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REVIEW

Cancer Focus

The current landscape of single-cell transcriptomics for cancer immunotherapy

Puneeth Guruprasad[1,2,3], Yong Gu Lee[2,3], Ki Hyun Kim[2,3], and Marco Ruella[1,2,3,4]

Immunotherapies such as immune checkpoint blockade and adoptive cell transfer have revolutionized cancer treatment, but further progress is hindered by our limited understanding of tumor resistance mechanisms. Emerging technologies now enable the study of tumors at the single-cell level, providing unprecedented high-resolution insights into the genetic makeup of the tumor microenvironment and immune system that bulk genomics cannot fully capture. Here, we highlight the recent key findings of the use of single-cell RNA sequencing to deconvolute heterogeneous tumors and immune populations during immunotherapy. Single-cell RNA sequencing has identified new crucial factors and cellular subpopulations that either promote tumor progression or leave tumors vulnerable to immunotherapy. We anticipate that the strategic use of single-cell analytics will promote the development of the next generation of successful, rationally designed immunotherapeutics.

Introduction

Immunotherapy marks a new era in cancer treatment. Clinical outcomes of immune checkpoint blockade (ICB) and adoptive cell therapies have led to breakthrough drug approvals for solid and hematologic malignancies. Checkpoint inhibitors such as anti–PD-1 or anti–PD-L1 antibodies, as well as chimeric antigen receptor (CAR) T cell therapies, are now routinely used in a subset of cancer patients (Braendstrup et al., 2020; Vaddepally et al., 2020; Hargadon et al., 2018). Despite these exciting results, most of the patients treated with novel immunotherapies do not respond or eventually relapse (Darvin et al., 2018; Ghilardi et al., 2020). Recent data suggest that as few as 10% of patients may respond to ICB therapy (Haslam and Prasad, 2019) and that 30–60% of patients who receive CAR-T therapy may experience a relapse (Majzner and Mackall, 2019; Ruella and Maus, 2016). Optimizing these immunotherapies to overcome resistance will depend on a deeper understanding of the behavior of cancer cells and the tumor microenvironment (TME) before, during, and after treatment.

Single-cell transcriptomics is emerging as a powerful process to deconvolute heterogeneous cell populations like those present in the TME, a complicated network of proliferating malignant cells, immune infiltrates, and tumor stroma (Hanahan and Weinberg, 2011; Saadatpour et al., 2015; Kunz et al., 2018; Valdes-Mora et al., 2018; Tirosh and Suvà, 2019; Lim et al., 2020). Technologies such as single-cell RNA sequencing (scRNA-seq) allow for the study of the gene expression profile of individual cells, unlike bulk RNA sequencing (RNA-seq), which provides an averaged expression profile across a heterogeneous population (Fig. 1). In doing so, scRNA-seq studies have characterized unique transcriptional programs in the TME, have discovered new cellular subsets, and have underscored the magnitude of "intertumoral" and "intratumoral" heterogeneity (Schelker et al., 2017; Andrews and Hemberg, 2018; Patel et al., 2014). Compared with bulk-level transcriptomics, detection of the nuances in single-cell gene expression that contribute to intratumoral heterogeneity is now feasible with scRNA-seq. However, it is important to note that scRNA-seq also has limitations compared with bulk RNA-seq (Lähnemann et al., 2020), which include low transcript capture efficiency, low sequencing coverage, bias of coverage to one end of the transcript, and overall cost. Nevertheless, paired with machine learning and advanced statistics (Luecken and Theis, 2019), scRNA-seq has enabled the rapid identification of novel factors that affect tumor progression and influence the outcome of patients undergoing immunotherapy.

In this review, we describe the critical insights that scRNA-seq has provided into both the TME and cancer immunotherapies.

[1]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA; [2]Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA; [3]Department of Medicine, Division of Hematology and Oncology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA; [4]Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA.

Correspondence to Marco Ruella: mruella@upenn.edu.

