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# Insights Gained from Single-Cell Analysis of Immune Cells in the Tumor Microenvironment

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## Abstract

Understanding tumor immune microenvironments is critical for identifying immune modifiers of cancer progression and developing cancer immunotherapies. Recent applications of single-cell RNA sequencing (scRNA-seq) in dissecting tumor microenvironments have brought important insights into the biology of tumor-infiltrating immune cells, including their heterogeneity, dynamics, and potential roles in both disease progression and response to immune checkpoint inhibitors and other immunotherapies. This review focuses on the advances in knowledge of tumor immune microenvironments acquired from scRNA-seq studies across multiple types of human tumors, with a particular emphasis on the study of phenotypic plasticity and lineage dynamics of immune cells in the tumor environment. We also discuss several imminent questions emerging from scRNA-seq observations and their potential solutions on the horizon.

## INTRODUCTION

Cancer is a complex disease of genetic alterations that threatens human health owing to loss of growth-control mechanisms and maladaptive cross talk with the human body, including the immune system. While the immune system can successfully protect the host from infections of most pathogens via its multidimensional innate and adaptive effector mechanisms, its role in eradicating cancers can be compromised by immunoediting of tumor neoantigens, recruitment of immunosuppressive myeloid cells, and hijacking of the T cell checkpoint pathways that control T cell responses (1, 2). Over the last 25 years, researchers have been focused on T cells and their immune checkpoint proteins, resulting in successful cancer immunotherapies that provide durable clinical responses to previously incurable diseases (3–5). Current cancer immunotherapies, including cancer vaccines, adoptive immune cell transfer, and in particular immune checkpoint blockade, are clinically effective for multiple cancers, such as melanoma, lymphoma, head-and-neck cancers, clear cell renal carcinomas, microsatellite-instable (MSI) tumors, gastric cancer, bladder cancer, and non-small cell lung cancer (6–15). However, the clinical benefit is only available for a fraction of patients. Understanding the precise cellular and molecular mechanisms underlying tumor immune escape remains a critical task to further improve the current immunotherapies or develop new therapeutic avenues.

As critical players in tumor immunity, $CD8^+$ T cells can directly recognize and kill cancer cells upon recognition of neoantigens and also respond to various cues to tune their developmental lineages and states (**Figure 1**). $CD4^+$ T cells, including highly suppressive $FOXP3^+$ regulatory CD4 T cells, can also orchestrate diverse immune responses to enhance or suppress immunity against cancer cells. Dendritic cells (DCs) initiate and regulate both innate and adaptive immune responses against tumors by presenting cancer antigens and secreting pro- and anti-inflammatory cytokines and chemokines. Macrophages promote cancer initiation, malignant progression, and even metastasis by stimulating angiogenesis, promoting nonproductive inflammation and suppressing antitumor immunity (16, 17).

The developmental diversity and phenotypic plasticity of immune cells enable their dynamic and specialized responses to various immunogens, and some of these properties can be explored using traditional approaches. Flow cytometric analysis of dozens of immune cell lineage and cell fate markers is now commonplace, augmented by bulk genomic profiling of tumor tissues and immune status deconvolution (18, 19). Even with the advances of flow cytometry and bulk tissue RNA sequencing (RNA-seq), technological limitations prohibit panoramic characterization of the phenotypes and functions of tumor-infiltrating immune cells and thus hinder our understanding of the inefficient elimination of malignant cells in most cancer patients. The rapid development of single-cell RNA-seq (scRNA-seq) technologies has provided powerful tools to dissect the heterogeneity of tumor-infiltrating immune cells and their interactions with the diverse normal and abnormal cell types in tumors (20–24). With scRNA-seq, the quantities and qualities of tumor-infiltrating immune cells of many cancers have been resolved at unprecedented resolution, including melanoma (25–31); lymphoma (32); glioma (33–37); cholangiocarcinoma (38); and nasopharyngeal (39), breast (40–44), head-and-neck (45), colorectal (46–49), gastric (50–52), liver (53–56), kidney (57, 58), pancreas (59–64), bladder (65, 66), ovary (67), and lung (68–72) cancers. These studies were either specifically designed for tumor-infiltrating T cells or broadly applied to the whole tumor immune microenvironments (**Figure 1**), bringing new knowledge and deep insights into the composition, heterogeneity, dynamics, and regulation of immune cells in tumor microenvironments. The studies also suggest novel gene targets to pursue as well as promising gene-based biomarkers to stratify patients for clinical actions.

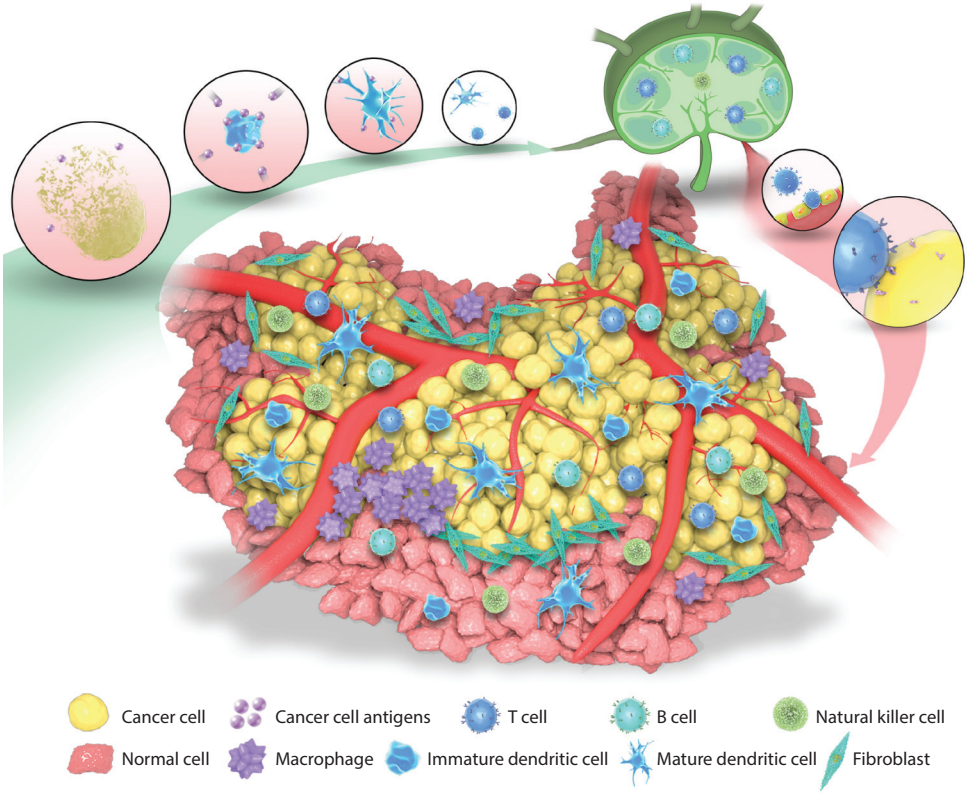


**Figure 1**

The architecture and surroundings of the tumor microenvironment. The tumor microenvironment is composed of multiple cell types including cancer cells, fibroblasts, endothelial cells, and various immune cells. The complicated and dynamic interactions among these cells together with the surrounding systems make the tumor microenvironment greatly heterogeneous. In particular, dendritic cells efferent from tumors can present cancer-related antigens to T cells after they enter lymph nodes. Then, activated T cells leave lymph nodes and are recruited to the tumor to clear cancer cells. Various mechanisms can interfere with this immune cycle and result in tumor immune escape. Single-cell RNA sequencing has been an ideal tool to dissect such heterogeneity; the underlying dynamics; and the immune interplay of these diverse cell types in the tumor microenvironment with the adjacent tissues, lymph nodes, and peripheral blood.

scRNA-seq methods can also track cell lineages and the dynamics of cell fate and differentiation in multiple ways. Variations on scRNA-seq methods allow sequencing and reconstruction of full-length and paired T cell receptor (TCR) α and β sequences for individual T cells (40, 73), allowing lineage identification and tracking (46), and corresponding antibody–B cell receptor sequences can also be obtained from B cells. Interrogation of mitochondrial mutations is another method that can identify and track individual cell lineages across a broader collection of cell types (74). Finally, the study of nuclear versus cytoplasmic RNA populations in individual cells, known as RNA velocity analysis (75), can give insights into the dynamics of cell fate. Lineage and cell fate analysis methods have provided promising biological insights and deserve a systematic review and discussion.

In this review we focus on tumor-infiltrating T cells, DCs, and macrophages and discuss their gene expression heterogeneity, compositional differences among cancer types, cell dynamics, and cell origins revealed by TCR sequencing (22, 73), mitochondrial mutation tracking (74), and RNA

velocity analyses (75), and their potential roles in tumor immunity and in response or resistance to immunotherapy. We also briefly discuss questions emerging from current scRNA-seq studies of tumor-infiltrating immune cells and possible future directions in the field.

## THE CURRENT STATE OF TUMOR-INFILTRATING IMMUNE CELL STUDIES BY scRNA-seq

The rapid development of single-cell sequencing technologies has progressed with a speed parallel to Moore's law in computer science (21–24, 76–93). With both technological advances and cellular throughput increasing exponentially, multiple layers of information including epigenomic, genomic, transcriptomic, and proteomic characteristics of individual cells and their combinations can be obtained at unprecedented resolution and cost. Such high resolution is ideally suited to studying the properties of immune cells, which are well-known for their diverse developmental lineages, antigen specificities, phenotypic plasticity, and adaptability to various microenvironments. The technological advances have been recently reviewed (94); thus, we focus on summarizing the insights gained from the wide application of scRNA-seq for understanding tumor-infiltrating immune cells across human cancers.

