Editorial on Immunotherapy and Tumor Microenvironment

Cancer patient stratification based on the tumor microenvironment

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Cancer immunotherapies targeting immune-checkpoints, which function through alleviating tumor-induced immunosuppression, have aroused as new therapeutic strategies in recent decades. The checkpoint blockade strategies have achieved significant treatment efficacy in thoracic cancers and other cancer types. Programmed cell death 1 (PD-1) and its ligand PD-L1, as well as cytotoxic T-lymphocyte antigen 4 (CTLA4) are the main immune checkpoints that have been targeted in lung cancer treatment (1). Interestingly, single agent checkpoint blockade (such as nivolumab, an anti-PD-1 antibody) is associated with around 27% objective response rate in patients with tumor PD-L1 expression ≥1%, whilst combination of nivolumab (anti-PD-1 antibody) and ipilimumab (anti-CTLA4 antibody) leads to a further increased response rate and duration of response compared to nivolumab or chemotherapy in advanced non-small-cell lung cancer (NSCLC) patients (2). It is worth noting that combination therapy with immune checkpoint blockade has significantly improved the overall response rate in lung cancer according to a recent meta-analysis (3). However, due to limited understanding in treatment mechanisms, it is difficult to decide which combination of immunotherapies to use in patients and which patient groups would benefit from the treatments.

The dynamic interactions between tumor and immune cells in the tumor microenvironment (TME) determine the effectiveness of immunotherapy (4). This suggests that investigation of the composition and heterogeneity of the TME will in principle enable more detailed patient stratification, novel biomarker discovery and eventually improved therapeutic strategies. Deeper insights into the TME also suggest diverse mechanisms of tumor immune evasion. The density of tumor infiltrating lymphocytes [TILs, e.g., $CD8^+$ T cells and $FOXP3^+$ regulatory T cells (Tregs)] and the expression of immune checkpoints (e.g., PD-1/PD-L1) in TME dramatically influence the immune response in cancer therapy, which is one of the key factors in enhancing immune response and restricting tumor immune evasion, and results in better survival outcomes of cancer patients. There are several aspects need to be considered in cancer immunotherapy, such as TME components including the density/expression of immune checkpoints in TME, as well as the distribution and subtypes of TILs. Besides, genomic backgrounds and aberrant activation of various pathways (such as inflammation-related pathways, metabolic pathways, or angiogenesis-related pathways) (5) are also important factors that need to be considered for cancer patients' stratification. However, in this editorial comment, we will mainly focus on TME components, especially on immune cells and immune checkpoints.

Density of TILs and expression of immune checkpoints

The density of TILs within the TME is now recognized as a predictive marker of patients' responses and overall survival outcomes. Since $CD8^+$ T cells are the main effectors of

anti-tumor immunity and enable the elimination of tumor cells, they have been reported to be a positive prognostic predictor in stage I NSCLC (6). However, PD-L1 and CD8 co-expression in EGRF-mutated and ALK-rearranged lung cancer has been reported as a poor prognostic biomarker (7). Additionally, low CD8 and high FOXP3 expression exhibited significantly worse overall survival compared to other groups in stage I lung adenocarcinoma (LUAD) (8). Moreover, PD-L1 expression was an important marker to stratify cancer patients for anti-PD-1/PD-L1 therapy initially, while a recent study revealed that patients with very low expression of PD-L1 also generated positive response to anti-PD-1/PD-L1 immunotherapy (9), indicating that other factors may also play a pivotal role in local immune response.

Furthermore, the combination of TILs density and PD-L1 expression is supposed to improve patient stratification, as such, patients with low level of TILs may require strategies to increase T cell infiltration into the tumors, while patients with a large number of TILs but high expression of PD-L1 may be more responsive to anti-PD-1/PD-L1 blockade. Therefore, to choose and apply a suitable treatment for each cancer patient, it is necessary to further categorize patients into certain subgroups based on the expression of PD-L1 and occurrence of TILs.