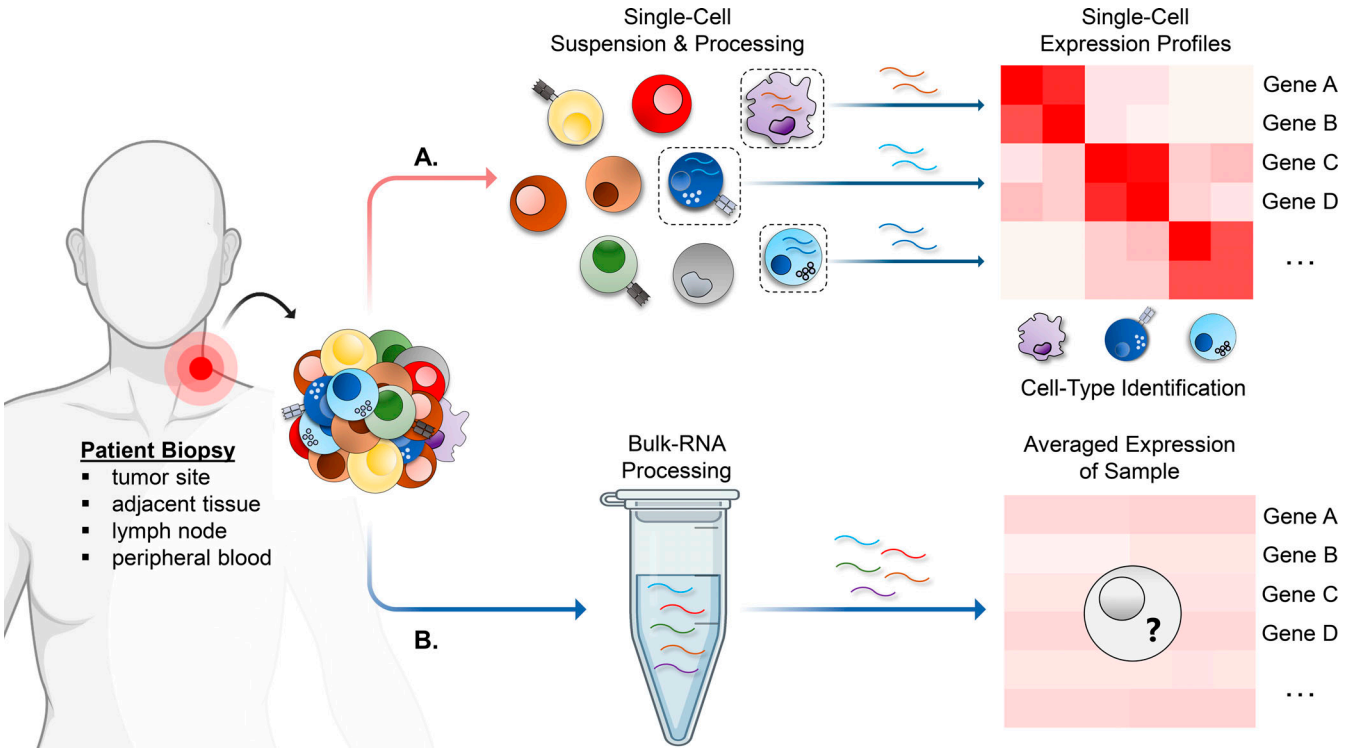

Figure 1. **scRNA-seq versus bulk RNA-seq for profiling the TME. (A and B)** Transcriptomic studies of patient biopsies can provide intimate details of important gene signatures involved in tumor progression or response to immunotherapy. **(A)** In the scRNA-seq workflow, a tumor biopsy sample is first disassociated into a single-cell suspension, and platforms like that of 10X Genomics Chromium are used to generate a uniquely barcoded cDNA library from reverse transcription of the isolated poly-adenylated mRNA within each individual cell. **(B)** Bulk RNA-seq instead generates cDNA directly without tagging unique transcripts of individual cells. After generation of cDNA via reverse transcription of mRNA, both platforms use PCR amplification, next-generation sequencing, and subsequent downstream informatics to process data. For scRNA-seq, visualization methods such as heat maps show expression of individual genes (rows) for individual cells (columns). Clustering cells with similar expression allows for identification of cell type. Bulk RNA-seq instead returns average gene expression values across the sample cell population, thus preventing cell classification.

We primarily focus on how these methods have shed light on the elusive, dual role of infiltrating immune cells (IICs) in the TME that can both eliminate and promote tumors (Hanahan and Weinberg, 2011; Lei et al., 2020). Profiling single cancer and immune cells has significant potential to reveal novel mechanisms of resistance, immune tolerance, and relapse in individual cancer patients, thus likely facilitating the development of personalized and effective targeted immunotherapies.

