phenotypes, tumor proliferation, and TAM activation in 15 FFPE tumors from TNBC patients and 5 tumors from ER-positive patients.

Results: Using a multiplex panel of 10 markers we found a significant increase in the number of T cells, M2 TAMs, and proliferating tumor cells in samples from TNBC patients compared to samples from ER-positive patients. Additionally, we found a positive correlation between the presence of M2 TAMs and proliferating tumor cells, and M2 TAMs and Tregs, while no positive correlation for M1 TAMs was observed. In a spatial nearest neighbor analysis M2 TAMs were found to be in closer proximity to both proliferating tumor cells and Tregs than M1 TAMs, regardless of hormone-receptor status. These data are suggestive of a possible pathway in which alternatively activated M2 TAMs in hormone receptor negative breast cancer tumors play a pro-tumorigenic role by stimulating proliferation of tumor cells, while also promoting an immunosuppressive tumor environment via promotion of Tregs.

MultiOmyx™ Hyperplexed IF Assay Workflow MultiOmyx™ Deep Learning Cell Classification

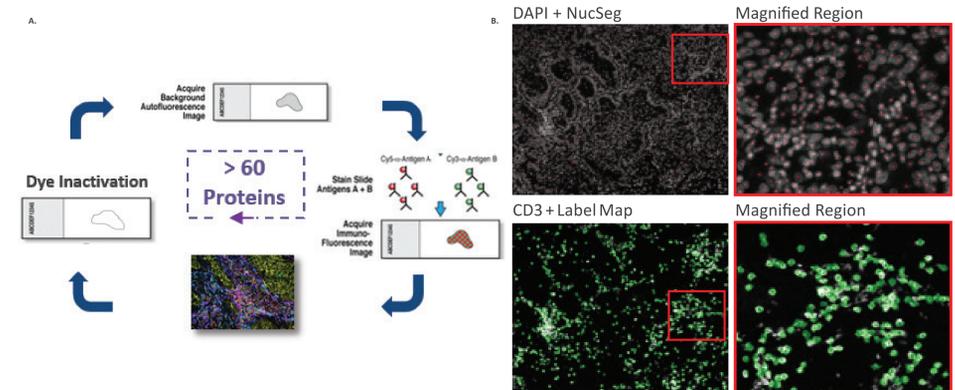


Figure 1. MultiOmyx™ Assay Workflow. Slides were prepared and stained using MultiOmyx™ multiplexing IF staining protocol. **A.** For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. **B.** 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 using via Label masks shown here for CD3.

Immune Marker Panel		
Round	Cy3	Cy5
1	CD34	Ki67
2	PanCK	HLA-DR
3	CD8	FoxP3
4	CD3	CD163
5	CD4	CD68

Table 1. Antibody Staining Sequence for MultiOmyx™ multiplexing staining.

Nomenclature	Tumor Tissue Phenotype
T helper cells	CD3+CD4+
T regulatory cells	CD3+CD4+FoxP3+
T cytotoxic cells	CD3+CD8+
TAMs	CD3-CD68+
M1 TAMs	CD3-CD68+HLA-DR+CD163-
M2 TAMs	CD3-CD68+HLA-DR-CD163+
PanCK+Ki67+	Proliferating 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.

