Recurrent Somatic Mutations affect Immune Cell Density in the Tumor-core

Fifty-six patients in the ccRCC cohort previously underwent whole exome DNA sequencing from which we identified recurrent somatic mutations: BAP1 (4/56 patients, 7.1%), KDM5C (9/56, 16.1%), MTOR (4/56, 7.1%), PBRM1 (22/56, 39.3%), PTEN (5/56, 8.9%), SETD2 (8/56, 14.3%), and VHL (42/56, 75.0%). Patients with MTOR alterations had significantly lower tumor-core cell density of T-bet+ T-cells (29 vs. 13 cells/mm^2; p < 0.05) and CD163+ macrophages (264 vs. 47 cells/mm^2; p < 0.05). Patients with SETD2 alterations had significantly lower tumor-core cell density of CD8+ T-cells (97 vs. 30 cells/mm^2; p < 0.05). PBRM1 alterations were not associated with significant differences in immune cell density. Comparisons for other alterations can be found in Supplemental Figure 2.

RNA-seq Immune Cell Gene Expression is Inconsistently Associated with mIF Density

Bulk RNA-seq data was obtained from tumor-core samples in 92 patients with ccRCC. Correlation was determined between tumor-core cell density as determined by mIF, and xCell score; an RNA-seq based gene expression score quantifying relative expression of 64 different cell types. Relevant comparisons were made between xCell immune cell-types and corresponding mIF marked cell types.

Within the ccRCC cohort, CD8+ xCell score and CD8+ mIF cell density were strongly correlated (Spearman’s R = 0.63). Moderate correlation was found between B-cell xCell score and CD-20+ mIF cell density (R = 0.42), T-reg xCell score and FOXP3+ mIF cell density (R = 0.38), and a generalized xCell macrophage score with CD-68+ mIF cell density (R = 0.34). Poor correlation was identified between M2 macrophage xCell score and CD163+ and CD206+ mIF cell density (R = -0.16), and Th1 T-cell xCell score and T-bet+ mIF cell density (R = -0.23). A correlation matrix for the ccRCC cohort is available in Supplemental Figure 3.

Low Angiogenesis and High T-effector Score Tumors are Infiltrated with CD8+ T-cells and CD68+ TAMs
Angiogenesis and T-effector gene signature scores, previously derived from the IMmotion150 trial data, were determined for 92 patients with ccRCC\(^1\). Patients with high angiogenesis scores had significantly lower mIF cell densities of CD68\(^+\) and CD8\(^+\) cells in the tumor-core (\(p = 0.002\) and \(p = 0.05\), respectively), stroma (\(p = 0.04\) and \(p = 0.05\), respectively), and tumor-stroma interface zones (\(p < 0.001\) and \(p = 0.001\), respectively). Patients with high T-effector scores had significantly higher tumor-core mIF cell densities of CD68\(^+\) (\(p < 0.001\)), CD8\(^+\) (\(p = 0.002\)), FOXP3\(^+\) (\(p = 0.04\)), and PDL1\(^+\) cells (\(p = 0.02\))

**Supplemental Figure 4.**

**Sensitivity Analyses**

Several analyses were conducted to confirm that the previous findings were robust. First, PD-L1 tumor-core density was correlated with Tumor/CD163 and Tumor/CD8 interface nK(75), confirming that these are not merely surrogate findings for PD-L1 density. Spearman’s correlation coefficients for PD-L1 density were -0.014 and 0.01 for Tumor/CD163 and Tumor/CD8 interface nK(75), respectively.

**Supplemental Figure 5.**

To confirm that the Tumor/CD163 interface nK(75) metric was not easily reproduced using a Tumor/Stroma density ratio, a Spearman’s correlation and Kaplan Meier analysis were conducted, revealing a weak positive relationship (\(R = 0.31\)) and no difference in overall survival when stratified by the CD163 Tumor/Stroma density ratio (log-rank \(p = 0.75\)). **Supplemental Figure 6.**

To confirm that the TCGA overall survival validation was not influenced by false-stratification, the CD163 Clustering Gene Signature was divided into tertiles instead of using a median cutoff, which confirmed a stepwise association with clinical outcomes along the tertile continuum. **Supplemental Figure 7.**

Ridgeline plots can be visualized in **Supplemental Figure 8**, detailing how many samples were excluded due to the 10 cell per ROI cutoff for spatial analysis.