An abridged history of single-cell transcriptomic technologies

In 2009, Tang et al. generated the first scRNA expression profile of a mouse blastomere (Tang et al., 2009). Their method, and all subsequent variations, follow a basic blueprint of isolation and lysis of single cells, generation of cDNA through reverse transcription, PCR amplification, and detection on next-generation sequencing platforms (Haque et al., 2017). In 2012, Ramsköld et al. developed Smart sequencing (Smart-seq), isolating single viable cells through manual pipette picking and introducing new dilution methods to improve transcriptome coverage (Ramsköld et al., 2012). Smart-seq became the first scRNA-seq technique applied to primary tumor cells, revealing heterogeneity in immune response, oncogenic signaling, and proliferation (Patel et al., 2014). Techniques that further improved transcript length and cell throughput include Smart-seq2 (Picelli et al., 2013), MARS-Seq (massively parallel RNA single-cell sequencing; Jaitin et al., 2014), and recently Smart-seq3 (Hagemann-Jensen et al., 2020). Other techniques such as CEL-Seq (Cell Expression by Linear amplification and Sequencing) introduced unique molecular identifier sequences in the primers for cDNA generation to pinpoint specific mRNAs (Chen et al., 2019; Hashimshony et al., 2012; Islam et al., 2014). Microfluidic platforms, like that of Fluidigm C1 (See et al., 2018), then became an attractive option to isolate single cells due to precise fluid control and broad input cell range (Sánchez Barea et al., 2019; Guo et al., 2012). Ultra-high-throughput droplet-based methods such as Drop sequencing (Macosko et al., 2015), inDrop (Klein et al., 2015), and the popular 10X Genomics Chromium platform (Zheng et al., 2017) then emerged thanks to their ability to encapsulate single cells into droplets with integrated barcodes.

Despite these platforms reducing costs per cell sequenced, the cost to sequence a sizable population of several thousand cells via microfluidic scRNA-seq remains expensive. Also, since companies like 10X Genomics have carved a niche in the transcriptomic tech space (Eisenstein, 2019), the market of competing scRNA-seq services remains limited. Microwell-based scRNA-seq systems, such as CytoSeq (Fan et al., 2015) and Microwell-Seq (Han et al., 2018) were developed in parallel to microfluidic platforms, but a lack of commercial infrastructure

has limited their global adoption (Zhang et al., 2019d). Bioinformatic analysis is an essential piece of the scRNA-seq pipeline. Downstream big data analysis following scRNA-seq is typically used to produce gene expression heat maps and cell-type cluster plots from algorithms such as t-distributed Stochastic Neighbor Embedding and uniform manifold approximation and projection (Kobak and Berens, 2019; Becht et al., 2019). These bioinformatic tools have also emerged at an impressive rate that parallels the development of scRNA-seq technologies, although the complexity of these computational tools often limits their accessibility to experimentalists (Poirion et al., 2016; Chen et al., 2019; Cakir et al., 2020).

The rise of scRNA-seq to study cancer

scRNA-seq has been employed to unveil different aspects of the cancer landscape—from the TME composition and phenotype to cancer cell heterogeneity. The composition of the TME has long been known to affect clinical outcomes of patients with cancer. In particular, high infiltrates of cytotoxic and memory $CD8^+$ T cells, $CD4^+$ T helper (Th) 1 cells, and natural killer (NK) cells are usually associated with good prognosis in several cancers (Fridman et al., 2012; Takanami et al., 2001; Ishigami et al., 2000; Tachibana et al., 2005). On the contrary, abundance of Th2 and Th17 $CD4^+$ populations, T regulatory cells (T reg cells), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) appears to disarm the cytotoxic immune response and drive tumor growth (Fridman et al., 2012; Wherry, 2011; Noy and Pollard, 2014; Galon and Bruni, 2020; Baghban et al., 2020). Understanding the role of IICs is of primary importance in guiding new immunotherapeutic strategies. Not surprisingly, the efficacy of both checkpoint inhibitors and engineered cells is also subject to myriad immune pressures in the TME. For example, the infiltration of $CD8^+$ T and NK cells into the tumor is predictive of response to ICB for many cancers, including skin, lung, colon, pancreatic, and breast cancers (BCs; Trujillo et al., 2018; Chen and Mellman, 2017; Sikandar et al., 2017; Barry et al., 2018; Zappasodi et al., 2018). In addition, TAM and T reg cells both suppress $CD8^+$ T cell functions and will thus limit the antitumor efficacy of checkpoint inhibitors (Cassetta and Kitamura, 2018). Since Patel et al. (2014) applied Smart-seq to clinical cancer biopsies, scRNA-seq has refined our understanding of tumor immune populations, adding multiple candidates to the regulatory network of each IIC (Fig. 2; Zhang et al., 2019c; Guo et al., 2018) and describing diverse states of polarization of IICs (Lambrechts et al., 2018; Durante et al., 2020; Azizi et al., 2018; MacParland et al., 2018). Besides understanding tumor composition, scRNA-seq studies may additionally include samples from the patient's peripheral blood or adjacent normal tissue. Indeed, cellular subsets such as those of naive, memory, and exhausted T cells demonstrate different tissue enrichment preferences. Thus, sampling strategies that include additional tissues besides the tumor can provide critical information on the origin of tumor-reactive T cells and immune responses and also tumor spreading.

Moreover, single-cell transcriptomics has been used to further probe tumor cells' evolution and their immune relations under direct pressure from immunotherapeutics (Gibellini et al., 2020; Chuah and Chew, 2020; Ren et al., 2018). Simultaneously pairing scRNA-seq with B cell receptor or TCR sequencing has also enabled the identification of B cell or T cell malignant clones and now allows us to monitor the expansion of specific T cell clones (Singh et al., 2019; De Simone et al., 2018), including those of CAR-T infusion products.

