Unmassked: Single-Cell Profiling of Immune Cell Populations in Tumors

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Extensive preclinical and clinical data accumulated over the last few decades have documented that host immunity can play paradoxical roles in both promoting tumor outgrowth and in promoting tumor control and sculpting the immunogenicity of tumors. T cells and, more specifically, CD8 T cells are considered to be essential for tumor killing, whereas the composition of the innate immune compartment serves to dictate T cell infiltration (secretion of chemokines), activation, phenotype, and functional capacity. Combined mapping of the innate myeloid compartment, primarily the monocytes/macrophages and dendritic cells, and the T cell compartment is thus a powerful method to dissect the immune ecosystem within the tumor microenvironment (TME). This integrated mapping of the composition and function of the immune infiltrate of tumors can set the pathway for both diagnosis and personalized treatment options. Two recent independent studies have now utilized multiplexed single-cell profiling on millions of immune cells derived from cohorts of 28 non-small cell lung cancer (NSCLC) patients and 73 clear cell renal cell carcinoma (ccRCC) patients.[1,2]

Chevrier et al.[2] used two panels of multiplexed antibodies targeting T cells and tumor-associated macrophages (TAMs) to perform mass-cytometry on patients with different grades of ccRCC and compared them to five healthy matched kidney samples. Not surprisingly, they found that T cells were the dominant immune cell population in ccRCC. To generate a comprehensive view of the T cell subpopulations, they used a combination of the non-linear dimensionality reduction technique, t-distributed stochastic neighbor embedding (t-SNE); and PhenoGraph, an algorithm that identifies subpopulations in high-dimensional single-cell data. Analysis of the CD8$^+$ populations expressing programmed death-1 (PD-1$^+$) demonstrated that the different subpopulations varied in the co-expression of various co-inhibitory and costimulatory receptors, which could thus represent T cell subpopulations along the spectrum of exhaustion. Among the various subpopulations that were identified, two of the more well-known subpopulations included the exhausted CD8$^+$ T cell cluster and the regulatory CD4$^+$ T cell cluster (T$_{reg}$). One of the novel findings of the deep multiplexed profiling was that CD8$^+$ PD-1$^+$ cells were also predominantly CD38 (activation marker) positive. Classification of the tumor-associated macrophages (TAMs) using the same approach identified 17 TAM subpopulations, and, based on the expression of most of the markers profiled, the findings support the existence of a continuum of macrophage phenotypes. One of the most interesting observations was the identification of a specific TAM cluster that is similar to the CD8$^+$PD-1$^+$ T cells outlined above, in that it also expressed high levels of CD38. The integrated profile of this subset is somewhat confounding in that it has properties that are considered both anti- and pro-tumor. Immunofluorescent staining of ccRCC tissue was also used to demonstrate that T cells and TAMs expressing CD38 could co-localize among these tumor sections. Finally, stratification of patients based on the combined frequencies of the identified TAM subpopulations showed an association with progression-free survival statistics. Collectively, a strong correlation was found among the exhausted CD8$^+$ T cell cluster, the T$_{reg}$ cluster, and the macrophage cluster, suggesting that the latter two cell types might be responsible for the exhaustion phenotype documented in the first cluster.

Lavin et al.[1] utilized a simple cellular barcoding strategy based on anti-CD45 antibodies conjugated to different heavy-metal isotopes to enable the matched profiling of lung adenocarcinoma lesions, matched normal lung (nLung), and peripheral blood immune cells by multiplexed mass cytometry. One of the major advantages of this study was that the cohort of patients was treatment naive and thus the organization, function, and phenotype of immune cells within the TME was unaffected by prior treatment. In addition to phenotypic markers, they expanded their antibody panels to include cytokine measurements in a subset of ten patients. tSNE was again used to visualize the different subsets of immune cells within the TME and to quantify differences to the nLung samples. As with the other study, T cells were the most abundant population within the TME. In order to determine the spatial distribution of immune cells within tissue, the authors used a tissue profiling method called multiplexed immunohistochemical consecutive staining on a single slide (MICSSS). Consistent with the known spatial localization of immune cells in tumors, these results illustrated that immune cells were primarily present in the stroma and the invasive margins surrounding the tumor islets. Similar to the results outlined in ccRCC above, populations of T$_{reg}$ with high expression of CTLA4 and CD38, and functionally exhausted T cells, leading to a reduced T-effector/T$_{reg}$ ratio, was a strong signature of a lung tumor lesion. PD-1 expression was documented on a small subset of CD8 and CD4 T cells. Analysis of natural killer (NK) cells indicated that their frequency and functional capacity was diminished compared to the nLung.