Immune Cell Phenotypes in ER-positive versus TNBC Tumor Microenvironment

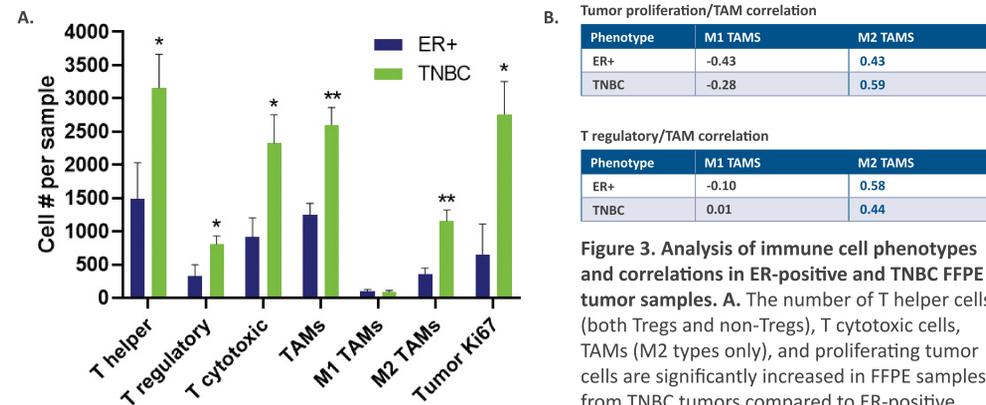


Figure 3. Analysis of immune cell phenotypes and correlations in ER-positive and TNBC FFPE tumor samples. **A.** The number of T helper cells (both Tregs and non-Tregs), T cytotoxic cells, TAMs (M2 types only), and proliferating tumor cells are significantly increased in FFPE samples from TNBC tumors compared to ER-positive tumors. **B.** In a Pearson correlation analysis, the presence of M2 TAMs in an ROI were found to be positively correlated to the presence of both proliferating tumor cells and Tregs, while no correlation (or a negative correlation) was observed for M1 TAMs.

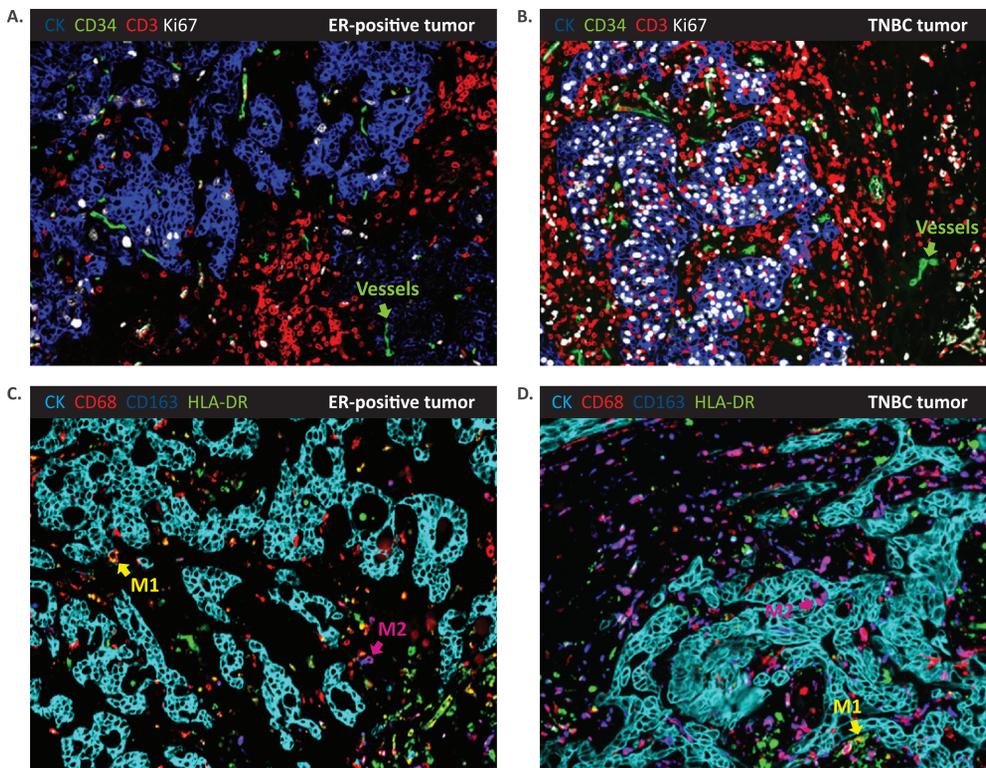


Figure 4. Immuno-profiling of FFPE breast cancer tumors using MultiOmyx. Multiplexed overlaid images of PanCK, CD34, CD3, CD68, HLA-DR, CD163, and Ki67. **A+B** (A = ER-positive tumor, and B = TNBC tumor). **T cells** (CD3+) are red, **vessels** are green (CD34+), and **proliferating tumor cells** are blue and white (CK+Ki67+). **C+D** (C = ER-positive tumor, and D = TNBC tumor). **TAMs** not expressing HLA-DR or CD163 are red, **M1 type TAMs** (CD68+HLA-DR+CD163-) are yellow, and **M2 type TAMs** (CD68+HLADR-CD163+) are magenta.