In the next sections, we discuss the key discoveries of scRNA-seq in several cancer types and describe its role in understanding responses to immunotherapy.

Hematologic malignancies

Leukemia, lymphoma, and myeloma

Blood cancers account for 10% of new cancer diagnoses and have been the chief beneficiaries of cell-based immunotherapies such as CAR T cells (Hay and Turtle, 2017). Several scRNA-seq studies have sought to elucidate the complex TME of leukemias, lymphomas, and myelomas.

One particularly studied blood cancer using scRNA-seq is acute myeloid leukemia (AML), a malignancy derived from myeloid hemopoietic cells that has a high relapse rate and overall poor outcome following standard chemotherapy regimens (Yilmaz et al., 2019). Xiong et al. (2020) used the Fluidigm C1 platform to study at the single-cell level the bone marrow of two AML patients. Of the 31 differentially expressed genes in the AML cells of the patient who relapsed after hematopoietic stem cell transplantation, the authors found three cancer genes (*ARID2*, *MLL*, and *SYNCRIP*) that served as prognostic biomarkers in a validation cohort of 52 AML patients analyzed by bulk RNA-seq. High expression levels of either *ARID2* (required for hematopoietic stem cell homeostasis) or *MLL* (associated with dysfunctional fusion proteins) in the patients' AML gene signatures of the bulk dataset correlated with poor patient outcomes. Conversely, high levels of *SYNCRIP*, a regulator of the myeloid stem cell program, correlated with a more favorable outcome. In another study, Petti et al. (2019) developed a unique method to detect in scRNA-seq data somatic mutations in AML cells that express single nucleotide variants, after first detecting these mutations using whole-genome sequencing. In this study, leukemic cells harboring a well-known AML mutation in the hematopoiesis regulator *GATA2* ($GATA2^{R361C}$) were restricted to the same space of a clustering projection, suggesting that malignant cells bearing the same mutation may have similar RNA expression. van Galen et al. (2019) also used scRNA-seq to generate gene signatures for various AML malignant clones of the same sample and provide insight into the functional significance of *FLT3* mutant AML cells. In one AML patient, a subclone with a mutation in the tyrosine kinase domain of *FLT3* (*FLT3*-TKD) primarily contained differentiated cells, while a *FLT3* internal tandem duplication (*FLT3*-ITD) mutated subclone contained progenitor-like cells and suppressed differentiation of AML cells in vitro. This scRNA-seq result reinforces the relationship between the *FLT3*-ITD genotype and the observed enhanced aggressiveness that leads to poor patient outcome (Leick and Levis, 2017). The authors also found that a $CD14^+$ monocyte-like AML cluster had up-regulation of the immunosuppressive TNF-α and IL-10 pathway genes that were associated with poor