To characterize the tumor-infiltrating myeloid compartment in an unbiased manner, single-cell RNA sequencing (RNAseq) of ~1,500 cells from tumor and nLung of a stage 1A adenocarcinoma patient was performed.

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These data were then used to classify the different subsets of dendritic cells, monocytes, and macrophages. These single-cell signatures revealed different macrophage populations present in the tumor lesions in comparison to the nLung samples. The macrophages in the tumor lesions displayed similarities to the TAM subset identified in ccRCC. Survival analysis based on the The Cancer Genome Atlas (TCGA) data suggested a significant survival disadvantage for patients with a high ratio of the tumor macrophages to nLung macrophages. The expression of programmed death-ligand 1 (PD-L1) was high but no different between the macrophages from either tumors or nLungs, providing a possible explanation for the increased rate of pneumonitis in patients treated with PD-L1 inhibitors.

Altogether, these results portray an atlas for immune cells in early untreated NSCLC. A unique population of tumor macrophages and T_{reg} promote an immunosuppressive environment that is enriched in exhausted T cells and functionally impaired NK cells. One of the significant findings of the study is that this immune contexture of the tumor is established early and is not significantly altered in later-stage tumors (stages II and III). This, in turn, argues that immunomodulatory agents, including PD-1 antibodies, could benefit even early stage tumors.

From a biological standpoint, both studies suggest a role for CD38, even if its expression differs between the cellular populations that were identified. CD38, classically defined as a T cell activation marker, is a multi-functional enzyme and receptor.[3] $CD8^+CD38^{hi}$ T cells have been shown to secrete high levels of IFNγ to suppress the effector $CD4^+$ T cell subsets.[4] In parallel, CD38 has been shown to synergize with the CD73 axis to generate extracellular adenosine, a well-documented immunosuppressant within the TME.[5] Thus, the functional and immunoregulatory status of $CD8^+CD38^{hi}PD-1^+$ subsets identi-

fied by these studies warrants further investigation. CD38 overexpression has been best characterized in leukemias and multiple myeloma. Targeting CD38 with a human IgG1κ monoclonal antibody, daratumumab, has shown efficacy in refractory multiple myeloma and is being evaluated in phase 2 clinical trials.[6] As seen with NSCLC cohort, if the overexpression of CD38 is restricted to T_{reg} then a validated, depleting monoclonal antibody is appealing. If the expression of CD38 is also documented on effector T cells, then a blocking antibody might be more appropriate to alleviate immunosuppression. Regardless, it is clear that the role of CD38 within the TME warrants further investigation.

Single-cell analyses have made rapid strides in the past decade. Although flow cytometry and live-cell microscopy are well established single-cell methods, the ability to perform highly multiplexed analyses in a high-throughput manner, as outlined in both these papers, has been made possible by advances in technology like mass cytometry and single-cell RNA-seq. It is, however, important to recognize that both these papers represent results from technically challenging experiments and paired bioinformatic analysis, and this expertise cannot be easily replicated in standard labs. While a lot of work in the past few years has demonstrated the utility of these single-cell techniques at documenting heterogeneity in research settings, the value of single-cell profiling can be justified if it leads to actionable biological insights. Both these papers aim to advance clinical questions with the use of in-depth single-cell profiling. The objective of such elaborate single-cell profiling, as these papers aim to demonstrate, is to enable the discovery of the relevant subpopulations that can be identified using a minimal set of markers in standard clinical or laboratory settings. These subpopulations need to be extensively validated in

large clinical cohorts to determine their clinical utility. As outlined above, Lavin et al. aim to advance immunotherapeutic treatment to earlier stages of NSCLC, and this approach to treatment, if validated, has the potential for great benefit if T cell exhaustion can be reversed earlier rather than later. As the suite of single-cell technologies from sequencing to functional interrogation matures,[7] the ability to better define immune subpopulations should lead to clinically actionable insights or biomarkers.

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