Current tumor-infiltrating immune cell studies frequently use two types of scRNA-seq technologies: one plate based (individual cells are sorted into plates and RNA libraries are prepared in the wells) and the other droplet based (individual cells are encased in an aqueous droplet inside an oil emulsion). Some platforms sequence the full RNA transcripts, while other methods sequence only a shorter, representative transcript region. It remains an elusive goal to produce a complete and unbiased library of RNA transcripts from single cells by most or all methods so as to produce reduced-complexity libraries and missing transcripts that theoretically should have been present (dropouts).

Plate-based scRNA-seq methods include SMART-seq2 (22), which has a low dropout rate parallel to that of bulk RNA-seq and thus allows comprehensive characterization of a broader spectrum of gene expression. The full-length mRNA coverage by SMART-seq2 also allows quantification of expression at the transcript isoform level and assembly of the full-length mRNA sequences with high abundance, e.g., TCRs (73, 95). However, plate-based scRNA-seq is high-cost and has a low cellular throughput compared with droplet-based technologies, which have been improved with the recently proposed combinatorial indexing strategy (96). Droplet-based scRNA-seq, represented by solutions provided by 10x Genomics (24), can obtain scRNA-seq data for thousands of cells in one experiment, while plate-based techniques are often limited to tens to hundreds. The higher cell throughput enables more comprehensive sampling of cell types in a tissue, particularly for rare cell types. However, droplet-based scRNA-seq has its own technical defects, with even moderately expressed genes prone to dropouts. Droplet-based scRNA-seq methods often sequence only the 3′ or 5′ ends of mRNAs (24, 40), resulting in issues for isoform quantification and full-length mRNA assembly. Despite the advantages and disadvantages of these two types of technologies, they have both been successfully applied to tumor microenvironment characterization, and efforts combining the complementary strengths have provided more comprehensive illumination of tumor microenvironments (48, 53).

Another distinct characteristic among recent tumor-infiltrating immune cell studies lies in different strategies for sample collection and selection of relevant control tissues within each study. Either all cells in the tumor microenvironment, $CD45^+$ immune cells, or specifically $CD45^+CD3^+$ T cells have been enriched and then subjected to scRNA-seq in different studies. Such different immune cell enrichment methods introduced study-specific biases, which can interfere with subsequent integrative analyses and knowledge synthesis due to the different cell

capture rates and the final cellular resolution in each patient. Because of the migratory nature of immune cells, tumor-matched immune-relevant tissues including tumor-adjacent normal tissues, lymph nodes, and peripheral blood can play an important role in illustrating the biology of tumor-infiltrating immune cells. Such distinction in cell collections of tumor-matched tissues among different studies would result in different interpretability of the scRNA-seq observations. The third dimension of variation between studies lies in the longitudinal nature of the study design, with many studies having only single-subject time points available and some studies including longitudinal changes (especially pre- and posttreatments). Despite such a multitude of differences, many consistent biological observations have been made across cancer types for tumor-infiltrating immune cells and thus shed important light on the underlying mechanisms of tumor immune escape.

## TUMOR-INFILTRATING CD8$^+$ T CELLS ARE CHARACTERIZED BY A MIXTURE OF DYSFUNCTIONAL AND EFFECTOR T CELLS

Recent tumor-infiltrating T cell studies cover a wide range of cancers, including melanoma (25–30); glioma (33–36); and breast (40–43), head-and-neck (45), colorectal (46–49), liver (53–56), gastric (50, 51), pancreas (59–61), kidney (57, 58), bladder (65), and lung (68–71) cancers. In these studies, various T cell subsets were identified (**Table 1**). CD8$^+$ T cells can be observed in varying states of dysfunction, with reduced ability to perform cytotoxic functions, a phenomenon known as T cell exhaustion (97). Dysfunctional or exhausted T cells in the tumor microenvironment are characterized by elevated expression of inhibitory receptors, including PD-1, CTLA-4, TIM-3, TIGIT, and LAG3. Although these cells can express a subset of cytotoxicity-associated genes including those encoding interferons, granzymes, and perforins (*IFNG*, *GZMB*, and *PRF1*), suggesting an activated effector state, the expression of IL-2, tumor necrosis factor alpha (TNF-α), and T-box transcription factor (*TBX21*) is absent or at a low level. Notably, a small proportion of the exhausted CD8$^+$ T cells show high expression levels of *MKi67*, a marker of active proliferation (98), across multiple cancer types including melanoma (30, 31) and liver (53, 54), lung (68–70), breast (40–43), colorectal (46–49), and bladder (65) cancers. The proliferative CD8$^+$ T cells show a slightly lower exhaustion signal compared with the nonproliferative exhausted CD8$^+$ T cells, indicating that the exhaustion process may lag behind T cell proliferation (31). TCR-based tracking in liver, lung, and colorectal cancers reveals that many of the proliferative exhausted CD8$^+$ T cells share the same TCR sequences (paired α and β chains) with cells with exhausted phenotypes (46, 54, 69), suggesting that they have the same origins but different states. The induction of the exhaustion states of these tumor-infiltrating CD8$^+$ T cells may be attributable to prolonged exposure to their respective antigens in the tumor microenvironment or the contributions of the immunosuppressive nature within the tumor due to inhibitory ligands, soluble mediators, the metabolic milieu, or suppressive immune and stromal cells (99).

Another common CD8$^+$ T cell subset revealed by scRNA-seq across multiple cancer indications is characterized by high expression levels of *GZMK* rather than *GZMB*. These GZMK$^+$ T cells express little or no T cell exhaustion–associated markers and have been identified in liver (53–56), lung (68–71), colorectal (46–49), breast (40–43), pancreas (59–61), kidney (57, 58), and head-and-neck (45) cancers and melanoma (25–30). The wide presence of this GZMK$^+$ T cell subset across different types of tumors suggests a role in tumor immunity. In fact, the ratio of this GZMK$^+$CD8$^+$ T cell subset to the exhausted T cells in tumors has been positively associated with better survival of patients with non-small cell lung adenocarcinomas (69) and with better response to immune checkpoint inhibition in melanoma (29). TCR analysis in deeply sequenced liver (54), lung (69), and colorectal (46) cancers reveals tight links between GZMK$^+$ and exhausted T cells,

**Table 1 Typical human CD8$^+$ and CD4$^+$ T cell subsets revealed by scRNA-seq**

| T cell subsets | Enriched tissues | Signature genes | Cancer types | Annotation |
|---|---|---|---|---|
| **CD8$^+$ T cells** | | | | |
| LEF1$^+$ Tn | Blood | *CCR7*, *LEF1*, *SELL*, *TCF7*, *S1PR1* | Melanoma (29–31)<br>HNSCC (45)<br>BC (40)<br>BCC (141)<br>HCC (53, 54)<br>NSCLC (69)<br>CRC (46, 48) | Tn/Tn-like cells |
| GPR183$^+$ Tcm | Blood | *CCR7*, *SELL*, *IL7R*, *PRF1*, *GZMA*, *CCL5*, *GPR183*, *S1PR1* | Melanoma (29)<br>NSCLC (68, 69)<br>BC (40)<br>CRC (46, 48)<br>HCC (54) | Tcm/Tn-like/Tm cells |
| CX3CR1$^+$ Temra/Teff | Blood | *CX3CR1*, *FCGR3A*, *FGFBP2*, *KLRG1*, *PRF1*, *GZMH*, *TBX21*, *EOMES*, *S1PR1*, *S1PR5* | Melanoma (29–31)<br>HNSCC (45)<br>NSCLC (68, 69)<br>BCC (141)<br>HCC (53, 54)<br>CRC (46, 48) | Teff/cytotoxic T/Temra cells |
| XCL1$^+$ Trm | Tumor-adjacent | *XCL1*, *XCL2*, *MYADM*, *CAPG*, *CD6*, *NR4A1/2/3*, *CD69*, *ITGAE* | CRC (46, 48) | Trm cells |
| CD160$^+$ IEL | Tumor-adjacent | *CD160*, *KIR2DL4*, *TMIGD2*, *KLRC1/2/3*, *NR4A1/2/3*, *IKZF2*, *ENTPD1*, *CD69*, *ITGAE* | CRC (46, 48) | IELs/intraepithelial T cells |
| SLC4A10$^+$ MAIT | Tumor-adjacent, tumor | *SLC4A10*, *ZBTB16*, *KLRB1*, *NCR3* | HCC (53, 54) | MAIT cells |
| GZMK$^+$ Tem | Tumor-adjacent, tumor | *GZMK*, *CXCR4*, *CXCR3*, *CD44* | Melanoma (29, 31)<br>TNBC (42)<br>BC (40)<br>BCC (141)<br>HCC (53, 54)<br>CRC (46, 48) | Tem/transitional T cells |
| ZNF683$^+$ Trm | Tumor | ZNF683, HOPX, ITGAE | NSCLC (68, 69) | Trm/pre-exhausted T cells |
| LAYN$^+$ Tex | Tumor | *HAVCR2*, *PDCD1*, *CTLA4*, *TIGIT*, *LAG3*, *LAYN*, *TNFRSF9*, *TOX*, *CXCL13*, *IFNG*, *GZMB*, *ITGAE* | Melanoma (29–31)<br>HNSCC (45)<br>BCC (141)<br>NSCLC (68, 69)<br>TNBC (42)<br>HCC (53, 54)<br>CRC (46, 48, 68) | Tex/dysfunctional T cells |
| **CD4$^+$ Th cells** | | | | |
| CCR7$^+$ Tn | Blood | *CCR7*, *LEF1*, *SELL*, *TCF7*, *S1PR1* | Melanoma (30, 31)<br>BC (40)<br>HCC (53, 54)<br>CRC (46, 48) | Tn cells |