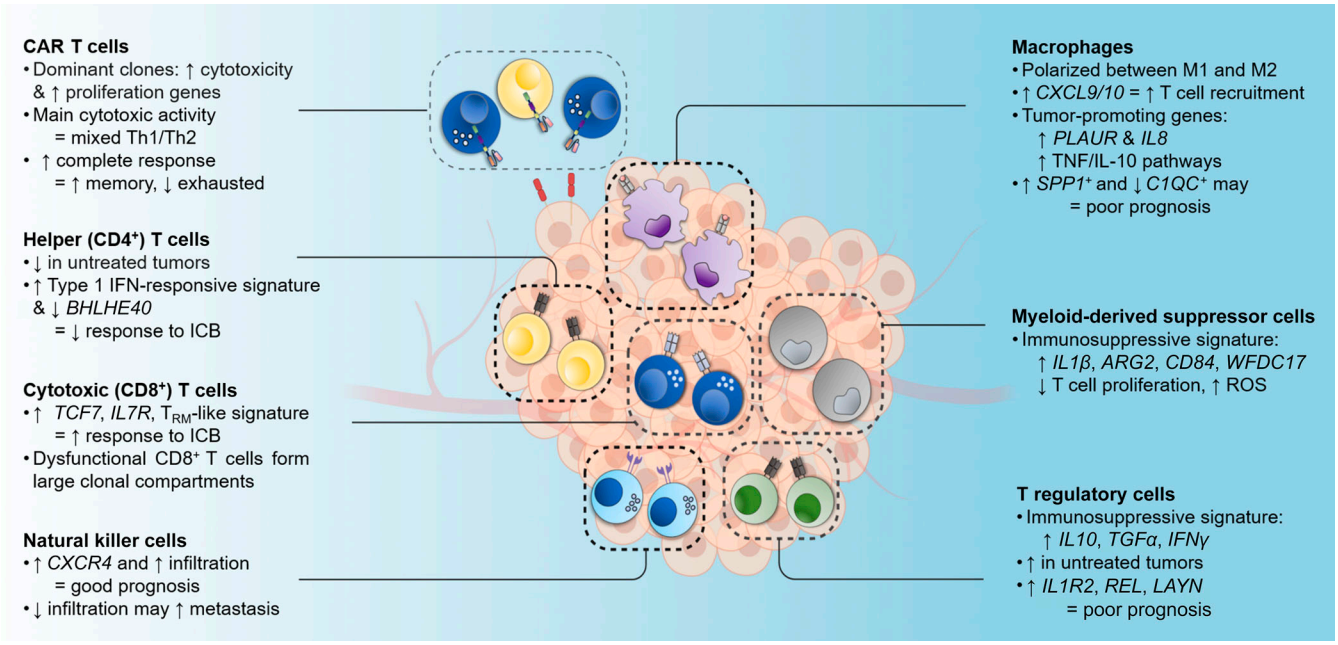

Figure 2. **Key findings from scRNA-seq studies of the TME.** scRNA-seq studies of tumor-IICs have vastly expanded our knowledge of the regulatory networks of these cells and thus provided a greater range of modulation routes for cancer treatment. Overall, most studies have confirmed the antitumor expression programs of NK, cytotoxic (CD8+) T, and Th (CD4+) cells, while noting the immunosuppressive nature of TAMs, MDSCs, and T reg cells. Other important cell types in the microenvironment include malignant cells, CAFs, CD14+ monocytes, and rare cells that may be cancer, organ, or patient specific. Identifying important genes that facilitate either cytotoxic or suppressive behaviors will allow us to harness and exploit new properties of the TME to enhance the efficacy of immunotherapies.

outcome in patients from The Cancer Genome Atlas (van Galen et al., 2019).

scRNA-seq has been of great value in understanding the immune transcriptional networks of non-Hodgkin lymphoma since technical challenges in the isolation and discrimination of neoplastic from normal B cells has prevented their analysis. Although sequencing of the B cell receptor has allowed for this discrimination, scRNA-seq can also simultaneously assess the expression profile of various malignant clones. Andor et al. (2019) used scRNA-seq in follicular lymphoma to distinguish malignant versus normal B cells through their restricted Ig light chain expression (either Igκ or Igλ) and demonstrated up-regulation of the anti-apoptotic *BCL2* gene and down-regulation of the MHC class II genes in the malignant clone. Interestingly, scRNA-seq analysis of tumor-infiltrating T cells showed that well-defined immune checkpoint markers (CTLA-4, PD-1) were also coexpressed with transcription factors and immune regulators such as *CEBPA* and *B2M* in T reg cells and *TNFRSF4/18* in CD4+ memory cells.

Hodgkin lymphoma (HL) is characterized by a peculiar TME where malignant cells make up ∼1% of the tumor mass. Thus, extensive infiltration of immunosuppressive immune cells into the tumor site makes the HL lymph node an attractive site to biopsy for scRNA-seq. Characterizing the HL lymph node should enable the understanding of important immunosuppressive resistance mechanisms that may blunt the efficacy of immunotherapies. Using the 10X Genomics platform, Aoki et al. (2020) carefully studied the TME of HL and, in particular, the T cells, finding a higher proportion of T reg cells compared with healthy lymph nodes. The authors also discovered a novel T reg cell subset characterized by LAG-3 expression that did not express classic T reg markers such as FoxP3. LAG-3+ T cells were found to mediate immunosuppression through their reduced proliferative activity and secretion of IL-10, TGF-β, and IFN-γ. Furthermore, removing LAG-3+ T cells from clinical HL samples induced T cell activation in vitro and, in a large validation cohort under first-line chemotherapy, higher numbers of LAG-3+ T cells associated with shortened overall survival. It is important to mention here that the 10X Genomics platform, with its cell size limitation of around 50 µm, may be unable to process and detect giant, malignant Hodgkin and Reed Sternberg cells from clinical samples that exceed 50 µm (Rengstl et al., 2013).