MultiOmyx™ Spatial Analytics – Nearest Neighbor Analysis

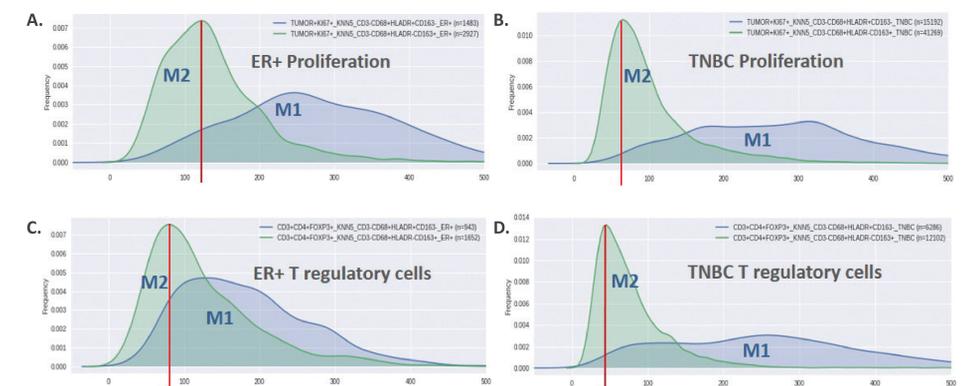


Figure 5. Nearest Neighbor Spatial Analysis. **A-D,** the average of the distance to the 5 nearest neighbors from any given phenotype is calculated. While M2 TAMs were found to be in closer proximity to proliferating tumor cells and Tregs compared to M1 TAMs in both ER-positive and TNBC tumor samples, they were found in closer proximity overall in TNBC samples compared to ER-positive samples.

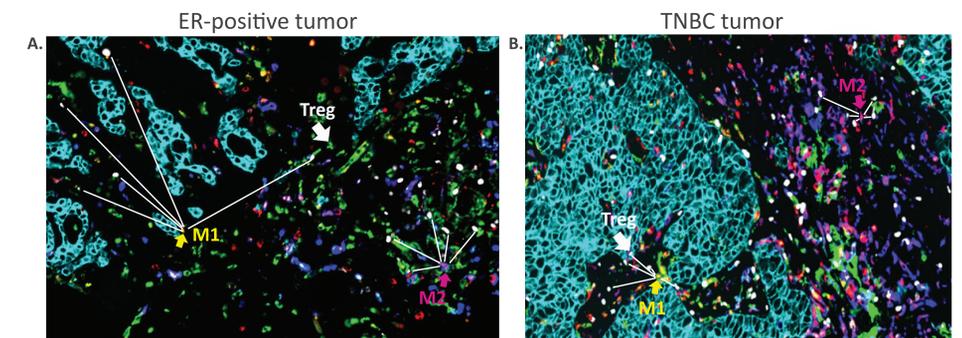


Figure 6. Multiplexed overlaid images of PanCK (cyan), CD68 (red), CD163 (blue), HLA-DR (green), and FoxP3 (white). The distance to the 5 nearest Tregs in ER-positive (A) and TNBC tumors (B) from an M1 and an M2 TAM is indicated as an example.

Conclusion

In this study, utilizing MultiOmyx™ technology, a platform offered exclusively by NeoGenomics Laboratories, protein expression in 20 patients with breast cancer were analyzed for a comparison of T cell phenotypes, TAM activation status, and tumor proliferation in ER-positive versus TNBC FFPE samples.

- Utilizing a panel of 10 antibody markers, we quantified the number of tumor-infiltrating T cells subtypes, M1/M2 TAMs, and Ki67-expressing tumor cells and found a significant increase in T helper cells (CD3+CD4+), Tregs (CD3+CD4+FoxP3+), T cytotoxic cells (CD3+CD8+), M2 TAMs (CD3-CD68+HLA-DR-CD163+), and proliferating tumor cells (PanCK+Ki67+) in TNBC tumors versus ER-positive tumors.
- We demonstrate a positive significant correlation between the presence of TAMs and Tregs, as well as between TAMs and proliferating tumor cells in the TME, for TAMs of the M2 subtype only.
- These data suggest a possible pathway in which TNBC cells induce TAM polarization toward a pro-tumorigenic M2 phenotype, thereby promoting an increase in tumor proliferation as well as indirectly creating an immuno-suppressive tumor environment via the recruitment of Tregs.