# Tumor-draining lymph nodes as (potential) hubs of anti-tumor immunity in early triple-negative breast cancer

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## Background

- Adding immune checkpoint inhibitors (ICIs) to neoadjuvant chemotherapy improves outcomes in early triple-negative breast cancer (eTNBC)
- ⅓ of patients lack major pathological response and face high recurrence risk
- Tumor-draining lymph nodes (TDLNs) are hubs of T-cell priming; their immune programs likely shape checkpoint inhibitor efficacy.
- Breast cancer surgery yields sentinel lymph nodes (SLNs), offering a unique window into TDLN immunity after chemo-immunotherapy

## Methods

- Prospective collection of sentinel lymph nodes from eTNBC patients treated with standard neoadjuvant chemoimmunotherapy (paclitaxel/carboplatin + pembrolizumab followed by epirubicin/cyclophosphamide + pembrolizumab)
- High-plex spatial immunophenotyping using Akoya PhenoCycler multiplex imaging with 51 marker antibody panel on full TDLN cross-sections
- Assessment of immune cell distribution, phenotypes, and spatial neighborhoods focusing on antigen-presenting cells (cDC1, cDC2, pDC), T cell subsets (CD4+, CD8+, Tfh, Treg, Tpex, Tex), and B cell populations.
- Marker intensity for each sample was exported and loaded into RStudio. Unsupervised analysis was performed using the R Package BANKSY, enabling us to perform spatial cell clustering and tissue domain segmentation, integrating individual cell features with their microenvironmental context.

## *Objective*

- Characterization of immune cell composition, spatial organization, and functional states within TDLNs in early TNBC to identify immunological signatures that distinguish responders from non-responders

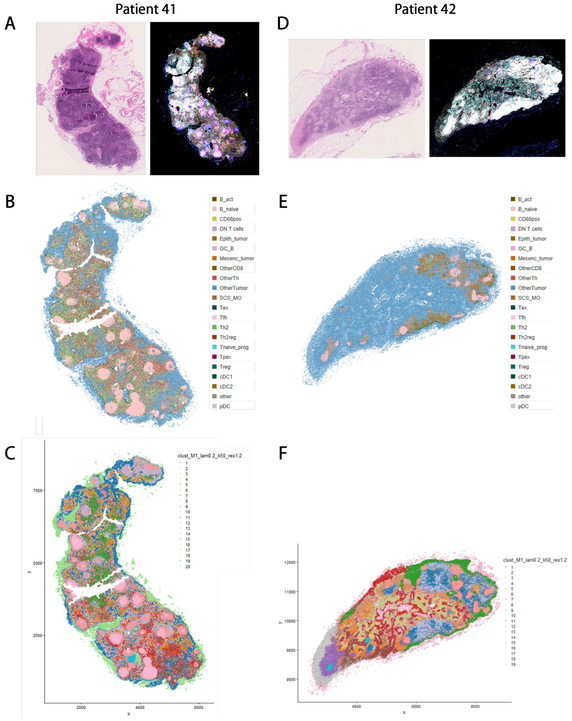



**Figure 1.** Analysis of two TDLNs from two eTNBC patients. (A,D) H&E staining – left panels – and multiplex immunofluorescence (PhenoCycler Fusion) – right panels. (B,E) Mapping of cell populations utilizing a decision-tree based supervised clustering method. (C,F) Mapping of unsupervised cell clusters (BANKSY script).

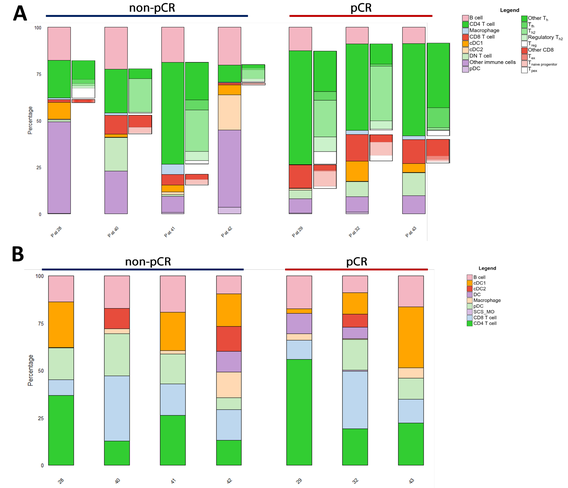



**Figure 2 .** Differential immune cell frequencies (% of all CD45 positive): (A) Cell classification based on supervised clustering methods. (B) Cell classification based on unsupervised clustering using BANKSY.

## Results

- We present preliminary findings of an ongoing study. Planned expansion to 30 patients with paired tumor and blood.
- Analyzed 7 patients: 3 pCR, 4 non-pCR *(Figure 1)*
- <u>Supervised: (Figure 2A):</u>
  - Responder TDLNs demonstrated significantly higher CD8+ (13.2% vs 4.6%) and CD4+ T cells (52.2% vs 26.8% of CD45+ cells) compared to non-responders
  - Within CD8+ compartment, pCR patients showed enrichment of precursor-exhausted (Tpex: TCF-1+PD-1+, 12.1% vs 4.0%) and exhausted (Tex: TCF-1-PD-1+, 7.0% vs 0.84%)
- <u>Unsupervised: (Figure 2B):</u>
  - Clusters were manually annotated based on their marker expression profiles, and clusters representing similar cell subtypes were then merged into broader cell-type categories
  - No unclassifiable CD45pos cells (10-50% in supervised)
  - Less pronounced difference of CD4 and CD8 cell dominance in pCR patients, compared to supervised classification

## Ongoing Work and Future Outlook

**Step 1** - Optimization
- Understand significant differences between supervised and unsupervised cell annotation, harmonize outputs (e.g. how were the CD45 unknown “reclassified” by BANKSY)
- Continue development of neighborhood analysis

**Step 2** – Cellular Activation States
- Comparison of activation markers in pre-specified cell population between defined neighborhoods
- Detection of pembrolizumab bound cells

## Conclusions

- Successful response to neoadjuvant chemoimmunotherapy in eTNBC associates with distinct TDLN immune architecture characterized by enhanced CD8+ T cell infiltration with Tpex/Tex phenotypes and optimal positioning of dendritic cells
- These preliminary findings nominate TDLN immune spatial phenotypes as potential predictive biomarkers

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