Multiple myeloma (MM) is a blood cancer that originates from plasma cells and, despite the development of several new therapeutic agents, still has a poor prognosis (Franssen et al., 2019; Rajkumar and Kumar, 2016). Recently, Zavidij et al. (2020) performed scRNA-seq on bone marrow biopsies of MM patients at various stages of disease. Here, the authors focused on NK cell infiltration into the bone marrow, which is considered a good prognostic factor. In patients with high NK cell bone marrow infiltration, precursor-stage NK cells had high expression of the chemokine receptor *CXCR4* while samples with fewer NK cells had a shift toward *CX3CR1* expression. This finding suggests that observed differences in the infiltration of NK cells are shaped by their chemotactic migration. Moreover, the authors observed that mature CD14+ monocytes in the bone marrow of MM overexpressed the E3 ubiquitin-protein ligase-encoding gene

MARCHF1, which internalizes MHC II molecules, potentially impacting T cell activation.

scRNA-seq has provided exciting insights on T cell function—particularly that of ex vivo engineered T cells—during immunotherapy for hematological malignancies. Clinical successes of CAR-T immunotherapy, however, are limited by heterogeneous responses, toxicities, and relapse, which may correlate to the heterogeneity of the CAR-T infusion product (Ruella and Maus, 2016; Bonifant et al., 2016; Shah and Fry, 2019). Bulk RNA-seq has previously highlighted the dependence of CAR-T efficacy on the degree of NK and $CD8^+$ infiltration (Zhang et al., 2019c). Bulk RNA-seq has also demonstrated that the preinfusion T cells of complete responders had a greater representation of genes involved in IL6/STAT3 responsiveness and an early memory-like phenotype compared with those of nonresponders (Fraietta et al., 2018). Compared with bulk RNA-seq, however, scRNA-seq is expected to provide deeper insights into the gene expression of the various components of CAR T cell products ($CD8^+$/$CD4^+$, memory subsets, and potentially novel subsets) and the TME that may affect CAR-T efficacy. Sheih et al. (2020) profiled $CD8^+$ CAR T cells from infusion products and peripheral blood of 10 leukemia patients at several time points during CD19-directed CAR-T immunotherapy. Notably, gene-set enrichment analysis revealed a transcriptional profile of $CD8^+$ CAR T cells consistent with an activated, effector T cell state that decreased in vivo during tumor elimination for each patient. Simultaneous sequencing of the TCR revealed that the clonal diversity of CAR T cells decreased as well, suggesting that selected CAR-T clones expanded more than others. The expanding clonotypes originated from the CAR T cells present in the infused product and were characterized by higher expression of cytotoxicity (granzymes H and K), proliferation (CD27), and activation (CCL4) markers. In another study, Xhangolli et al. (2019) seeded individual CAR T cells with Raji B cell lymphoma cells in microchambers. Using scRNA-seq, the authors found equal killing effectiveness in both $CD4^+$ and $CD8^+$ CAR T cells and, interestingly, that the predominant cytotoxic response came from T cells displaying highly mixed Th1/Th2 signatures.

Recently, researchers at MD Anderson Cancer Center performed scRNA-seq on anti-CD19 CAR T cells obtained from the infusion bags of the clinical product axicabtagene ciloleucel (Deng et al., 2020). After 3 mo, patients with large B cell lymphoma were classified based on response (progressive disease, partial response, or complete response). In the infusion products of patients with partial response/progressive disease, the authors found significant enrichment of exhausted $CD8^+$ T cells with an abundant coexpression of LAG-3 and TIM-3. On the other hand, the infusion products of patients who achieved complete response had significant enrichment of memory $CD8^+$ T cells, noted by the classic memory gene signature that includes expression of *CCR7*, *CD27*, and *SELL*. Reduced frequencies of exhausted $CD8^+$ T cells and increased frequencies of exhausted $CD4^+$ T cells were also associated with the development of high-grade cytokine release syndrome. Importantly, the authors also identified a rare monocyte-like cell population present in the infusion product that significantly associated with the development of high-grade neurotoxicity. This population had significantly higher expression of *IL1B* and *IL8*, two markers that have been implicated in the pathophysiology of neurotoxicity. However, the direct mechanism of action of monocyte-like CAR T cells in neurotoxicity remains unclear. Overall, the study underlined the increased efficacy of CAR-T products that are enriched for the memory gene signature and identify a monocyte-like CAR T cell population that may be responsible for observed toxicities during CAR-T therapy.

To further probe the potential factors that lead to neurotoxicity in anti-CD19 CAR-T therapy, Parker et al. (2020) analyzed scRNA-seq data generated from human brain. In these samples, the authors revealed CD19 RNA expression in brain mural cells, in particular, pericytes. The authors speculated that CAR-T neurotoxicity could be driven in part by this off-tumor CD19 expression. While the functional and clinical relevance of this finding must be validated, the identification of CD19 RNA in pericytes would not have been possible by bulk RNA-seq due to the low frequency of these cells in clinical samples.

Collectively, the presented findings of scRNA-seq in hematological malignancies suggest new mechanisms of immune invasion and factors that influence the patient response to CAR-T therapy. Overall, these studies have underscored new factors that form the regulatory gene network for hematologic malignancies that also may be exploited through immune modulation or improved CAR-T engineering.

Non–small cell lung cancer (NSCLC)

NSCLC is the leading cause of cancer deaths (Schabath and Cote, 2019). In the last few years, promising clinical responses have been achieved with ICB (Memon and Patel, 2019). Several PD-1–blocking antibodies (nivolumab and pembrolizumab) and anti–PD-L1 antibodies (atezolizumab, durvalumab) are approved by the US Food and Drug Administration as first-line treatment for metastatic NSCLC. Despite these promising advancements, the overwhelming majority of patients do not respond to ICB or eventually relapse (Haslam and Prasad, 2019). One of the dominant factors preventing the success of ICB is the immunosuppressive TME characterized by up-regulation of checkpoint ligands that can substantially curtail T cell cytotoxic activities (Thungappa et al., 2017).

Several recent scRNA-seq studies have focused on the study of the NSCLC TME. Guo et al. (2018) used Smart-seq2 on NSCLC biopsies to define unique subsets of "pre-exhausted" T cells—those with low expression of genes from a predefined signature of exhausted $CD8^+$ T cell infiltrates. One pre-exhausted subset included $CD8^+$ T cells with high expression of *ZNF683* (or *Hobit*), a central regulator of tissue residency (Mackay et al., 2016); these cells may precede the resident memory T cells, which are well associated with exhaustion and limited capacity for expansion (Ando et al., 2020). Another preexhausted subset was composed of granzyme K (*GZMK*)–high T cells. *GZMK* associates with the "effector-memory" phenotype (Wu et al., 2020), but *GZMK* memory T cells coexpress exhaustion markers and are also prone to give rise to exhausted T cells (Chu et al., 2020). The authors reported that a high ratio of "pre-exhausted" to "exhausted" T cells in the tumor was associated with better

prognosis in lung adenocarcinoma (Guo et al., 2018). Moreover, activated antigen-experienced T reg cells with expression of IL-1 receptor type II (*IL1R2*) and genes associated with immunosuppressive functions such as *REL* and *LAYN* correlated with poor prognosis. These distinct T cell signatures may serve as potential clinical biomarkers for NSCLC patients. Lambrechts et al. (2018) then confirmed high levels of T reg cells and exhausted (LAG-3, TIGIT) $CD8^+$ T cells in untreated tumors, while also finding low levels of $CD4^+$ T and NK cells. Thus, T reg cells that undermine T cell cytotoxic function hold potential as immunomodulation targets in NSCLC. In another key scRNA-seq study, Clarke et al. (2019) reported that a PD-1^+ TIM-3^+ tissue-resident memory T cell (T_{RM}) subset was enriched in NSCLC patients responding to PD-1 inhibitors and that it associated with tumors with a greater magnitude of cytotoxic responses (IL-2, IFN-γ, and TNF-α). Although previous bulk RNA-seq studies had found that high PD-1 expression on lymphocytes predicted ICB response and survival in a small NSCLC cohort (Thommen et al., 2018), scRNA-seq enabled detection of this unique PD-1^+ TIM-3^+ T_{RM} subset that reflected a functional and cytotoxic state rather than a dysfunctional state. Importantly, this finding confirms that not all infiltrating T cells with high exhaustion marker expression are dysfunctional and that defined T cell subsets are essential for response to ICB (Xia et al., 2019; Huang et al., 2017).

Sequencing of single NSCLC TAMs in treatment-naive patients has found populations with up-regulation of transcription factors *IRF2* and *STAT2* that decrease immune activation and down-regulation of inflammatory enhancers such as *Fos/Jun*, suggesting macrophage polarization toward a classically immunosuppressive M2 phenotype (Lambrechts et al., 2018). Maynard et al. also used scRNA-seq to find that TAMs of treatment-naive patients primarily display an M2 state (Maynard et al., 2019 *Preprint*). Macrophages in patients on systemic targeted chemotherapy, however, expressed inflammatory and T cell–recruiting cytokines such as *CXCL9/10* as well as *IDO1*, which promotes a tolerogenic TME (Munn and Mellor, 2016; Liu et al., 2018). In treatment-naive patients, Zilionis et al. (2019) also found a similar T cell–recruiting macrophage subset expressing *CXCL9/10* and *CXCL11*, but they further identified additional subsets like one with expression of neutrophil chemoattractant *CXCL5*. This suggests that heterogeneity in macrophage subsets is shaped partly by their expression of migration factors toward other immune cell types, such as T cells. Single-cell analysis of other NSCLC tumor-infiltrating myeloid cells has also uncovered a rare neutrophil population expressing type I IFN response genes (*IFIT1*, *IRF7*, *RSAD2*) and a unique "activated" dendritic cell (DC) population (Zilionis et al., 2019). Since both neutrophils and DCs have been implicated as regulators of cancer growth, the impact of *IFIT1*$^+$ neutrophils and activated DCs on tumor progression and treatment response requires further investigation.