(*Continued*)

**Table 1 (*Continued*)**

| T cell subsets | Enriched tissues | Signature genes | Cancer types | Annotation |
|---|---|---|---|---|
| ANXA1$^+$ Tcm | Blood | *CCR7*, *SELL*, *PTGER2*, *ICAM2*, *ANXA1*, *ANXA2*, *S1PR1* | Melanoma (29)<br>BC (40)<br>HCC (53, 54)<br>CRC (48, 54)<br>NSCLC (69) | Tcm/Tn-like cells |
| GNLY$^+$ Temra/Teff | Blood | *CX3CR1*, *KLRG1*, *NKG7*, *PRF1*, *GNLY*, *GZMH*, *TBX21*, *CTSW*, *S1PR1*, *S1PR5* | Melanoma (30)<br>HCC (53)<br>CRC (48)<br>NSCLC (69) | Teff/cytotoxic T/Temra cells |
| TCF7$^+$ Tn | Tumor-adjacent | *CCR7*, *TCF7*, *RGS1*, *CD69*, *CXCR5* | TNBC (42)<br>CRC (46, 48) | Tn-like T cells |
| Tfh | Tumor-adjacent | *CXCR5*, *BCL6*, *ICA1*, *TOX*, *TOX2*, *IL6ST*, *MAGEH1*, *BTLA*, *ICOS*, *PDCD1*, *CD200* | Melanoma (31)<br>BCC (141)<br>CRC (46, 48) | Tfh cells |
| CXCR6$^+$ Trm | Tumor-adjacent, tumor | *CXCR6*, *CD69*, *KLRB1*, *PTGER4*, *IL7R*, *NR4A1/2/3*, *MYADM* | CRC (46, 48)<br>HCC (54)<br>NSCLC (69) | Trm cells |
| GZMK$^+$ Tem | Tumor-adjacent, tumor | *GZMK*, *GZMA*, *CCL5*, *IFNG*, *RUNX3*, *EOMES*, *CXCR3*, *CXCR4*, *CD44* | BC (40)<br>CRC (46, 48)<br>HCC (54)<br>NSCLC (69) | Tem cells |
| Th17 | Tumor | *IL17A*, *IL23R*, *RORC*, *FURIN*, *CTSH*, *CCR6*, *KLRB1*, *CAPG*, *ITGAE* | BCC (141)<br>CRC (46, 48) | Th17 cells |
| CXCL13$^+$ Th1-like cells | Tumor | *CXCL13*, *IFNG*, *CXCR3*, *BHLHE40*, *GZMB*, *PDCD1*, *HAVCR2*, *ICOS*, *IGFLR1*, *ITGAE* | Melanoma (31)<br>TNBC (42)<br>HCC (53, 54)<br>CRC (46, 48)<br>NSCLC (69) | Th1-like/CD4$^+$ Tex/dysfunctional T cells |
| **CD4$^+$ Tregs** | | | | |
| Treg | Blood | *FOXP3*, *IL2RA*, *IL10RA*, *IKZF2*, *RTKN2*, *CDC25B*, *S1PR4* | HCC (54)<br>NSCLC (69)<br>CRC (46) | Blood Treg cells |
| IL10$^+$ Tfr | Tumor-adjacent | *FOXP3*, *IL2RA*, *CXCR5*, *PDCD1*, *IL10*, *CCR4*, *CD69* | CRC (46) | Tfr cells |
| CCR8$^+$ Treg | Tumor | *FOXP3*, *CTLA4*, *CCR8*, *LAYN*, *TNFRSF9*, *TNFRSF18*, *IKZF2*, *RTKN2*, *BATF* | Melanoma (29–31)<br>HNSCC (45)<br>BC (40)<br>NSCLC (69, 70)<br>TNBC (42)<br>HCC (53, 54)<br>CRC (46, 48) | Tumor Treg cells |

Abbreviations: BC, breast cancer; BCC, basal cell carcinoma; CRC, colorectal cancer; HCC, hepatocellular carcinoma; HNSCC, head-and-neck squamous cell carcinoma; IEL, intraepithelial lymphocyte; MAIT, mucosal-associated invariant T; NSCLC, non-small cell lung cancer; scRNA-seq, single-cell RNA sequencing; Tcm, central memory T; Teff, effector T; Temra, effector memory recently activated T; Tex, exhausted T; Th, T helper; Tfh, follicular helper T; Tfr, follicular regulatory T; Tn, naive T; TNBC, triple-negative breast cancer; Treg, regulatory T; Trm, tissue-resident memory T.

suggesting that these T cells may target the same antigens but undergo different state transitions due to the differences of individual cell niches. As this GZMK$^+$ T cell subset is also CD45RO$^+$ and has high expression of *IFNG* and *PRF1*, it is regarded as a group of CD8$^+$ effector memory T cells. Of note, the distinction between effector memory and exhausted T cells appears to be not binary but a continuum (100).

The relative proportions of exhausted and effector memory T cells vary in patients and across cancer cell types, with non-small cell lung cancers showing a relatively low frequency of exhausted T cells and a relatively higher proportion of effector memory T cells (46, 69). However, the proportion of exhausted T cells is much higher in hepatocellular carcinomas (46, 54). The relative composition of a tumor regarding the ratio of effector memory and exhausted T cells in its tumor microenvironment may be a promising prognostic marker for patient survival and a predictive marker for the responses to immunotherapies (29), and it deserves thorough investigation across all cancer types.

## DYNAMICS OF TUMOR-INFILTRATING CD8$^+$ T CELLS INFORMED BY TCRs

The diversity of TCRs provides a natural molecular marker to identify T cell clones and track dynamics of clonal populations (101). scRNA-seq technologies such as SMART-seq2 and 10x Genomics 5′+VDJ methods allow both gene expression and TCR identification (40, 73), and their application to tumor-infiltrating T cells has brought profound insights into the dynamics of CD8$^+$ T cells.

Four types of T cell dynamics can be quantitatively evaluated with a bioinformatics method named as STARTRAC (46), including the degree of clonal expansion, the propensity of transcriptomic states, the enrichment in a specific tissue, and the inclination to migrate to other tissues. For various human cancers, clonal expansion is observed for both the effector and exhausted tumor-infiltrating CD8$^+$ T cells, with the latter generally having a higher expansion level (30, 40, 46, 54, 65, 69). Such distinction may originate from their clonal composition differences, as the clonality of different T cell clones is uneven in tumors and probably follows a power-law distribution (69). It may also indicate that the phenotypic differences, i.e., effector memory versus exhaustion, may be TCR dependent. Despite the differences in both phenotypes and the clonal expansion levels, T cell clones including cells from both the GZMK$^+$ effector memory and the exhausted CD8$^+$ T cells are frequently observed in multiple tumors (30, 40, 46, 54, 69), indicating that GZMK$^+$ effector memory T cells may differentiate or switch their phenotypes into the exhausted states. Such observations in tumors are consistent with the finding of a group of stem-like T cells in both viral infection and tumor treatment settings (102–106) that are characterized by high expression of *CXCR5* and *TCF1*/*TCF7* and that are thought to be progenitors of exhausted CD8$^+$ T cells and a subset of the GZMK$^+$ effector memory T cells.

One notable distinction between GZMK$^+$ effector memory CD8$^+$ T cells and the exhausted T cells is their differences in tissue preference and mobility across tissues. In liver, lung, and colorectal cancers that have multiple autologous control tissues available (46, 54, 69), GZMK$^+$ effector memory CD8$^+$ T cells are enriched in both tumors and the tumor-adjacent normal tissues, while the exhausted T cells are enriched in tumors only. T cell clones spanning tumor-adjacent normal tissues and peripheral blood or tumors are frequently found in GZMK$^+$CD8$^+$ T cells, but few clones are found spanning all three tissues. These trends are also observed in other cancer types, although the numbers of autologous control tissues vary (31, 40). As systemic immunity is necessary for effective immunotherapies in both mouse and human (107), the infrequent TCR sharing between T cell clones in the peripheral blood and tumors, in contrast to the frequent TCR

sharing of the GZMK$^+$CD8$^+$ T cells among all tested locations, may indicate a mechanism of tumor immune escape. In liver cancer patients with ascites data, GZMK$^+$ effector memory CD8$^+$ T cells, but not those exhausted T cells, are more frequently found in ascites (53).

