Perivascular Accumulation of Immunosuppressive Cells in the Stroma of Human Triple Negative Breast Carcinomas: Implications for Immunotherapy

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Background

- A distinct subset of tumor-associated macrophages (TAMs) accumulate around blood vessels in mouse and human tumors. In mice. They have been shown to stimulate tumor angiogenesis and various steps in the metastatic pathway.
- These perivascular (PV) cells also limit tumor responses to chemotherapy and anti-vascular agents, and dampen anti-tumor immunity by recruiting regulatory T cells (via their release of CCL17) and suppressing the proliferation of T cells[1].
- However, the presence and phenotype of such PV TAMs - and their association with other immune cells in the PV niche -- have yet to be investigated in human tumors.
- In this study we have used **Neogenomic's MultiOmyx** multiplex immunofluorescence coupled to advanced analytics to compare the distribution and phenotype of TAMs, CD4+ and CD8+ T cells, CD4+FOXP3+ regulatory T cells (Tregs) and CD56+ NK cells in PV vs non-PV areas in the stroma and tumor cell islands (TCIs) of **40 human triple negative breast carcinomas (TNBCs),** 20 of which were from untreated patents and 20 from those treated with neoadjuvant chemotherapy.

Methods

(i) Multiplex Immunosfluorescence Staining

MultiOmyx analysis was performed as described previously[2]. Whole FFPE tissue sections were baked at 65 °C for 1 h, deparaffinized and treated with a two-step antigen retrieval process, blocked against nonspecific binding with 10% donkey serum and 3% BSA in PBS for 1h at RT and stained with DAPI for 15 min. Directly conjugated primary antibodies were diluted in PBS supplemented with 3% (wt/vol) BSA (to working concentrations optimized previously) and applied for 1h at RT on a Leica Bond III Stainer.

A total of 11 rounds of paired - antibody staining were performed in sequence on the FFPE slides. CTLA-4 and CD56 were stained in round 1, followed by PanCK and CD66b in round 2, SMA and LAG-3 in round 3, CD3 and arginase in round 4, CD4, and CD31 in round 5, CD8 and PD-L1 1 in round 6, CD11b and FoxP3 in round 7, CD68 and CXCR4 in round 8, PD-1 in round 9, TIM-3 in round 10, and CD163 in round 11 (**Figure 1**).

After each round of staining with two antibodies, high resolution images were collected from 20 viable PV and non-PV areas in both PanCK-rich areas (TCIs) and PanCK-negative areas (stroma) using a 20x objective on an INCell analyzer 2200 microscope (GE Healthcare Life Sciences). Slides were then washed in PBS/0.3% TritonX-100 and dye inactivation performed by immersion in an alkaline solution containing H2O2 for 15 min with gentle agitation at room temperature[2]. Slides were washed again in PBS, imaged to check the efficacy of the dye in-activation, and stained with the next round of antibodies.

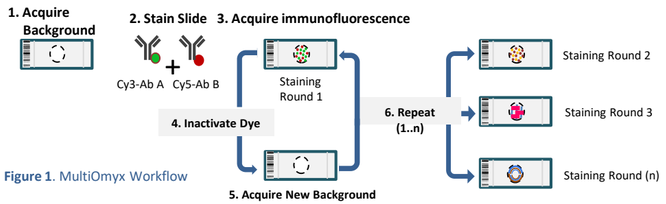

Figure 1. MultiOmyx Workflow

(ii) Quantitative Image Analysis

An AI-based advanced analytics platform, proprietary to NeoGenomics, called 'NEO Image Analysis', was used to quantify and analyse subsets of immune cell types in TNBCs including algorithms that could differentiate between them in TCIs vs the tumor stroma (**Figure 2**a), and within PV (within 50um from a CD31+ blood vessel) or non-PV areas (> 50um) of these regions (**Figure 2b**). Cells were segmented and tracked through each staining round; deep learning models were used to classify positivity value for each biomarker stain, as well as to classify regions as within TCIs or stroma. Stain co-expression analysis was used to define specific cell phenotypes.

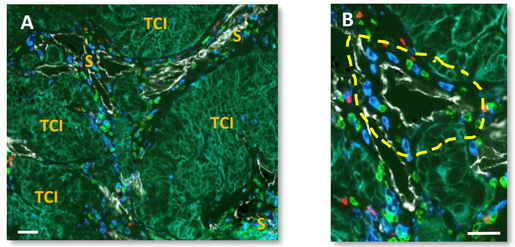

Figure 2. (A) MultiOmyx Image of a human TNBC. TCI = tumor cell island; S = stroma. (B) Definition of PV (50um from a vessel – within yellow dashed line) and non-PV areas (>50um from a vessel). *[PANCK (turquoise). CD31 (white), CD163+ TAMs (blue), CD8+ T cells (green) & FOXP3+ Tregs (red). No nuclear stain shown. Bar = 50um.]*