TME may be divided into four subtypes regarding the composition of TILs and immune checkpoints expression in the previous study: type I with high level of both PD-L1 expression and TILs, type II with low level of both PD-L1 expression and TILs, type III with high PD-L1 but low level of TILs, and type IV with low PD-L1 expression but high level of TILs (10). This helps stratify cancer patients into different categories with distinct immune responses, which can be applied for further personalized therapy. Our group has recently stratified gastric cancer (GC) patients into three different clusters by using the ratio of CD8/PD-L1 and CD8/FOXP3. We further confirmed that PD-L1 expression showed a positive prognostic value in the cluster of GC patients with low CD8/PD-L1 but high CD8/FOXP3 expression, which was validated at both protein and RNA level (11). The TME of this cluster, representing high PD-L1 and CD8 TILs expression, may correspond to type I TME. Furthermore, this classification has also been applied in LUAD and squamous cell carcinoma (SCC) patient cohorts, showing that LUAD patients with type III TME or SCC patients with type I TME had better survival outcomes compared to other types (12). These indicate that patients with high PD-L1 expression probably have better prognosis in certain patient group, despite the density of TILs in TME. But more studies are needed to refine the patients subgroup.

All the evidence suggested that the level of TILs, especially $CD8^+$ T cells, $FOXP3^+$ Tregs, as well as the expression of immune checkpoint (e.g., PD-L1) could be used for patients' stratification and further personalized treatment. The levels of TILs and PD-L1 in the TME, also indicate the level of T cell inflammatory responses and tumor mutational burden, and enable the stratification of patients based on TME. High expression of $CD8^+$ T cells with high levels of immune checkpoint (PD-L1) in TME usually represent positive immune response and better prognosis in cancer immunotherapies. Additionally, the ratio of $CD8^+$ T cells within the tumor and its invasive margin (the ratio is also known as Immunoscore), was found to better classify some cancers compared to the classical TNM stage, and has been validated in the past few years worldwide.

Distribution of TILs and immune checkpoints

In the previous reports, high density of $FOXP3^+$ Tregs in tumor were associated with an inferior prognosis potentially due to the inhibition of anti-tumoral immunoreaction (13). Recent studies on different immune cell distribution within either epithelial or stromal compartment showed that high densities of $FOXP3^+$ Tregs were associated with the favorable outcome (13). This further inspires the research on the spatial distribution of $FOXP3^+$ Tregs, $CD8^+$ cells and their cell-to-cell interactions. The development of multiplex-immunohistochemistry (mIHC) staining with the tyramide signal amplification enables the visibilization of local TME (14), which allows us to further investigate the spatial distribution of immune cells and the adjacent cancer cells. Besides the levels of TILs and immune checkpoints expression, the spatial distributions of these cells in lung cancer have been suggested to highly correlated with cancer patients' prognosis and survival outcomes (15,16).

Closer distance between $CD8^+FOXP3^+$ cells ($CD8^+$ Tregs) and tumor cells indicated poorer overall survival outcomes in NSCLC patients (15). The increased infiltration of Tregs into tumor region was a negative prognostic marker while high infiltration of $CD8^+$ T cells among Tregs was highly associated with better survival outcome in NSCLC (16). Interestingly, in Hodgkin lymphoma, $PD\text{-}L1^+$ TAMs were in close contact with T cells and Hodgkin Reed-Sternberg cells were close to PD-

1$^+$CD4$^+$ T cells (17), which indicated that anti-PD-1 could be used as a potential therapy.

Our group also unveiled the diversity of TME using mIHC technique, suggesting different distribution and distances between TILs and PD-L1$^+$ cells in GC (14), pancreatic cancer (unpublished data) and oral cancer (unpublished data). The diverse distribution of PD-L1$^+$ cells could also be found in various cancer types: some samples showed evenly distributed PD-L1 expression while some exhibited locally dense PD-L1 expression in TME (14,17). PD-L1 expression and distribution is also heterogeneous in different cancer patients, varying in different race, gender, smoking history and subtypes of lung cancer (18).

The diversity of PD-L1 distribution can be further studied and be used as an important criterion for selecting personalized treatment in clinical practice.

Subtype of TILs and other immune cells

A high density of TILs, especially CD8$^+$ T cells, is associated with a good prognosis in various cancers; however, it remains to be elucidated why the infiltration by TILs differs substantially even between individuals with the same cancer. Understanding the different subtype of TILs and the molecular basis of those TILs could help to identify both novel biomarkers for patient stratification and novel immunological pathways or components targeted by immunotherapy. Recently, some sub-populations of T cells in TME has been widely studied. Among them, tissue-resident memory CD8$^+$ T cells (T_{RM} cells), characterized by CD8$^+$CD69$^+$CD103$^+$, have been considered to play an important role in immune equilibrium in melanoma (19). Similar to CD8$^+$ cytotoxic T cells, T_{RM} cells can prevent and suppress the growth of solid tumor, which suggests in depth characterization of CD8$^+$ TILs in TME.

Other immune cells such as myeloid-derived suppressor cells (MDSCs) and neutrophils also play an important role in cancer immunology, but due to their low abundance, the study regarding these cell types remains difficult. MDSCs were shown to exhaust T cell activity and promote immune repressive TME in many tumor types and associated with poor prognosis (20). In NSCLC, tumor tissues had higher frequency of circulating monocytic MDSCs (M-MDSCs), PD-L1$^+$ MDSCs and C-C motif chemokine receptor 5$^+$ (CCR5$^+$) MDSCs compared to healthy donors (21). However, MDSCs frequency in peripheral blood but not in tumor tissues was reported to associate with poor prognosis (21). Thus, PD-L1 blockade has the potential to target MDSCs in lung cancer, however, detailed TME analysis regarding MDSCs are still required to understand their roles in cancer immunology.

With the advances in single cell RNA-seq (scRNA-seq), we are now able to investigate the heterogeneous tumor samples and dissect the roles of various immune cells in TME at the same time. Guo *et al*. identified seven CD8 and nine CD4 sub clusters of T cells in patient samples using blood and lung tissues. Exhausted CD8$^+$ T and CD4$^+$ Tregs were enriched in tumor samples, while naive and effector CD8$^+$ and CD4$^+$ T cells were found mainly in blood (22). This suggests that T cells in TME was distinct from peripheral blood in cancer patients, and biopsy is a reliable approach to define patient immune subtype. Exhausted T cells were marked with high expression of layilin (LAYN), immune checkpoint PD-1, hepatitis A virus cellular receptor 2 (HAVCR2), lymphocyte activating 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and CTLA4 (22,23). Two other CD8$^+$ T cells were defined as pre-exhausted with medium expression of these markers. Higher pre-exhausted/exhausted CD8$^+$ T cell ratio was associated with better prognosis in LUAD from The Cancer Genome Atlas (TCGA) database (22). This highlighted the necessity of further stratifying patients according to detailed TIL subtypes.

Besides FOXP3$^+$ Tregs and CD8$^+$ T cells, B cells and M2 macrophages were also found enriched in tumor tissues compare to normal lung tissues, while other CD4$^+$ T cells, NK cells and dendritic cells (DCs) appeared to be less abundant in tumor samples (23).

Analyses of tumor sample currently rely on the expression of a few marker genes that do not uniquely distinguish subtype of TILs, which makes it challenging to study the landscape of TILs in the tumor tissue. Combined application of high-throughput techniques, such as scRNA-seq and mass cytometry by time-of-flight (CyTOF), will undoubtedly yield more-comprehensive and reliable insights into understanding complex TIL subtypes. One limitation of scRNA-seq and CyTOF is that it can only provide the expression profiles of the TILs but cannot capture the spatial relationship of them. An alternative approach is to use spatial transcriptomics. It has been applied to melanoma and revealed a complex TME which is hard to discover using traditional histological approach (24). Although not performed on single cell, the resolution of this technique is able to provide fine TME structure coupled with gene expression profile of each spots. This makes the investigation of detailed immune cells possible.

Conclusion and future directions

Immune response is one of the most important factors that affects cancer development and treatment. Accurately dissecting the components and characteristics of local TME in each individual patient is the essential premise for precision medicine. In advanced/metastatic cancer, immune equilibrium is a dynamic state that prevent tumor cells from overgrowth and escape. Keeping metastatic tumors in an immune equilibrium status is necessary to restrain disease progression.

To achieve this, it is necessary to precisely decipher the landscape of the TME with the help of new technologies, such as mIHC staining, scRNA-seq and spatial transcriptomics. The three main aspects need to be considered are: (I) to reveal the expression of different immune cells and immune checkpoints, (II) to characterize the spatial distribution of these cells; and (III) to elucidate novel subtypes of these cells and their potential roles in TME.

Moreover, how to establish and optimize a novel and valuable methodology to stratify patients for personalized treatment is still under investigation. By using different combinations of immune checkpoints, as well as different immune cell markers, there are numerous ways to stratify patients. Importantly, patient stratification method should be closely linked to the patients' survival outcomes or prognosis, revealing a potential clinical application. Furthermore, other elements can be also further explored, such as genomic background of different patients, including novel mutations, neoantigens, etc.

Nevertheless, effective patient stratification and matched therapeutic strategies will enhance effector immune responses and overcome the immunosuppressive TME. The understanding of cancer patient stratification based on the tumor TME will eventually benefit cancer patients substantially.

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Footnote

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