I. Abstract

Background: While treatment with immune checkpoint blockade (ICB) has markedly improved outcomes in advanced melanoma patients and other malignancies, predicting response remains a challenge. Predictive biomarkers including tumor mutational burden (TMB), T-cell infiltration, and PD-L1 expression, have been identified but remain inadequate. Other components of innate and adaptive immunity, including B-cells and tertiary lymphoid structures (TLS), have been implicated in the prognosis and response to other cancer therapies, and preclinical data suggests B-cells may contribute to the response to combinatorial immunotherapy. Here, we use targeted protein expression profiling via NanoString digital spatial profiling (DSP) technology (research use only) to demonstrate a role for B-cells in the response to ICB in patients with high-risk resectable melanoma; furthermore, we characterize the B-cell subsets that enable this function.

Methods: We conducted a phase 2 clinical trial of neoadjuvant ICB therapy in patients with high-risk resectable melanoma (PD-1 blockade monotherapy or combinatorial CTLA-4-PD-1 blockade) (NCT02519322). Longitudinal tumor samples were taken during therapy and have been analyzed as possible by RNAseq, immunohistochemistry, CYTOF, and single-cell RNAseq. Formalin-fixed paraffin-embedded tissue sections from tumor samples exhibiting response by RECIST 1.1 criteria (n=5) were analyzed by NanoString DSP GeoMx technology and stained with a cocktail of CD108, CD45, CD19 and a 40-protein cocktail of antibodies conjugated to UV-photocleavable DNA barcodes. Regions of interest (ROI) were selected by a pathologist focusing on TLS-like structure and areas of tumor without significant TLS. This was followed by UV-activation of defined ROIs, releasing DNA barcodes for downstream quantitation on the NanoString nCounter® platform. Utilizing masking strategies, we further define the unique expression pattern within discrete subsets of immune cells.

Results: We identify higher expression of B-cell related genes within responder (R) as compared to non-responder (NR) patients receiving neoadjuvant ICB by RNAseq. Intratumoral B-cell density and TLS number and density are associated with response. B-cells are a major component of TLS and are closely integrated with CD4 and CD8 T-cells and follicular dendritic cells; activated, effector B-cells are enriched in R. TLS within the WLS exhibit a more activated phenotype as determined by NanoString DSP GeoMx technology.

Conclusion: NanoString DSP GeoMx data complement our deep molecular and immune profiling of tumors from melanoma patients treated with ICB, together they provide a novel predictive role for B-cells and TLS in the response to ICB, and importantly, provide mechanistic insight into the potential contribution of these cells in the response to cancer therapy.

II. Clinical Trial Data

Figure 1. Results of Phase 2 clinical trial of neoadjuvant ICB therapy in patients with high-risk resectable melanoma (PD-1 blockade monotherapy or combinatorial CTLA-4-PD-1 blockade) (NCT02519322). (a) Study schematic demonstrating arms as well as longitudinal tumor samples taken during therapy. (b) waterfall plots of ORR by RECIST 1.1 at 8 weeks and surgical pCR for the patients treated with nivolumab monotherapy (n=12), 25% RECIST ORR and pCR rate) and ipilimumab and nivolumab (n=11, 73% RECIST ORR and 45% pCR rate). (c) Kaplan-Meier estimates of PFS and overall survival by 80多年 log-rank test and are shown for each treatment group. For PFS, survival rates of 82% (95% confidence interval 45–95%) at 17.2 months with ipilimumab and nivolumab treatment versus 58% (27–80%) at 22.6 months with nivolumab treatment. P=0.19. For overall survival, rates of 100% (95% 100–100%) at 24.4 months with ipilimumab and nivolumab treatment versus 76% (31–94%) at 22.6 months with nivolumab treatment, P=0.18.

III. B-cells and TLS are Predictive of Response

Figure 2. B-cells and Tertiary lymphoid structures (TLS) containing B-cells, T-cells, and follicular dendritic cells are predictive of response to immune checkpoint blockade (ICB). (a) Volcano plot depiction of most differentially-expressed genes by response to neoadjuvant ICB in melanoma by RNA sequencing as defined by complete or partial response by RECIST 1.1 and NR, and partial response by RECIST 1.1 and NR, and partial response by RECIST 1.1 and NR. (b) Quantification of CD30 expression in tumor by single immunohistochemistry and association with complete response (n=11 NR and 7 R). (c) Orchestration of CDS and TLS in areas occupied by TLS and correlation to treatment response (n=10 NR and 8 R, and 7 NR and 7 R, respectively). (d) Orchestration of CD30 expression in tumor by single immunohistochemistry and association with complete response (n=10 NR and 9 R). Factors include median values of expression for DNA barcodes for CD30 comparisons made by two-sided Mann-Whitney U tests. (e) Density of TLS and ratio of tumor area occupied by TLS and correlation to treatment response (n=10 NR and 8 R, and 7 NR and 7 R, respectively). (f) Representative case of R with TLS associated HAE and R, and single HAE as indicated. (g) CYTOF, SNE plots demonstrating PBMC and intratumoral B-cell populations. In R vs NR (n=4 R and n=4 NR for PB and n=5 R and n=3 NR for tumor)

IV. NanoString DSP GeoMx™ Technology

Figure 3. NanoString DSP GeoMx technology offers imaging and profiling in single assay. (a) NanoString’s GeoMx™ Digital Spatial Profiler was employed to perform high-pixel proteomic analysis with spatial resolution. A cocktail of antibodies conjugated to UV-photocleavable DNA barcodes recognizing immunotherapy-related proteins is employed. Immunofluorescence followed by UV excitation of the defined ROIs releases the DNA barcodes for downstream quantitation on the NanoString nCounter® platform. Utilizing masking strategies, we further define the unique expression pattern within discrete subsets of immune cells. (b) Regions of interest (ROI) can be delineated via multiple different modes. (c) 40-protein cocktail of antibodies with proteins associated with the intratumoral immune response. All subsequent assays derived from this panel.

V. Patient and ROI Selection

Figure 4. Patient and ROI Selection. (a) Metadata for the subset of tumors from patients exhibiting robust response to ICB therapy and high density and number of TLS from which further analyses derive. RECIST and pathologic response is noted. (b) Example of ROI selection (200 x 200 µm) from representative patients including ROI containing TLS and ROI outside of a TLS. ROI selection was completed using H&E staining and confirmed with immunofluorescence as shown using SI008, PMLI, S103, 1303, and C200. Masking for B-cells and T-cells as indicated based on CD3 and CD20 staining.

VI. T- and B-cells within the TLS are unique

Figure 5. B- and T-cells within the TLS differ from those outside the TLS. PCA plots for all (a) T-cells and (b) B-cells per patient/slides as indicated. Individual PCA plots per slide demonstrating overall differences for (c) T-cells and (d) B-cells within the TLS as compared to those found outside the confines of a TLS as indicated. Data was normalized by positive ERCC controls and area (counts per 1000 µm²) and then by average log2 controls. Targets below signal to noise threshold of 2 were dropped from analysis. PCA plots calculated using singular value decomposition examining the covariances/correlations between individuals.

VII. TLS associated with markers of T-cell Activation

Figure 6. B-cell-rich TLS are associated with markers of T-cell activation and response. (a) Fold change in expression of various markers of T-cell activation and response in TLS-associated B-cells as compared to T-cells found outside of the TLS per individual slide. (b) Average fold-change of expression for TLS-associated T-cells as compared to non-TLS-associated T-cells. Individual dots represent individual patients/slides. (c) Fold change in expression of various markers of B-cell activation and response in TLS-associated B-cells as compared to B-cells found outside the tumor per individual slide. (d) Average fold-change of expression for TLS-associated B-cells as compared to non-TLS-associated B-cells. Individual dots represent individual patients/slides. For plots in a and c, data shows individual TLS ROI value divided by the average non-TLS ROI value of that slide. For plots b and d, data shows the average TLS ROI value divided by the average non-TLS ROI value per slide. Error bars indicate 95% confidence intervals.

VIII. Conclusion and Future Directions

• The presence of intratumoral B-cells and tertiary lymphoid structures (TLS) are predictive of response to immune checkpoint blockade in patients with bulky Stage III or oligometastatic Stage IV melanoma. Single cell analysis methods (CYTOF and SNE) may allow us to better understand the role of B-cells in melanoma.
• NanoString DSP GeoMx technology can be utilized to characterize the B- and T-cells within a tumor as compared to that found outside of the TLS.
• There is some heterogeneity of B- and T-cells amongst patients exhibiting response to ICB, but currently, B and T-cells within the confines of a TLS are distinct from their counterparts in other portions of the tumor outside the TLS.
• NanoString DSP GeoMx technology suggests that B-cell rich TLS structures are associated with markers of T-cell activation and response, suggesting that B-cells within the TLS may contribute to enhanced T-cell activation.
• B-cells within the TLS are more variable with the expression of a limited selection of markers of B-cell activation. Additional characterization of the B-cells themselves is warranted.
• NanoString DSP GeoMx data complement our deep molecular and immune profiling of tumors from melanoma patients treated with ICB. Together, they provide a novel predictive role for B-cells and TLS in the response to ICB and, importantly, provide mechanistic insight into the potential contribution of these cells in the response to cancer therapy.

IX. References