## TUMOR MICROENVIRONMENTS ARE SHAPED IN PART BY REGIONAL IMMUNITY OF SPECIFIC TISSUES

It is increasingly evident that tumors with different anatomical origins show distinct T cell properties. In scRNA-seq studies of T cells from patients with hepatocellular carcinoma (54), non-small cell lung cancer (69), or colorectal cancer (46) that have multiple autologous control tissues available, similar T cell compositions are observed in peripheral blood for all three cancer types (46). In contrast, distinct T cell subsets emerge when different tumors that have distinct tissue origins are compared. Mucosal-associated invariant T (MAIT) cells are significantly enriched in hepatocellular carcinomas (46, 54), with high expression of perforins and diverse granzymes. However, in tumors of non-small cell lung cancers, MAIT cells are seldom found, but a CD8$^+$ T cell subset characterized by the transcription factor *ZNF683* emerges (46, 69). CD8$^+$ZNF683$^+$ T cells highly express *PRF1*, *GZMB*, and *PDCD1* but have relatively low expression levels of *IFNG* and other exhaustion markers, including *CTLA-4*, *HAVCR2*, *TIGIT*, and *LAG3*, compared with the exhausted CD8$^+$ T cells (69). Interestingly, tight TCR links can be observed between this CD8$^+$ZNF683$^+$ T cell subset and those exhausted T cells (69), suggesting that tumors may hijack and reprogram this lung-resident CD8$^+$ T cell subset to escape immune attacks. In colorectal cancers, a CD6$^+$CD8$^+$ T cell subset is uniquely observed (46), characterized by high expression levels of *IFNG*, *GZMB*, and *PRF1*; low expression levels of *PDCD1*, *CTLA4*, and *HAVCR2*; relatively high levels of *TIGIT* and *LAG3*; and TCR sharing with exhausted T cells. In breast cancer, γδ T cells are abundant in the tumor microenvironment (40, 42). Some of these tumor-histology-specific T cell subtypes have been associated with patient prognosis. For example, the abundance of the CD8$^+$ZNF683$^+$ T cell subset is associated with better survival for non-small cell lung cancer patients (69), and these contributions of tissue-regional T cell subsets will likely be relevant to success or failure of immunotherapies (10, 108, 109).

## THE ORIGINS OF TUMOR-INFILTRATING CD8$^+$ T CELLS INDICATED BY scRNA-seq

The life cycle of T cells, which originate in the thymus, mature in lymph nodes, migrate through blood and lymphatic vessels, and function in the target tissue, has been difficult to assess by traditional methods. The application of scRNA-seq to simultaneously chart the cell identity and TCRs of individual T cells together with various autologous immune-relevant controls provides an opportunity to estimate the sources of tumor-infiltrating T cells in patients for the first time (46, 53, 54, 69).

The high degree of clonal expansion of exhausted CD8$^+$ T cells and GZMK$^+$ effector memory CD8$^+$ T cells within multiple tumor types clearly demonstrates that local expansion is a major source of tumor-infiltrating CD8$^+$ T cells (**Figure 2**). The presence of a small group of proliferating T cells that have tight TCR links with exhausted T cells and sometimes with GZMK$^+$ T cells suggests ongoing local expansion (31, 46, 54, 69), but it is still not clear how local expansion of CD8$^+$ T cells is regulated in tumors, even though a few indications are available (110–115). The shared TCRs of GZMK$^+$ effector memory CD8$^+$ T cells in tumor-adjacent tissues with exhausted CD8$^+$ T cells suggest that tumor-infiltrating CD8$^+$ T cells may also originate from the migration of GZMK$^+$ effector memory T cells, followed by phenotypic transitions to the exhausted state.

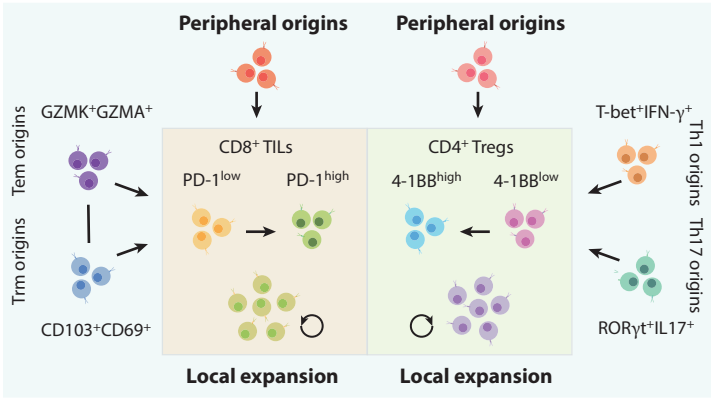


**Figure 2**

Origins and dynamics of tumor-infiltrating CD8$^+$ exhausted T cells and CD4$^+$ Tregs. The phenotypes of tumor-infiltrating CD8$^+$ T cells show great heterogeneity and can generally be categorized into a continuum, from preexhausted (PD-1$^{low}$) to terminally exhausted (PD-1$^{high}$). Local expansion is the main source of CD8$^+$ T cells within tumors, while CD103$^+$CD69$^+$ Trms and GZMK$^+$GZMA$^+$ Tems are the main external sources for multiple cancer types. State transitions frequently occur after the migration of Trms and Tems from tumor-adjacent tissues to tumors due to the immunosuppressive tumor microenvironments. Although peripheral origins of CD8$^+$ T cells are observed at low frequency, they are relevant to patient responses to cancer immunotherapies in various cancer types. The phenotypes and origins of Tregs show similarity to those of tumor-infiltrating CD8$^+$ T cells, with local expansion as the main source and peripheral origins and conversion with Th1 and Th17 cells in the tumor and tumor-adjacent tissues as the external sources. Such phenotypic and dynamics similarity between CD8$^+$ T cells and Tregs in tumors may partially be spatially instructed, as revealed by single-cell RNA sequencing plus spatial transcriptomics and ligand-receptor-based computational modeling (159, 161, 162). Abbreviations: IFN-γ, interferon gamma; Tem, effector memory T cell; Th1, type 1 T helper cell; Trm, tissue-resident memory T cell; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.

As the enrichment of GZMK$^+$ effector memory T cells in tumors and their adjacent tissues is observed in multiple cancer types, including melanoma (29) and liver (54), lung (69), colorectal (46), breast (40, 42), and gastric (50, 51) cancers, infiltration of such cells into tumors may be a general mechanism for providing the external sources of CD8$^+$ T cells in tumors in addition to the local expansion mechanism (**Figure 2**).

A subset of GZMK$^+$ effector memory CD8$^+$ T cells also shares TCRs with blood-enriched CD8$^+$CX3CR1$^+$ T cells showing effector or effector memory phenotypes in multiple cancer types (46, 53, 54, 69, 116). In particular, T cell clones that span these three subsets, although with low frequency, have been observed across these cancer types. Such clones may indicate some of the origins of tumor-infiltrating effector memory or exhausted T cells, and the low frequency may indicate failure of systemic immune responses to tumor neoantigens and thus result in tumor immune escape. It has been recently shown that peripheral T cell expansion can predict tumor infiltration and clinical responses to anti-PD-L1 immunotherapies in renal and lung cancer patients (117), highlighting the importance of such T cells of peripheral origins. Such low frequency of T cell clones spanning these three states may also provide an explanation for the mutually exclusive TCR sharing of GZMK$^+$ T cells with blood-origin CD8$^+$CX3CR1$^+$ T cells and exhausted T cells in multiple tumor types (46, 54, 69). However, it is still unknown whether these T cell clones are tumor reactive, and further investigations are needed.

It is worth noting that although the CD8$^+$CX3CR1$^+$ T cells have no or low exhaustion signatures (PD-1$^-$) and are blood enriched, they exhibit the highest migratory potentials across blood,

tumors, and tumor-adjacent normal tissues for liver, lung, and colorectal cancers (46, 54, 69, 116). When transitioning from blood to solid tissues, e.g., liver, lung, and colon, $CD8^+CX3CR1^+$ T cells tend to undergo state transitions to $GZMK^+$ effector memory $CD8^+$ T cells, evidenced by the highest state transition score evaluated by STARTRAC (46). Such T cells are also highly expanded, but they are rarely observed to harbor the signature of cell proliferation (46). Such disparity between clonal expansion and cell proliferation highlights the necessity of identifying the sources of this group of T cells, as well as their antigenicity and fates after tumor infiltration.

Studies equipped by scRNA-seq also demonstrate that tissue-resident T cells are important origins of tumor-infiltrating T cells. In the lung, the group of T cells characterized by high expression of *ZNF683*, *ITGAE*, and *CD69* share more TCRs with those exhausted T cells than $GZMK^+$ effector memory T cells (69). Recent scRNA-seq studies of COVID-19 patients demonstrate the presence of $ZNF683^+$ T cells in lung and their possible roles in eliminating SARS-CoV-2 infections (118), confirming the lung-resident nature of this T cell subset and its role in maintaining lung homeostasis. The shared TCRs between $ZNF683^+$ and exhausted T cells, together with the better survival advantage of lung cancer patients with high infiltration of $ZNF683^+$ T cells, highlight the importance of tissue-resident T cells as antitumor agents. However, the antitumor effects of tissue-resident T cells vary among tissues and the T cell subtypes. In the liver, MAIT cells are tissue-resident (119, 120), but they tend to be depleted from the tumor microenvironment of hepatocellular carcinomas (54). In colorectal cancers, two groups of tissue-resident T cells are found, i.e., $CD160^+CD8^+$ T cells (resembling intraepithelial lymphocytes) and $CD6^+CD8^+$ T cells (46). But $CD160^+CD8^+$ T cells are depleted from the tumor microenvironment of colorectal cancers and have few TCRs shared with exhausted T cells. In contrast, $CD6^+CD8^+$ T cells are enriched in both tumor and adjacent mucosa tissues, and they have high TCR sharing with both exhausted T cells and the $GZMK^+$ effector memory $CD8^+$ T cells. In breast cancer, γδ T cells are tissue-resident and frequently found in tumors by scRNA-seq studies (40–43). Although shared TCRs are also frequently observed between $GZMK^+$ effector memory T cells and tissue-resident T cells such as $ZNF683^+$ T cells in lung (69) and $CD6^+$ T cells in colon (46), the roles of such transitions between tissue-resident and effector memory T cells in clearing tumor cells are unknown.

In a nutshell, scRNA-seq studies of various tumors provide important insights into the origins of tumor-infiltrating T cells. A consistent paradigm across multiple cancer types is revealed for $CD8^+$ T cells (**Figure 2**), with (*a*) self-expansion being the primary source, (*b*) migration of $GZMK^+$ effector memory T cells from adjacent tissue being the primary external source, and (*c*) tissue-resident T cells forming the third source type but varying among tumor types. The relative contributions of these three mechanisms may be relevant to the immune state of tumor microenvironments. Evidence has shown the benefits of higher levels of T cell infiltration originated from effector memory and tissue-resident memory cells (29, 42, 69, 108), particularly when systemic T cell responses are invoked (107, 117). However, the regulatory mechanisms underlying $CD8^+$ infiltration still need more investigation.

## COMPARISON OF REGULATORY T CELL PROPERTIES TO THOSE OF TUMOR-INFILTRATING $CD8^+$ T CELLS

While consistent patterns are observed for tumor-infiltrating $CD8^+$ T cells across diverse cancer types, the situations of $CD4^+$ T cells are more complicated. Overall, $CD8^+$ T cells have higher clonal expansion than $CD4^+$ T cells, with higher ratios of clonal $CD8^+$ T cells than $CD4^+$ T cells detected when the same sequencing depth is applied (46, 54, 69). An exception is $CD4^+FOXP3^+$

regulatory T cells (Tregs), which show clonal expansion patterns and other biological behaviors similar to those of tumor-infiltrating $CD8^+$ T cells (46, 54, 69).

In contrast to the globally low degree of clonal expansion of tumor-infiltrating $CD4^+$ T cells, tumor-resident Tregs demonstrate higher clonal expansion levels across a diverse set of cancer types (**Figure 2**), and this population shows markers consistent with higher proliferation (40, 46, 54, 69). Similar to the case of tumor-infiltrating $CD8^+$ T cells, local expansion may be the main source of tumor-infiltrating Tregs. Shared TCRs are also observed between tumor-infiltrating Tregs and conventional $CD4^+$ T cells (Tconvs) [type 1 T helper (Th1)-like and Th17] and at low frequency with Tregs in the tumor-adjacent normal tissues (**Figure 2**), suggesting that natural and induced Tregs may both play important roles in providing the external sources of tumor-infiltrating Tregs.

In addition to clonal expansion, tumor-resident Tregs also demonstrate phenotypes partially similar to those of exhausted $CD8^+$ T cells in tumors, which express high levels of inhibitory receptors including *CTLA4*, *TIGIT*, *PDCD1*, *HAVCR2*, and *LAG3*. Similarly, these cells tend to be restricted to tumors, showing low migration scores according to STARTRAC analysis (46). With in silico sorting by 4-1BB, tumor-resident Tregs can be divided into two subgroups (69), with one subgroup expressing fewer inhibitory receptors than the other, similar to the relationship between $GZMK^+$ effector memory $CD8^+$ T cells and the exhausted T cells in tumors. However, such similarity is absent in blood-enriched Tregs, which show lower expression levels of genes associated with Treg activation, including *FOXP3*, *CCR8*, and *IL2RA*. The enrichment of tumor Tregs, particularly the $4\text{-}1BB^+$ subgroup, has been linked to poor prognosis of non-small cell lung cancer patients (69), underscoring their immunosuppressive role. Moreover, the identification of a group of $CD8^+FOXP3^+$ T cells in hepatocellular carcinoma and other cancers further highlights the potential connections of tumor $CD8^+$ T cells and Tregs (54).

## EMERGING PATTERNS OF CONVENTIONAL T CELLS IN TUMOR MICROENVIRONMENTS

Compared to Tregs and $CD8^+$ T cells, Tconvs show greater heterogeneity, in terms of both gene expression and TCR repertoire, in scRNA-seq studies across patients with different cancer types. Interesting patterns are beginning to emerge. A specific subset of Th1-like $CD4^+$ T cells is significantly enriched in the tumor microenvironment of MSI but not microsatellite-stable (MSS) colorectal cancer patients (46). This $CD4^+$ T cell subset is characterized by *CXCL13* and *BHLHE40* positivity as well as high expression levels of *IFNG* and *GZMB*, suggesting their effector functions and regulatory roles. These $CXCL13^+BHLHE40^+$ cells are clonally expanded and share TCRs with $CD4^+GZMK^+$ helper T cells. The enrichment of such Th1-like cells in MSI patients may explain their favorable response to immunotherapies (12, 13), similar to the relevance of specific cytotoxic $CD4^+$ T cell subsets to the effective responses in melanoma (107) and bladder cancer (65). It is still unclear whether these $CD4^+$ T cell subsets are functionally equivalent. Compared to those in MSI patients, Th17 cells are enriched in MSS patients and show TCR clonality overlap with Tregs (46), suggesting roles in tumor immunosuppression and the ineffective responses of MSS patients to immunotherapies. As previously discussed, Th1 and Th17 cells both have TCRs shared with Tregs and may serve as the external origins of tumor Tregs (46, 69). The balance between Th1 and Th17 responses and their conversion with Tregs may form an important switch determining the immune status of tumor microenvironments and the response to immunotherapies for multiple cancer types, as evidenced by a mouse model of prostate cancer with bone metastasis (121).

## THE IMMUNE MODULATORY ROLES OF LAMP3$^+$ DENDRITIC CELLS IN TUMOR MICROENVIRONMENTS

scRNA-seq not only provides important insights into the biology of tumor-infiltrating T cells but also sheds critical light on the myeloid microenvironments and their cross talk with tumor-infiltrating T cells. As professional antigen-presenting cells, DCs are a crucial component that induces and maintains antitumor immunity (122, 123). Traditionally, DCs can be categorized into CD11C$^+$ conventional DCs (cDCs) and plasmacytoid DCs (pDCs), with cDCs further separated into CD141$^+$ type 1 cDCs (cDC1s) and CD1C$^+$ cDC2s based on cell surface markers and their distinct abilities to prime CD8$^+$ and CD4$^+$ T cells (124–126). cDC1s have been shown to undergo a process of Toll-like receptor (TLR)-induced immunogenic maturation, with the upregulation of Ccr7 and Cd40 (127). To delineate the development and functional roles of mature DCs in cancer, several scRNA-seq studies have been applied to map the tumor-infiltrating DC landscape, and a conserved maturation module has been characterized in multiple human and mouse tumors (**Figure 3*a***) (48, 53, 128, 129).

A study of human and mouse non-small cell lung cancers reveals a subpopulation of CCR7$^+$ DCs showing an activated DC phenotype but lacking the expression of key cDC or pDC genes (129). In contrast to other cDCs, a mature and migratory DC subset has *FSCN1*, *CCR7*, *CCL19*, *CCL22*, and *CD274* as conserved marker genes shared between human patients and mouse models