Results

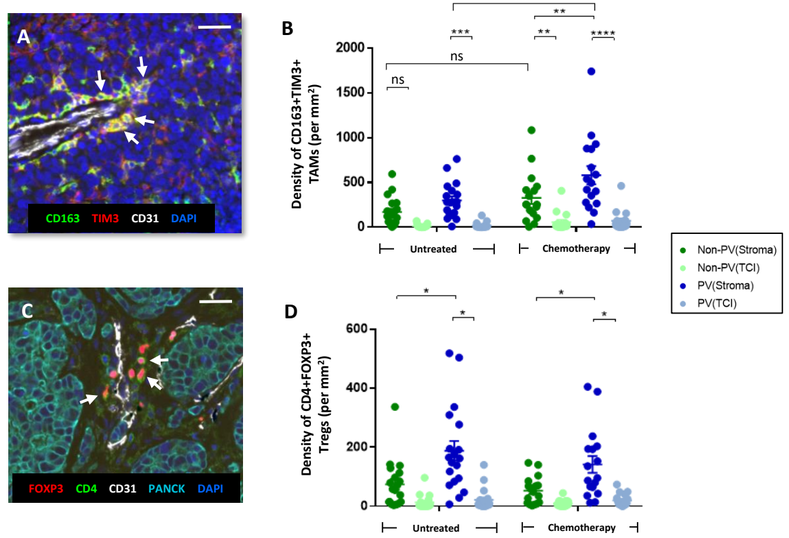

Figure 3. Perivascular Accumulation of CD163+TIM3+ TAMs and **FOXP3+ Tregs (both white arrows) in untreated and chemotherapy-treated human TNBCs.** MultiOmyx Images of human TNBCs showing **(A) CD163+TIM3+ TAMs** and **(C) CD4+FOXP3+ T regs.** Quantification of these two cell subsets in different tumor regions (**B, D**) – ie. PV and non-PV areas of the stroma vs tumor cell islands (TCIs). *P<0.05. Bars = 50um.

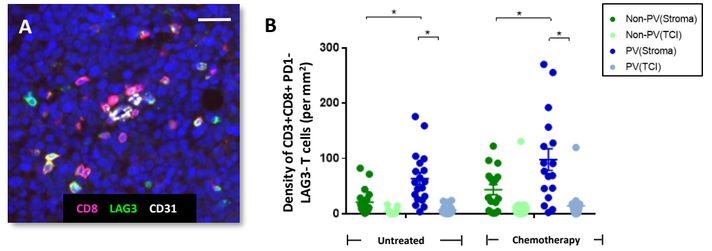

Figure 4. Perivascular Accumulation of CD3+CD8+PD1-LAG3- T Cells (white arrows) in untreated and chemotherapy-treated human TNBCs. (A) MultiOmyx Image of a human TNBCs showing **CD3+CD8+PD1-LAG3- T Cells.** (**B**) Quantification of these two cell subsets in different tumor regions (**B, D**) – ie. PV and non-PV areas of the stroma vs tumor cell islands. *P<0.05. Bars = 50um.

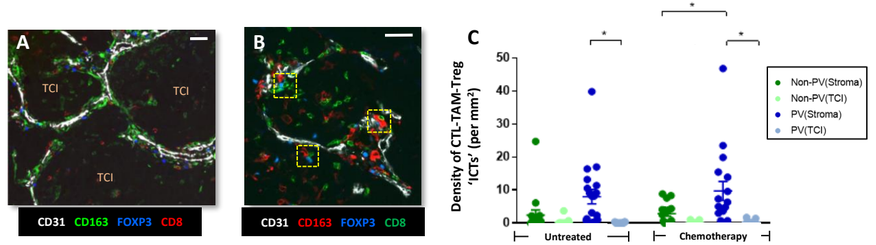

Figure 5. Perivascular Accumulation of 'Immunosuppressive Cell Triads' ('ICTs' = CD3+CD8+PD1-LAG3- T Cells in direct contact with CD163+TIM3+TAMs and CD4+FOXP3+ Tregs) in the stroma of untreated and chemotherapy-treated human TNBCs. (A,B) MultiOmyx Images of a human TNBCs showing these ICTs (no DAPI included; ICTs highlighted in yellow boxes in B) **(A;** low magn. **B;** higher magn**)**. **(C)** Quantification of ICTs. *P<0.05. Bars = 50um.

Key Findings

1. **CD163+ TAMs** were more abundant throughout the stroma than the TCIs of TNBCs (data not shown). Around blood vessels in the stroma, these cells upregulate the anti-metastatic receptor, **TIM3**, especially after chemotherapy (**Figure 3**).

2. Both **CD4+FOXP3+ Tregs** and **inactive (PD1-LAG3-) CD8+ T cells** also preferentially accumulate in PV stromal areas of untreated TNBCs - and were retained after chemotherapy (**Figures 3 and 4**).

3. In PV areas of the stroma, the above 3 cell types frequently made direct contacts forming cell 'triads' which could suppress the function of CD8+ T cells as they enter tumors. These potentially **immunosuppressive cell triads (ICTs)** increased after chemotherapy (**Figure 5**) and could potentially limit the efficacy of T cell-mediated immunotherapies.

4. If so, the frequency of PV ICTs could have utility in the stratification of patients for immunotherapy.

References Cited

[1] Lewis et al. 2016. *Cancer Cell* 30:18-25. [2] Gerdes et al. 2013. *PNAS USA*. 110:11982-7.

Funding: