Cytometric analysis of immune cell populations in clinical tumor biopsy tissue microarrays for immuno-oncology

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Introduction

Despite significant advances in the medicinal field, cancer remains one of the deadliest and most complex diseases worldwide that affects millions of people yearly. The variety of genetic, epigenetic, and environmental factors that affect the etiology of cancer gives rise to a multitude of cancer types and subtypes that are clinically observed. Due to this variability, it is essential for healthcare providers to be able to gain better insights into the extent of a patient's cancer to tailor treatments for a better prognosis. Current methods of diagnosis are either qualitative or semi-quantitative, which may not give a full picture of the patient's tumor microenvironment, which plays a vital role in cancer progression (Hinshaw et al.). Therefore, we have developed a method utilizing 12-plex fluorescence imaging, quantitative image analysis, as well as cytometric analysis of immune cell populations to better categorize patients and determine the best treatment strategy for the individual patient.

Methods

Multiplex labeling was completed on human breast cancer tissue microarray (TMA) (XBra089-01) using conventional immunofluorescence labeling techniques (Figure 1) combined with the EasyPlex™ antibody stripping technique. The TMA was rehydrated through decreasing ethanol concentrations, followed by antigen retrieval in citrate buffer. Labeling of the TMA was completed in 4 panels counterstained with DAPI. Panels were carefully designed and validated to reduce cross-reactivity and promote labeling sensitivity. Removal of antibody labeling between panels was completed using Visikol Inc's proprietary stripping reagent EasyPlex™. Imaging was completed at 40x on the Leica Versa 8 slide scanner.

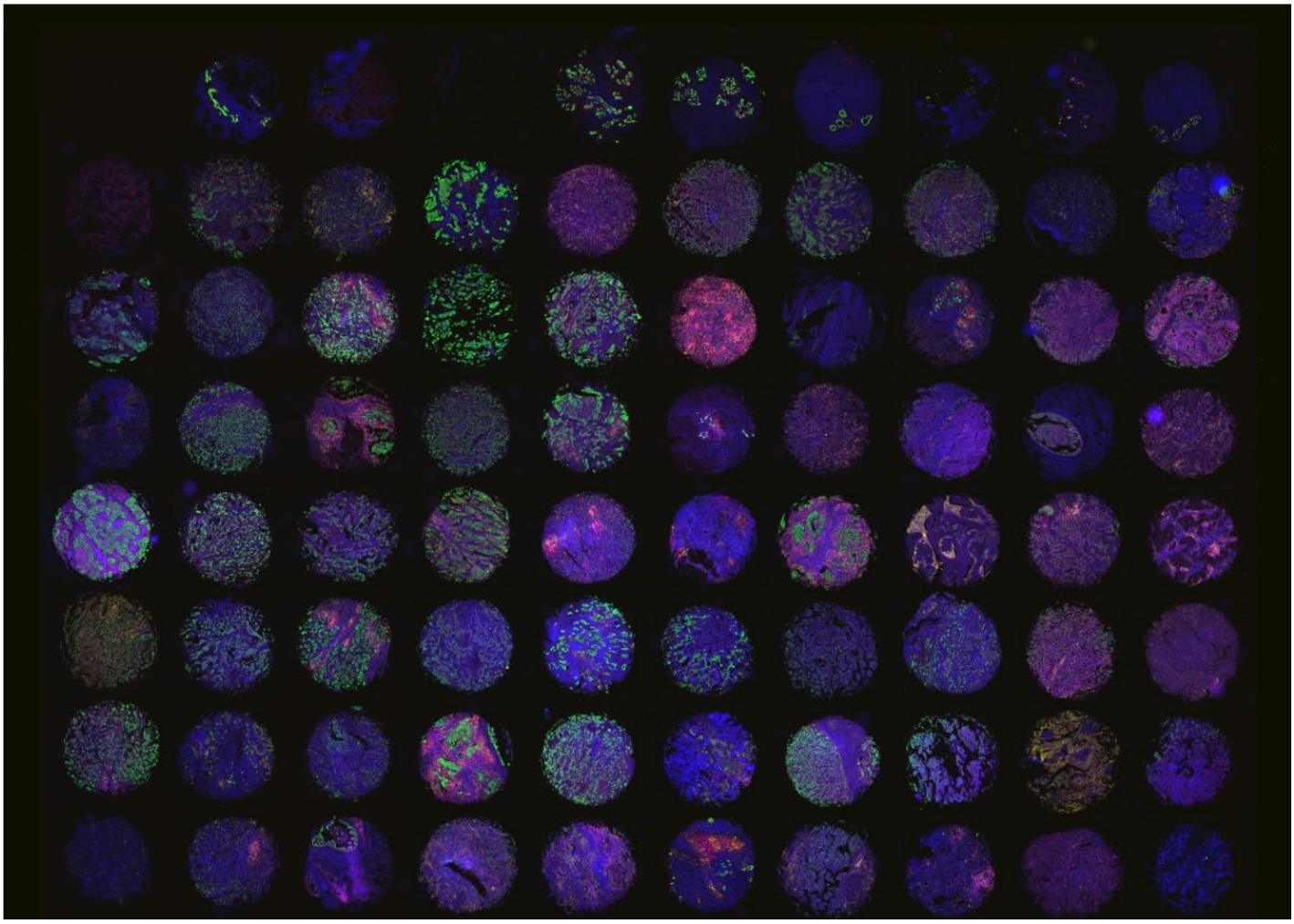

Figure 1. An image of the TMA panel showing a few of the markers with DAPI colored in blue.

Once the fluorescence imaging was complete, the HALO analysis software was utilized for extracting data from the TMA cores. Pre-processing involved registration of the 4 panels using ImageJ to align the cells from multiple imaging rounds and tiling the TMA cores using the HALO TMA module so that each core could be individually analyzed. Once the images were pre-processed, cell segmentation and marker colocalization was performed using the HALO Highplex module to determine the number of cells positive for each marker and combinations of markers to ascertain the cell phenotypes present within each core. Subsequently, a proximity analysis was performed using the HALO Spatial Analysis module to gain insights into the proximity of cell phenotypes from one another. Once the analysis was complete, the data was exported as a .csv file where subsequent data analysis was performed utilizing well-established python libraries (Pandas, SciPy, and Scikit-Learn).

The quantitative data was normalized using the mean-centered standard scaling technique resulting in mean-centered values representing the number of standard deviations from the mean to ensure that the data is not skewed by uneven scaling of the different features. To evaluate trends in the data, clustering was used to create a correlogram to evaluate relationships between the extracted features as well as a clustered heatmap to evaluate trends in the features among the patients clustered by cancer stage. To select a subset of features based on their contribution to the variation between groups, feature selection was performed using the F-test. To visualize patient population-wide variation and clustering from the selected feature subsets, principal component analysis (PCA) was conducted. Clustering of samples and

measurements was conducted using hierarchical agglomerative clustering of the Euclidean distance matrix from selected feature subsets.

Results and Discussion

The patient cohort represented by the TMA cores shows a good spread in the samples available for the various breast cancer stages, TNM scores, node statuses, and patient age range as seen in Figure 1. This spread in the data allows for a good representation of the various progressions of breast cancer for analysis.

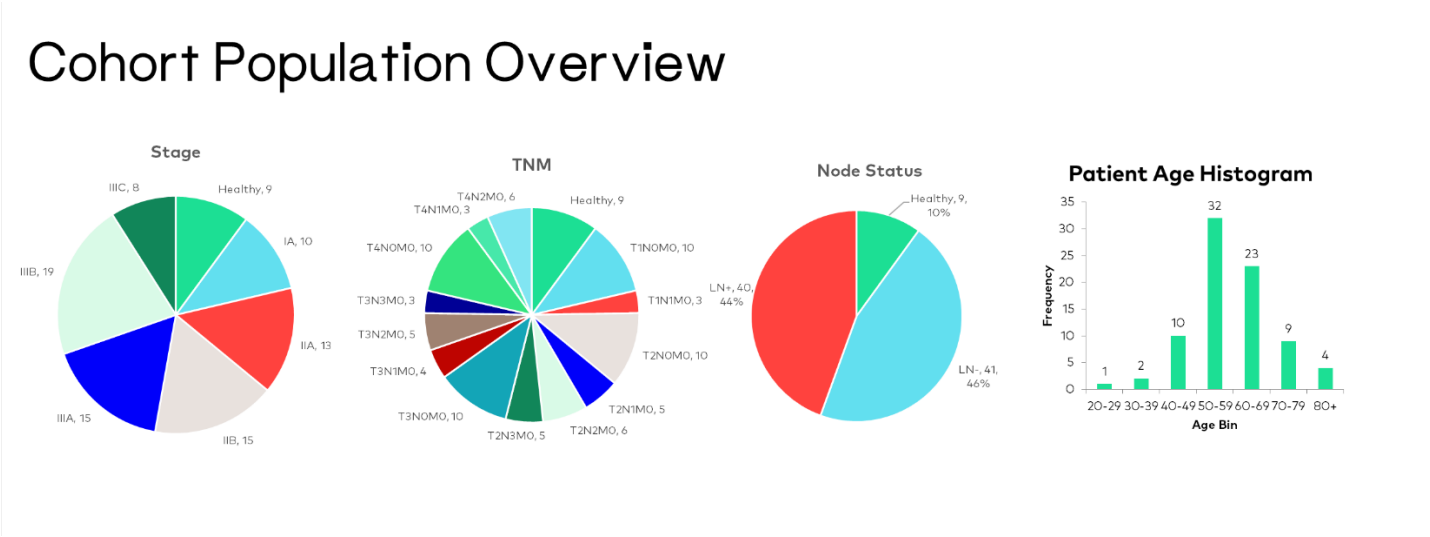

Figure 2. An overview of the patient cohort information from the TMA showing a breakdown of the populations present by cancer stage, TNM score, node status, and by binned patient ages.

Intercommunication between immune cells and the tumor microenvironment (TME) is a dynamic process that consists of complex feedback between immunosurveillance and tumor progression, known as immunoediting. Immunoediting progresses to impact aspects of tumor biology in three distinct phases: elimination, equilibrium, and escape (Tower et al.). During the “elimination phase,” the TME consists of innate and adaptive immune responses to tumor cells, which in earlier stages contribute to the elimination of tumor cells. However, adaptive pressure upon the tumor cells which survive causes shifts in the phenotype of the tumor cells towards the “equilibrium phase”—during which the TME transitions towards a non-immunogenic phenotype, promoting tumor progression. Cells that survive by acquired resistance to elimination enter the “escape phase”, promoting cancer cell growth and expansion in an uncontrolled manner. At this stage, the tumor immunophenotype is non-immunogenic, very few immune cells are detected, and the tissue resembles healthy tissue from an immunological perspective.

Utilizing 12-plex fluorescence imaging of TMA, quantitative image analysis, as well as cytometric analysis of immune cell populations we showed discrete subpopulations of breast cancer patients exhibiting immunological signatures across the transitional phases of immunoediting (Figure 3). Several distinctive immunological phenotypes were observed, ranging from highly inflamed patients with significant T-cell-macrophage interactions, to many patients with immunological phenotypes indistinguishable from healthy patients (see groupings in Figure 3). To better understand correlations between cell-cell interactions within the TME, a correlogram was generated, and as indicated on Figure 4, there was a high degree of correlation between the coincidence of interactions between macrophages and various T-cells/NK-cell subtypes, and proximity to immune checkpoint inhibitor PD-L1, promoting the treatment of this subgroup of patients with immune checkpoint inhibitors such as PD-L1 inhibitors.

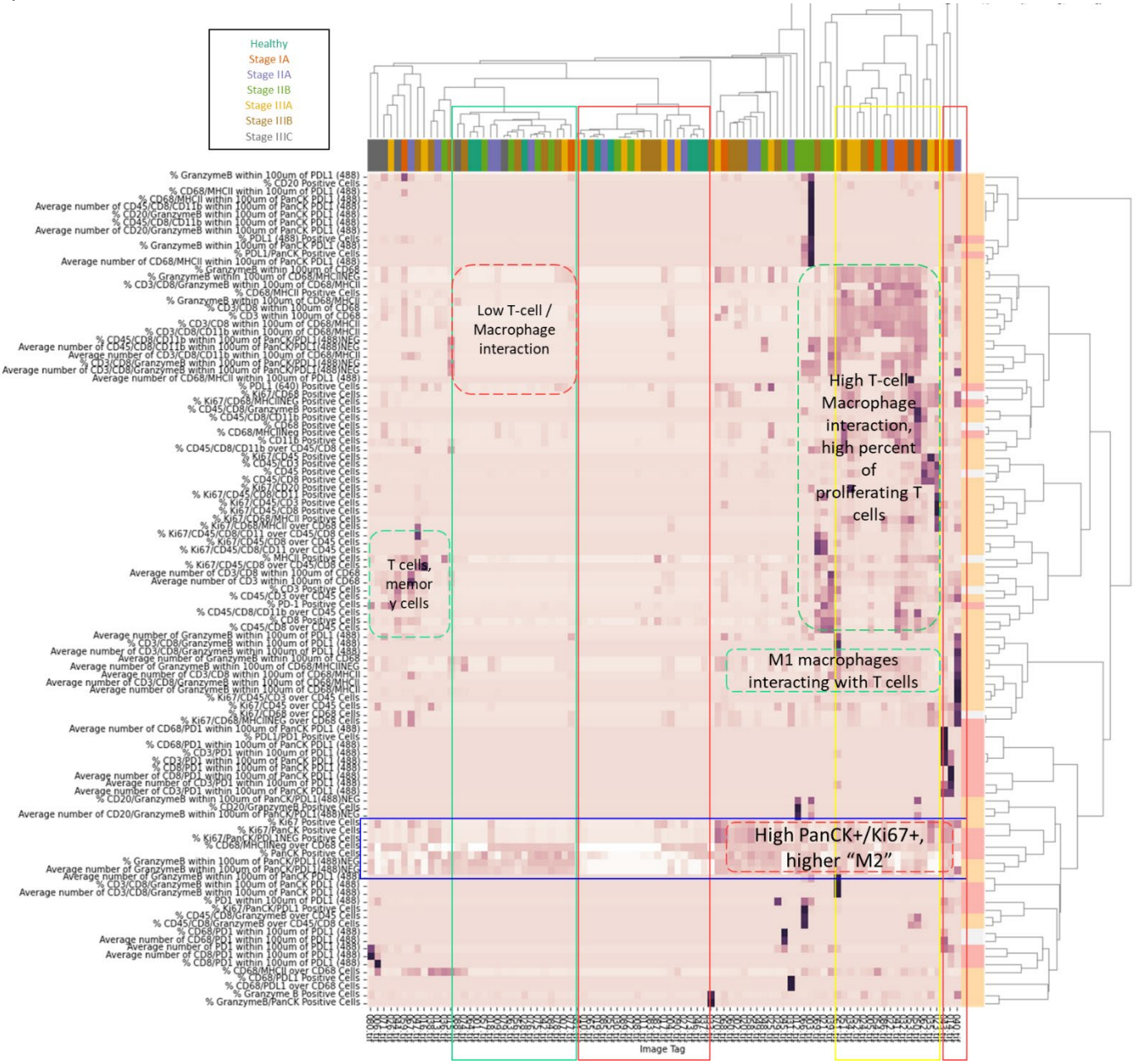

Figure 3. Clustered Heatmap of patients (color coded along top x-axis by stage, see legend) for all measured immunophenotypical features measured in the cohort. As indicated, several clear subcategories emerge, representing a spectrum from highly active immune response (green dashed outlines) and immunosuppressive response (red dashed outlines).