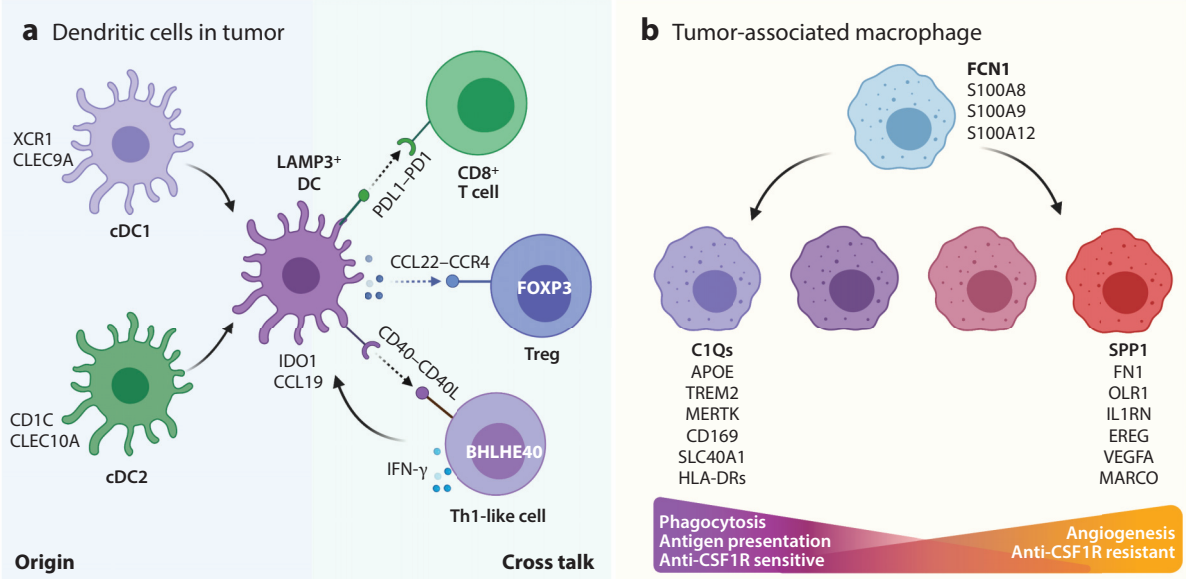


**Figure 3**

Tumor-infiltrating cDCs and TAMs. (*a*) The fundamental properties, origin, and cross talk of LAMP3$^+$ DCs in the tumor microenvironment. LAMP3$^+$ DCs are characterized by downregulation of both cDC1 and cDC2 hallmarks (e.g., XCR1, CLEC9A, and CD1C) and upregulation of migration-related (e.g., CCR7) and activation-related (e.g., CD40) molecules. LAMP3$^+$ DCs in multiple tumor types including liver, lung, breast, and colorectal cancers together with mouse tumor models are characterized by high levels of immunoregulatory molecules such as PD-L1 (48, 53, 128, 129), and they can interact with various T cells via diverse ligand-receptor interactions such as PD-L1–PD-1 and CD40–CD40L. (*b*) Phenotypic plasticity of TAMs. While C1Q$^+$ TAMs show polarization toward phagocytosis and antigen presentation and are conserved in multiple cancer types (48, 53, 71), SPP1$^+$ TAMs are enriched in colorectal cancers and resistant to anti-CSF1R blockade (48). Abbreviations: cDC1, type 1 conventional DC; DC, dendritic cell; IFN-γ, interferon gamma; TAM, tumor-associated macrophage; Th1, type 1 T helper cell; Treg, regulatory T cell. Figure adapted from images created with BioRender.com.

(**Figure 3*a***) (129). This mature DC subset is also enriched in the tumor microenvironment and lymph nodes of hepatocellular carcinoma and can migrate from tumors to adjacent lymph nodes (53), probably along the gradient of CCL19, as demonstrated in vitro (53). Notably, *LAMP3*, a gene reported to be associated with DC maturation under CD40L stimulation (130), is highlighted as an intriguing maturation marker to separate this DC subset from naive primary cDCs and pDCs (53). Similarly, such mature LAMP3$^+$CCR7$^+$ DCs were also identified in breast cancer (131), colorectal cancer, and mice bearing Renca and MC38 tumors (48), while another study of lung cancer defined a cluster of mature DCs enriched in immunoregulatory molecules (mregDCs) that remarkably resembles LAMP3$^+$ DCs in hepatocellular carcinoma, with the expression of similar gene signatures (128).

The origins of LAMP3$^+$ DCs appear to be heterogeneous. RNA velocity analysis and mitochondrial mutation tracking in hepatocellular carcinoma suggest that both cDC1s and cDC2s in tumors can differentiate into LAMP3$^+$ DCs (53), consistent with the observations of both cDC1 and cDC2 markers on this subset in lung cancer at both the RNA and protein levels by CITE-seq (128). The induction of cDC1-derived mregDCs has been reported to be correlated with tumor-associated antigens (128). Compared with migratory DCs in normal lungs, tumor-infiltrating mregDCs generally express higher levels of PD-L1 and PD-L2, which can bind to PD-1 expressed by various tumor-infiltrating T cells (53, 128). In fact, compared with other tumor-resident myeloid cells, LAMP3$^+$ DCs tend to express more types of chemokine, cytokine, and costimulatory/inhibitory ligands, and they regulate the functions of tumor-infiltrating T cells (**Figure 3*a***), which express high levels of the corresponding receptors (53). Phenotypically, signals from cDC1-derived LAMP3$^+$ DCs can drive naive T cells toward Tregs and activated CD8$^+$ states (48, 128), and the infiltration levels of LAMP3$^+$ DCs positively correlate with the infiltration of exhausted CD8$^+$ T cells and Tregs in liver cancer (53). In mouse models bearing MC38 tumors, following anti-CD40 treatment LAMP3$^+$ DCs can stimulate the clonal expansion of Th1-like cells (48). Mechanically, the expression of *PD-L1* by LAMP3$^+$ DCs can be downregulated by *Axl* and upregulated by CD40 and TLR-3, while their expression of *IL-12* can be downregulated by interferon gamma (IFN-$\gamma$) and IL-4 (128). In summary, the identification of LAMP3$^+$ DCs by scRNA-seq across multiple cancer types may reveal a common mechanism that can modulate the function and infiltration of specific T cell subsets. However, further investigations are warranted to fully illustrate their role in tumor immunity and immunotherapies considering the complex coexpression pattern of activating and inhibitory molecules.

## PHENOTYPIC PLASTICITY OF TUMOR-ASSOCIATED MACROPHAGES AND ITS IMMUNOLOGICAL IMPLICATIONS

Tumor-associated macrophages (TAMs) constitute another major component of the tumor immune microenvironment (**Figure 1**). The functional phenotypes of these cells were often contextualized with the M1/M2 macrophage polarization system, introduced in 2000, to mimic the Th cell nomenclature based on the in vitro activation of macrophages (132). While the classically activated (M1) macrophages are characterized as proinflammatory, the alternatively activated (M2) macrophages are thought to promote tumor growth by their anti-inflammatory function. Although the M1 and M2 dichotomy fits a variety of in vitro mouse models under different stimuli, this model may be too simplistic to explain the properties of TAMs in cancer patients (17). Accumulating single-cell studies have begun to reveal the heterogeneity of TAMs in cancer patients (**Table 2**). For example, a recent scRNA-seq study of stromal cells in human lung tumors demonstrates an M2-like polarization of TAMs, showing hallmarks of M2 macrophages described in murine tumor models, including the strong reduction of inflammatory responses and

**Table 2 Human TAM subsets identified based on single-cell studies**

| TAM subset/state | Tumor type/data set | Tissue enrichment | Platforms | Gene signature | Annotation | Reference |
|---|---|---|---|---|---|---|
| FCN1$^+$ macrophages | CRC | Tumor | SMART-seq2 and 10x Genomics | *FCN1*, *HSPA1A*, *HSPA1B*, *CXCR4*, *FCGR2A*, *FCGR3A*, *S100A8*, *S100A9*, *S100A12* | FCN1$^+$ monocyte-like cells | 48 |
| | HCC | Tumor | SMART-seq2 and 10x Genomics | *FCN1*, *S100A6*, *VCAN*, *S100A8*, *S100A9*, *S100A12* | MDSC-like macrophages | 53 |
| | NSCLC | Tumor | inDrop | *CD14*, *FCN1* | $Mono_1$ | 129 |
| | | | | *S100A8*, *S100A9*, *S100A12*, *VCAN* | $Mono_3$ | |
| C1Q$^+$ TAMs | CRC | Tumor, normal tissue | SMART-seq2 and 10x Genomics | *C1QA/B/C*, *MERTK*, *FPR3*, *TREM2*, *MS4A4A*, *SLCO2B1*, *NRP1*, *SLAMF8*, *FCGR1A*, *SIRPA*, *MAF*, *MAFB*, *SLC40A1*, *SIGLEC1* | C1QC$^+$ TAMs | 48 |
| | HCC | Tumor | SMART-seq2 and 10x Genomics | *C1QA/B*, *APOE*, *TREM2*, *GPNMB*, *SLC40A1* | TAM-like macrophages | 53 |
| | NSCLC | Tumor, normal tissue | CyTOF and MARS-seq | *C1QA*, *MAFB*, *CSF1R*, *CCL3*, *JUN* | Macrophages | 71 |
| | NSCLC | Tumor | CyTOF and MARS-seq | *TREM2*, *CD81*, *MARCO*, *APOE*, *CALR*, *CD63*, *SPP1* | Tumor-specific macrophages | 71 |
| | BC | Tumor | inDrop | *APOE*, *SPP1*, *FN1*, *CD276*, *CD9*, *TREM2*, *C1QB*, *CHIT1*, *CCL18*, *MARCO*, *CD81*, *NRP2* | TAMs | 40 |
| SPP1$^+$ TAMs | CRC | Tumor | SMART-seq2 and 10x Genomics | *SPP1*, *IL1RN*, *OLR1*, *VEGFA*, *EREG*, *FN1*, *C15ORF48*, *PHLDA1*, *AQP9*, *TNS3*, *NDRG1* | SPP1$^+$ TAMs | 48 |
| Microglial TAMs | Gliomas | Tumor | SMART-seq2 | *CX3CR1*, *P2RY12*, *P2RY13*, *SELPLG* | Microglia-like cells | 171 |
| | Gliomas | Tumor | SMART-seq and 10x Genomics | *CX3CR1*, *P2RY12*, *NAV3*, *SIGLEC8*, *SLC1A3* | Microglial TAMs | 133 |
| Blood-derived TAMs | Gliomas | Tumor | SMART-seq2 | *CD163*, *IFITM2*, *IFITM3*, *TAGLN2*, *F13A1*, *TGFBI* | Macrophage-like cells | 171 |
| | Gliomas | Tumor | SMART-seq and 10x Genomics | *TGFBI*, *IFITM2*, *FPR3*, *S100A11*, *KYNU*, *ITGA4* | Blood-derived TAMs | 133 |

Abbreviations: BC, breast carcinoma; CRC, colorectal carcinoma; CyTOF, cytometry by time-of-flight; HCC, hepatocellular carcinoma; MARS-seq, massively parallel RNA single-cell sequencing; MDSC, monocyte-derived suppressor cell; $Mono_1$, subset 1 monocyte; NA, not available; NSCLC, non-small cell lung carcinoma; TAM, tumor-associated macrophage.

TNF-α-induced proliferation pathways (70). This M2-like polarization of TAMs was confirmed by an independent scRNA-seq analysis of myeloid cells from human and mouse lung cancers (129). However, such a phenotype model of TAMs was not generalized in broader cancer types. In breast cancer patients, TAMs studied by scRNA-seq express markers associated with both M1 and M2 phenotypes, suggesting limitations to the M1/M2 polarization model (40). The mixed expression of M1 and M2 markers was also observed in single-cell studies in TAMs from colorectal

cancer (48), liver cancer (53), renal cancer (58), and glioma patients (133), further suggesting more complex phenotypes of macrophages in the tumor microenvironment.

scRNA-seq studies demonstrate that TAMs, unlike DCs, whose phenotypes are relatively conserved among cancer types and between human and mouse (129), show much higher phenotypic plasticity and heterogeneity between cancer types (48, 53, 129). In a liver cancer study that applied SMART-seq2 and 10x Genomics methods to characterize the cellular phenotypes, both platforms suggested a continuum rather than discrete subsets for the phenotypes of TAMs (53). Similar trends are also observed in breast and lung cancers and repeated with the inDrop platform (40, 71). A group of TAMs characterized by high expression of complement family proteins and MHC class II molecules appear to be universally present in a wide spectrum of cancers (**Figure 3*b***), including liver (53), lung (71), colorectal (48), and breast (40) cancers. The core signatures of this type of TAM can be represented by expression of *C1Q*s, *TREM2*, *SLC40A1*, and *APOE*. These macrophages highly expressing *C1Q*s have an essential role in clearance of apoptotic cells to prevent autoimmunity (134). Recent studies demonstrate that C1Qs can enhance the phagocytosis of macrophages and reduce the production of proinflammatory cytokines in response to tissue damage (135). Similarly, *TREM2* has been associated with maintaining tissue-level lipid homeostasis (136) and promoting macrophage survival in lung disease after acute viral infection (137). Consistent with the function of these key genes, $C1Q^+$ TAMs demonstrate a phenotype of polarized phagocytosis and antigen processing and presentation (48). In both liver and colorectal cancer studies that have multiple autologous control tissues available, $C1Q^+$ TAMs tend to be more abundant in tumors compared with other tissues (48, 53), suggesting their relevance to modulating the tumor immune microenvironments. Interrogation of the potential cell-cell relationships by either ligand-receptor interactions or TCGA-based coenrichment in colorectal carcinomas (CRCs) suggests that $C1Q^+$ TAMs have roles in recruiting and regulating various T cell subsets, including $CD8^+$ exhausted T cells, Tregs, and Th1-like cells (48). This $C1Q^+$ TAM subtype seems conserved between human and mouse tumors and is sensitive to CSF1R blockade (48). However, markers for this subset have varying prognostic power among cancer types. In liver cancer, its core signature and the marker gene *SLC40A1* are associated with poor survival (53). But decreased expression of *SLC40A1* promotes breast cancer and myeloma progression and has been linked to the poor prognosis of these patients (138, 139). In vitro knockout experiments reveal that *SLC40A1* regulates proinflammatory cytokines, including IL-6, IL-23, and IL-1β, under different TLR stimuli (53).

Another TAM subset is characterized by high expression of *FCN1*, *VCAN*, *S100A8*, *S100A9*, and *S100A12* and can be identified in liver (53), lung (129), and colorectal (48) cancers. In contrast to the $C1Q^+$ TAMs, which are tumor-preferred, $FCN1^+$ TAMs are also enriched in tumor-adjacent tissues (48). Phenotypically, this TAM subset exhibits signatures of both blood-derived classical monocytes (CD14 and FCN1) and macrophages (CD68), and it highly expresses neutrophil-associated genes, including *S100A8*, *S100A9*, and *CSF3R*. Such phenotypes suggest a monocyte origin of this specific TAM subset and an intermediate state during the monocyte maturation into macrophages in tumors (48, 129). Further investigations are needed to elucidate its role in tumor immunity.

An additional subset of TAMs illuminated by scRNA-seq has been defined by high expression of *SPP1*, initially found in colorectal cancers (48). Although the expression of *SPP1* can also be detected in TAMs from lung and breast cancers (40, 71), clustering studies could not identify a corresponding homogeneous subset in those tumor types. Similar to $C1Q^+$ TAMs, $SPP1^+$ TAMs are more abundant in tumors compared to nontumor tissues. $SPP1^+$ TAMs are characterized by high expression of angiogenesis-related genes (**Figure 3*b***), including *VEGFA*, *VCAN*, *CXCL8*, and *ANGPTL4*, and loss of MHC class II expression (48). Similar $SPP1^+$ TAM subsets were

identified in mouse tumor models, and those cells are resistant to treatment by CSF1R blockade and potentially contribute to tumor progression (48). Common gene signatures can also be identified between SPP1$^+$ and C1Q$^+$ TAMs, e.g., *GPNMB*, which is associated with poor survival in liver cancer (53). Different from C1Q$^+$ TAMs, ligand-receptor-based and TCGA-based analyses suggest that SPP1$^+$ TAMs have tight associations with cancer-associated fibroblasts and endothelial cells and that they have different roles in modulating the status of tumor microenvironments (48). A recent study of renal clear cell carcinomas suggests that high levels of IL-8 and downregulation of antigen-presentation machinery in myeloid cells correlate with decreased efficacy of PD-L1 blockade in patients with metastatic renal clear cell carcinoma and urothelial carcinoma (140), which is consistent with the phenotypes of SPP1$^+$ TAMs and highlights the necessity to investigate the impacts of SPP1$^+$ TAMs on immune checkpoint inhibition.

## INSIGHTS INTO CANCER IMMUNOTHERAPIES BY scRNA-seq

scRNA-seq methods are being exploited to identify individual markers or specific cell type subsets that are associated with response or resistance to immunotherapy of cancer. These approaches have been applied to melanoma (29, 141); glioblastoma (33); and lung (117), renal, and urothelial cancers (140), resulting in novel insights (**Figure 4**). For example, scRNA-seq can be used to resolve the relative contributions of reinvigoration of preexisting T cells versus recruitment of novel T cells to the responses of cancer patients to anti-PD-1 therapies. TCR-based tracking supports a clonal replacement mechanism during the T cell responses to anti-PD-1 therapies in basal and squamous cell carcinomas, suggesting that recruitment of new T cells may be more important than reinvigoration of preexisting exhausted T cells in mediating antitumor activity in these tumor types (141). Increasing evidence suggests that T cells from the adjacent or even peripheral tissues are important for effective responses to cancer immunotherapies (107, 117) (**Figure 2**).

Diverse mouse studies suggest the importance of a CXCR5/TCF1$^+$CD8$^+$ T cell subset for a durable response to immune checkpoint inhibition (29, 142–146). This T cell population is regarded as having a progenitor or stem-like phenotype, expresses low levels of PD-1 and TIM-3 inhibitory receptors, and can self-renew and differentiate into terminally exhausted T cells characterized by TCF1$^-$ and high expression of inhibitory receptors (56, 91, 105, 145). Phenotypically, this CD8$^+$ T cell population is similar to a subset of the CD8$^+$GZMK$^+$ effector memory T cells residing in tumor and adjacent tissues, or the MKi67$^+$ proliferative CD8$^+$ T cells in tumors, suggesting an effector memory origin (46, 54, 69). The observation that immune checkpoint inhibition can still control tumor growth in murine models when TCF1$^+$ T cells are depleted suggests that such CXCR5/TCF1$^+$CD8$^+$ T cells are not the only mediators of response to immunotherapy in these models (145). In addition to effector memory T cells, tissue-resident memory and peripheral T cells can also be important pools to support the reinvigoration and replenishment of tumor-infiltrating T cells (**Figure 4**), as supported by recent studies demonstrating the importance of lung-resident memory T cells and peripheral immune responses to cancer immunotherapies (107, 117, 147–149).

Emerging evidence suggests that, in addition to cytotoxic CD8$^+$ T cells, CD4$^+$ T cells are important for effective antitumor immunity and responses to immunotherapy. As mentioned above, Th1-like cells are significantly enriched in immunotherapy-sensitive, MSI CRC patients, while Th17 cells are enriched in immunotherapy-resistant MSS patients (46). The balance between Th1-like cells and Th17 cells in tumor microenvironments is also associated with the responsiveness of prostate cancer to immune checkpoint inhibitors (**Figure 4**), wherein Th1 populations are associated with better response while Th17 populations are associated with resistance (121). A

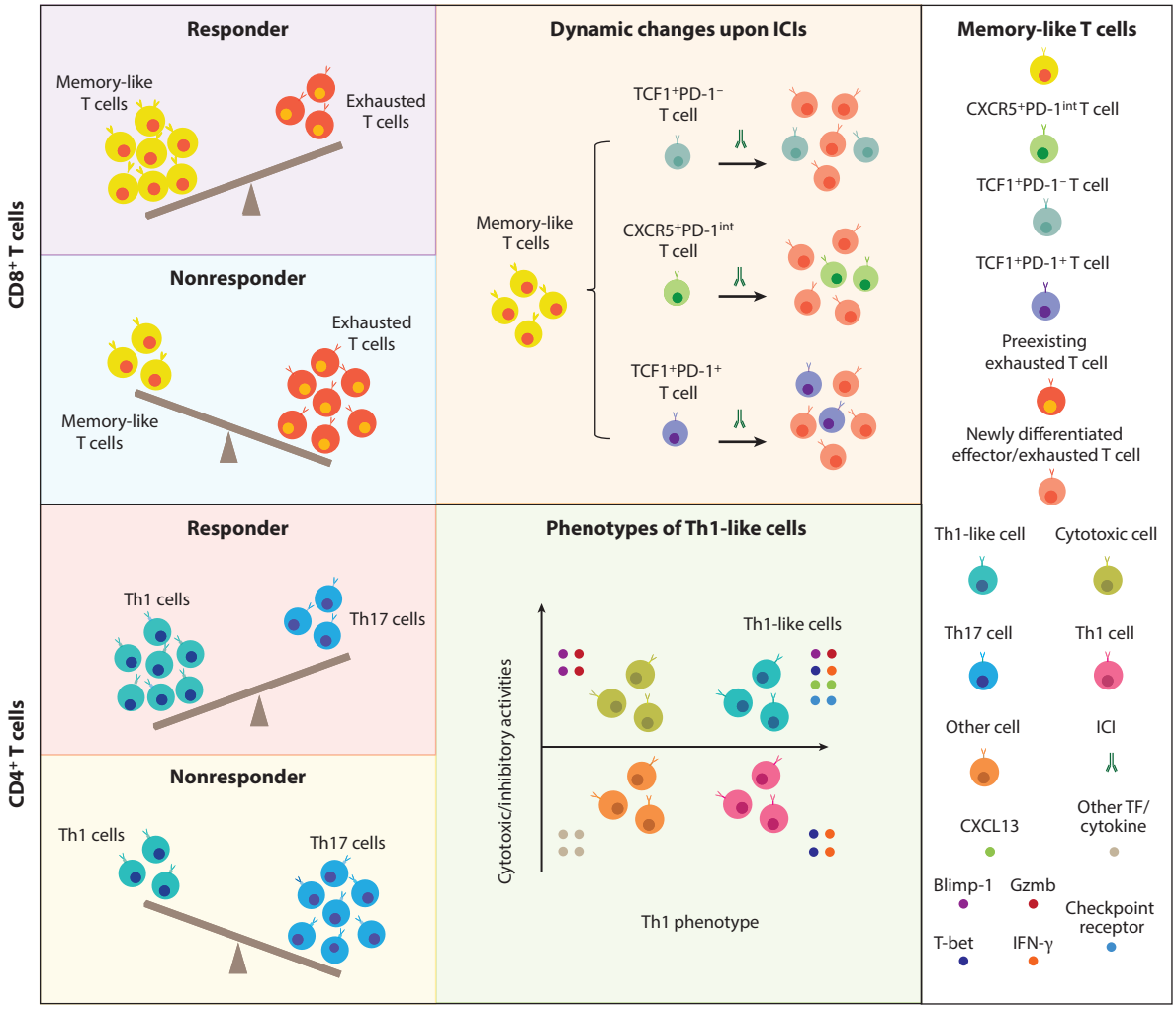


**Figure 4**

Emerging paradigms of different CD8+ and CD4+ T cell subsets shaping patient responses to immunotherapies. For CD8+ T cells, the balance between preexhausted and terminally exhausted T cells seems to be predictive of patient responses to immunotherapies. The CXCR5+/TCF1+ subsets having memory- and stem-like phenotypes are of particular relevance. For CD4+ T cells, the balance between Th1 and Th17 cells is important for patient responses to immunotherapies. In particular, Th1-like cells with a Th1 phenotype and high levels of cytotoxic and inhibitory activities are highly enriched in MSI patients, who have better responses to ICIs than MSS patients. Abbreviations: ICI, immune checkpoint inhibitor; IFN-γ, interferon gamma; MSI, microsatellite instable; MSS, microsatellite stable; TF, transcription factor; Th1, type 1 T helper cell.

recent scRNA-seq study in bladder cancer has suggested a critical role of cytotoxic CD4+ T cells in mediating the antitumor effects of anti-PD-L1 therapy in an MHC class II–dependent manner (65). Studies in human colon cancer and mouse models bearing MC38 tumors have suggested a critical role of a Th1-like CD4+ T cell subset in mediating the response to anti-CD40 therapy (48). These cytotoxic and Th1-like CD4+ T cell subsets have similar phenotypes but with some notable differences. The Th1-like cells have higher expression of CXCL13 and demonstrate more significant enrichment in tumors than cytotoxic CD4+ T cells (46, 65). In addition to tumor CD4+

T cells, peripheral CD4$^+$ T cells are also implicated in mediating antitumor immunity, with evidence from both mouse models and melanoma patients treated with anti-CTLA4 antibodies (107). The relevance of peripheral CD4$^+$ T cells to cancer immunotherapy is supported by studies in lung cancer where specific subsets of CD4$^+$ T cells in the peripheral circulation were predictive of good prognosis in non-small cell lung cancer patients (117). In mouse models, such polyfunctional helper CD4$^+$ T cells with cytotoxic activity, characterized by high expression of T-bet, IFN-γ, GzmB, and TNF-α, are regulated by transcriptional factors *Blimp-1* and *T-bet* independently, with signals from both required for maximal antitumor immune response (150). Of note, the BLIMP1$^+$CD4$^+$ T helper cells exhibit more inhibitory properties in human cancers, with high expression of coinhibitory receptors including *PDCD1*, *CTLA4*, and *TIGIT*, as well as chemokine ligand *CXCL13* (46, 54, 69). Thus, these Th1-like cells may be different from the conventional Th1 cells due to their specific characteristics of both cytotoxic and inhibitory activities.

## ADDING THE SPATIAL DIMENSION TO FURTHER RELEASE THE POWER OF scRNA-seq IN ILLUMINATING TUMOR-INFILTRATING IMMUNE CELLS

While scRNA-seq has brought important insights into the cellular and molecular landscapes of multiple cancer types, the cellular spatial relationships are lost during the tissue dissociation process. The emergence of spatial transcriptomics, where transcript libraries are created and studied across intact tissue geographies, as well as technologies interrogating cell-cell interactions, has provided a valid technique to complement scRNA-seq in dissecting the composition and architecture of tumor microenvironments (151–160). The combination of scRNA-seq and spatial transcriptomics has been applied to pancreatic and human squamous cell carcinomas and resulted in novel insights into the cross talk between tumor and immune cells (161, 162). Both studies reveal that cancer cells tend to localize within a fibrovascular niche while immune cells tend to be spatially restricted in different compartments, suggesting a spatial mechanism that cancer cells may employ to escape immune surveillance. In particular, tertiary lymphoid structures and the spatial proximity of CD8$^+$ T cells to tumor cells may be important predictors of patient responses to immunotherapies (143, 163–166). Spatial transcriptomics also suggest that tumor-infiltrating CD8$^+$ T cells tend to be colocalized with Tregs (161, 162, 167, 168), providing a spatial explanation of their phenotypic similarities and implying potential regulatory relationships between each other. Computational modeling by CSOmap, a bioinformatics tool that can reconstruct cellular spatial organizations de novo from scRNA-seq data via ligand-receptor interactions (159), has revealed that tumor-resident Tregs may be recruited by CD8$^+$ T cells via the CCL4-CCR8 axis (169), consistent with observations of combined scRNA-seq and spatial transcriptomics in human squamous cell carcinoma (162). In silico depletion of Tregs from the micromilieus of exhausted CD8$^+$ T cells by CSOmap suggests that the infiltration of originally blood-enriched CD8$^+$ effector T cells and CXCR6$^+$ tissue-resident CD4$^+$ T helper cells can be enhanced via the CCL5-CXCR3 axis (159), a result that is also supported by a recent study in melanoma (170).

## CONCLUDING REMARKS

Illustrating the biology of tumor-infiltrating immune cells is pivotal to understanding tumor immunity and improving cancer immunotherapies. The applications of scRNA-seq to the tumor microenvironment of multiple cancer types have revealed important biology of the phenotypic heterogeneity and the origins and dynamics of tumor-infiltrating T cells, DCs, and macrophages, including both common patterns and histology-specific alterations across cancers. It is increasingly evident that the detailed composition and functional states of multiple cell types influence

the differential responses to cancer immunotherapies, and our further understanding of the complex tumor microenvironment through scRNA-seq has great potential to identify not only reliable predictive biomarkers but also novel therapeutic strategies that will complement current therapeutic agents.

## DISCLOSURE STATEMENT

Z.Z. and N.S. have ownership interest in Analytical Bio-Sciences, Limited, and Abiosciences, Inc. Z.Z. is also a consultant/advisory board member for InnoCare Pharma and ArsenalBio. Other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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