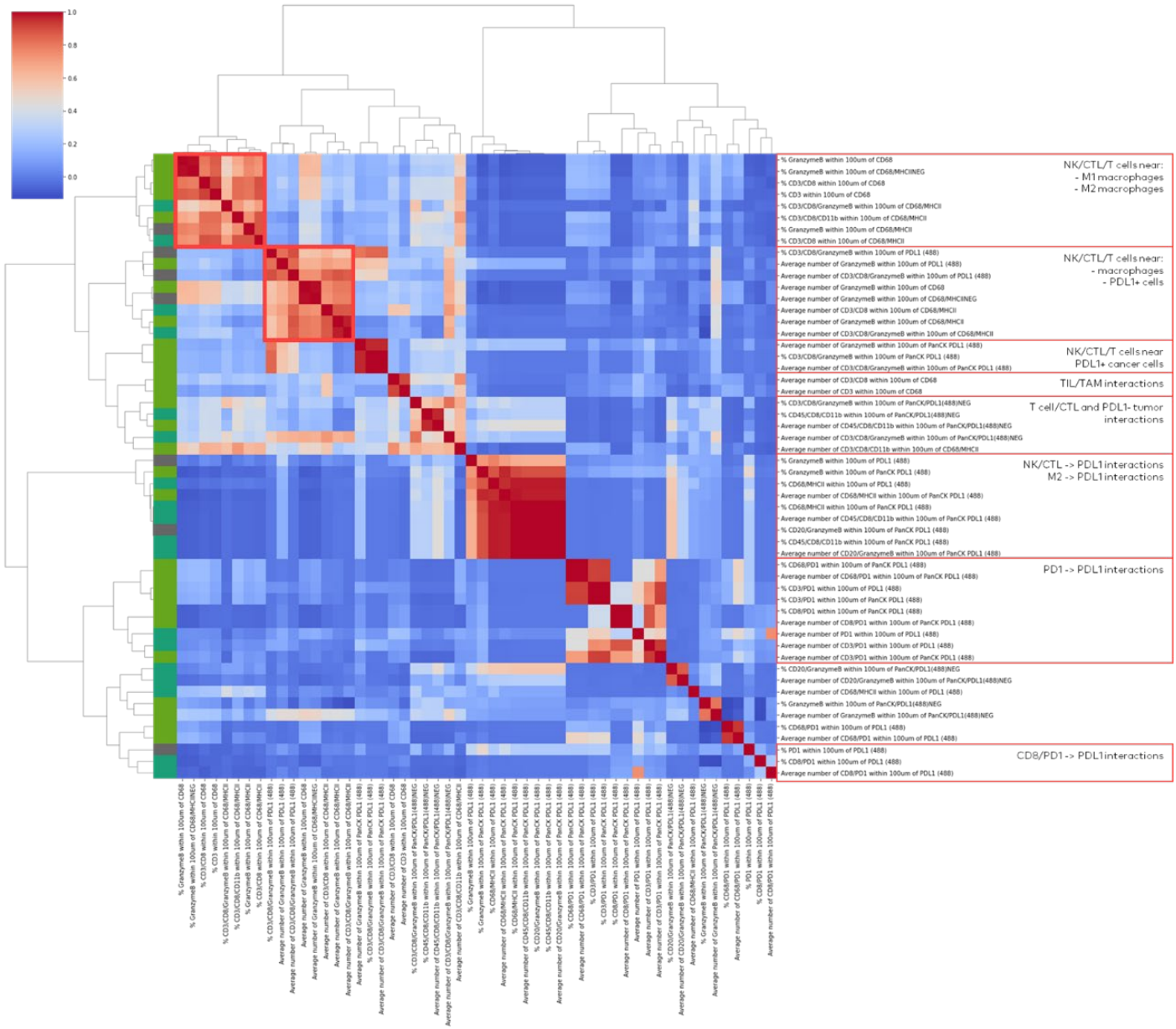

Figure 4. Correlogram depicting correlational trends between interacting subtypes of immune cells within the tumor immune microenvironment measured within the cohort. Several distinct relationships were detected, highlighted in red outlines on the figure.

Using statistical techniques, the most significant measurements (i.e., “features”) of differences in immunological phenotype between patients in different stages of cancer progression were ranked using the F-test (Figure 5A) and selecting measurements based on a p-value threshold. The resulting subset of measurements (highlighted in orange on Figure 5A). was used for subsequent analyses. To visualize the contributions of the features to the population-wide variation among patients, principal component analysis (PCA) was conducted, selecting the first and second component for plotting on the x and y axis, respectively. The factor-loadings map, shown in the left panel of Figure 5B, illustrates the contributions of each measurement to the variation across the population. The PCA plot shown in the right-side panel of Figure 5B is colored by the patient’s stage, with confidence ellipses around the patients in each stage, illustrating significant heterogeneity of immunophenotype within each stage, as seen

by the high degree of overlap between the stages on the plot. Figure 5C depicts the same plot as Figure 5B, however the plot is colored by the proposed phase of immunoediting which the phenotype corresponds to, as determined by the clustering analysis described in Figure 6. Figure 5C illustrates the clear delineation of the transition from the "elimination" phase to the "escape" phase. The proper determination of the immunophenotype corresponding to each phase of the immunoediting transition is critical to personalized medicine and to properly identify suitable therapeutic treatments for a given patient.

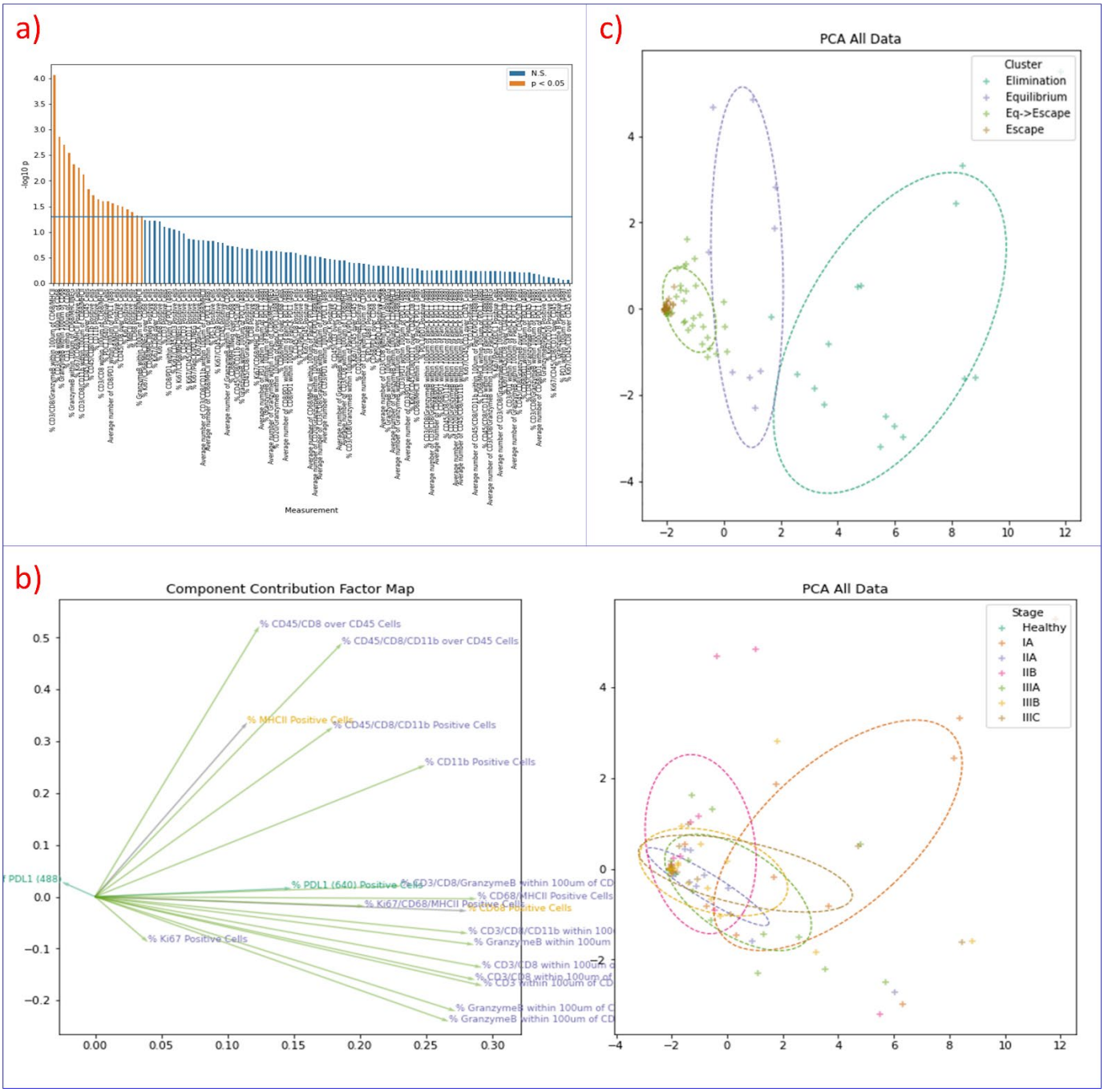

Figure 5. a) Rank-order of feature-importance measured via the F-test illustrating features most significant to accounting for differences between stages of patients within the cohort. **b)** Factor loadings map (left) and plot depicting 1st and 2nd principal component (right) calculated for each sample within the cohort using selected immunophenotypical features depicted in **Figure 5A** colored by cancer stage at diagnosis. Dotted ellipses depict 95% confidence interval for cancer stage. **c)** Principal component plot depicting same points as **Figure 5B**, colored by immunological stage determined by immunophenotypical fingerprinting of cohort population.

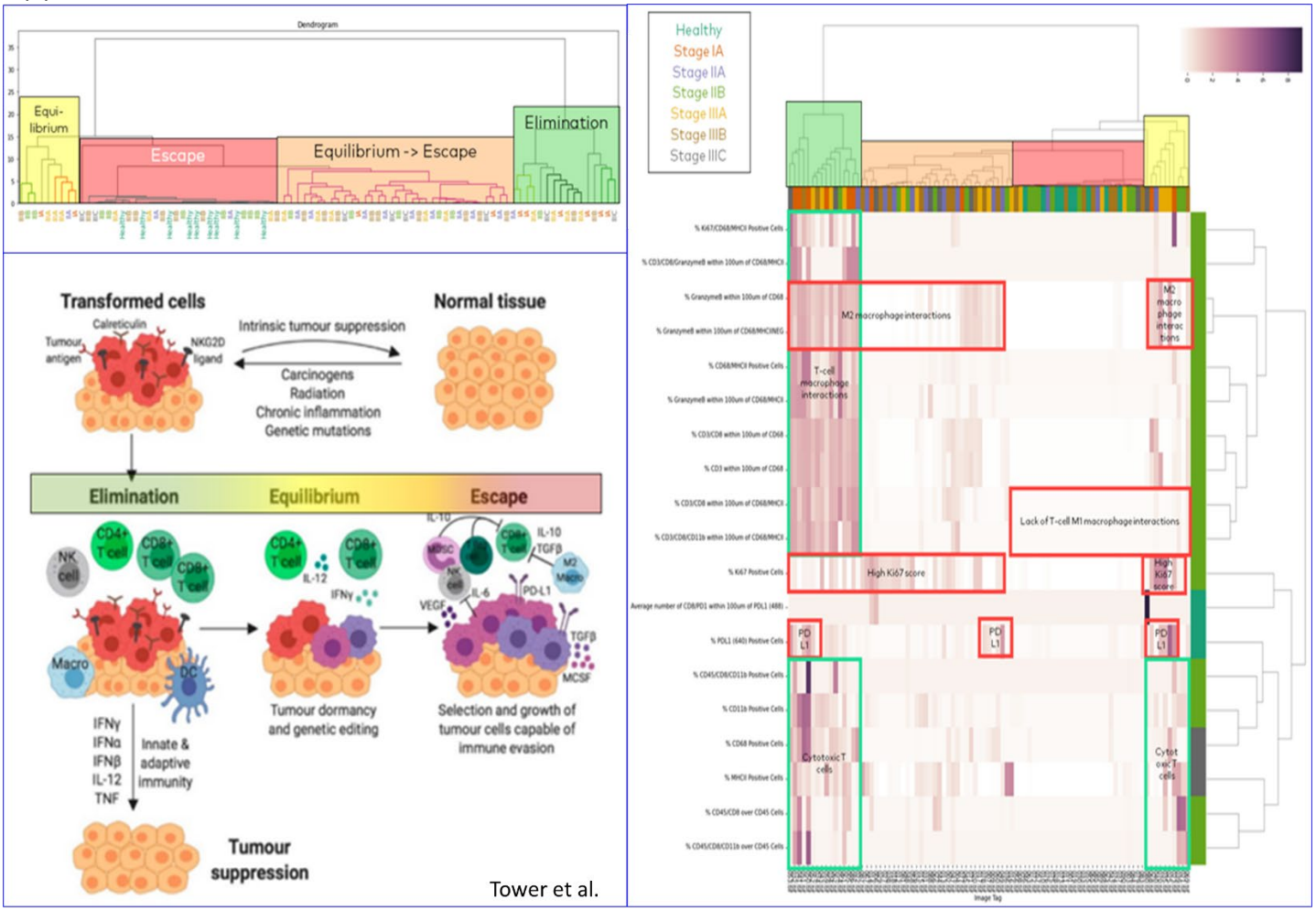

Figure 6. An illustration of the immune microenvironment response within the tumor (bottom left) (Tower et al.), which shows how tumor cells are eliminated at first by immune cells, then go into equilibrium with the immune system, and finally escape the immune system altogether. The dendrogram (top left) shows how the patients fit this pattern, and the clustered heatmap (right) further shows this by highlighting features prevalent at each of the stages in the immune microenvironment response.

Conclusions

In conclusion, the cytometric analysis of immune cell populations in clinical tumor biopsy tissue microarrays provides valuable insights into the immuno-oncology of tumors. This technique offers a powerful tool to categorize patients by immune system interactions that play crucial roles in tumor progression and can offer more pertinent information to guiding treatment thus providing better patient outcomes. As the field of immuno-oncology continues to evolve, cytometric analysis of immune cell populations in clinical tumor biopsy tissue microarrays is likely to become an important tool for advancing our understanding of the complex interactions between the immune system and cancer and improving patient prognosis.

References

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