Some studies have focused on the NSCLC cancer cells instead of the immune cells associated with them. Indeed, gene expression programs in malignant NSCLC cells can influence the immune system: in this context, single-cell analysis of cancerous cells is crucial to paint a detailed portrait of the TME. The single-cell analysis of primary lung adenocarcinoma cells and cell lines showed that down-regulation of genes in the IFN-γ signaling pathway correlated with reduced expression of MHC II as well as resistance to the multiple kinase inhibitor vandetanib (Ma et al., 2019a). In preclinical models focused on NK cell therapy, Laughney et al. (2020) found that resistance to NK-mediated killing was conferred by expression of *SOX9* in cancer cells. In their murine NSCLC metastasis model, depleting NK cells using anti-GM1 antibodies led to increased and accelerated metastatic outbreaks that were enriched in *SOX9*. This finding suggests an intimate, dynamic interplay of NK cell and NSCLC progression, a relationship that may prove useful for future immune exploitation. In summary, scRNA-seq of both the NSCLC immune microenvironment and cancer cells has uncovered new transcriptional signatures of both the NSCLC immune microenvironment but also cancer cells that will provide important clues on how to develop effective next-generation immunotherapies.

Melanoma

Melanoma is a highly aggressive tumor of the skin characterized by the asymmetrical, unusual growth of transformed melanocytes. Melanoma is diagnosed in around 100,000 people per year in the United States (Siegel et al., 2020). Novel agents target common melanoma-associated genetic abnormalities such as the *BRAF* mutation that affects half of the patients (Cheng et al., 2018). However, even combination therapies including *BRAF* inhibition are tarnished by high rates of cancer recurrence (Desvignes et al., 2017). Melanoma's high mutational burden has made the disease exceptionally responsive to immunotherapy. Dr. Steven Rosenberg's pioneering work at the National Cancer Institute on tumor-infiltrating lymphocytes (TILs) for the treatment of metastatic melanoma led to the first objective clinical responses from a cellular immunotherapy in the 1990s (Geukes Foppen et al., 2015). In 2011, the first ICB drug, ipilimumab, an anti–CTLA-4 antibody, was approved by the US Food and Drug Administration for the treatment of melanoma, sparking a series of new immunotherapeutic strategies, including the use of nivolumab and pembrolizumab, that have been at the forefront of clinical trials (Larkin et al., 2019; Azijli et al., 2014).

Recently, scRNA-seq has begun to dissect the immune infiltrates of metastatic melanoma, noting, in particular, the abundance of exhausted or dysfunctional T cells. Tirosh et al. (2016) identified a core T cell exhaustion signature with up-regulation of inhibitory (TIGIT) and costimulatory (4-1BB, CD27) receptors, in addition to conventional exhaustion markers (PD-1, TIM-3, CTLA-4). Li et al. (2019) used dual scRNA- and TCR-seq in tumor samples of 25 melanoma patients to uncover a gradient of $CD8^+$ T cell modules between dysfunctional (LAG-3) and cytotoxic (*FGFBP2*) programs. Additionally, the authors describe several factors that were not previously associated with a dysfunctional T cell phenotype, including *RBPJ*, a major mediator of the canonical Notch pathway. Up-regulation of the Notch pathway was also observed in T cells from studies of NSCLC by Guo et al. (2018).

In line with the scRNA-seq data in NSCLC and breast tumors (Guo et al., 2018; Azizi et al., 2018), exhausted $CD8^+$ T cells in melanoma formed highly proliferative and large clonal

compartments. Moreover, active cytotoxic T cells in melanoma were characterized by a high expression of the inhibitory receptor *KLRG1* and were unconnected to the exhausted, dysfunctional T cell gradient. Recently, Durante et al. (2020) studied samples of both primary and metastatic lesions from uveal melanoma, known to be highly metastatic and unresponsive to checkpoint blockade, under no therapeutic intervention. The researchers found LAG-3 as the dominant exhaustion marker expressed in $CD8^+$ T cells, low levels of NK cells, and a spectrum of M1- to M2-polarized macrophage states. Lastly, scRNA-seq has demonstrated that IFN-γ can hinder antitumor immunity through induction of suppressor-of-cytokine-2 (*SOCS2*) expression on infiltrating monocytes and DCs in melanoma (Nirschl et al., 2017). *SOCS2*, a member of the JAK-STAT regulators, limits DC-based priming of T cells in vivo; limiting *SOCS2* expression through immune modulation may enable robust immune-mediated tumor rejection.

scRNA-seq has also been used to study the melanoma TME during immunotherapy. Sade-Feldman et al. (2018) profiled immune cells from 48 melanoma tumor samples of patients undergoing ICB therapy, identifying two effector $CD8^+$ T cell states associated with responsive tumor regression (memory, activation, cell survival) or nonresponsive tumor progression (exhaustion, cell cycle). They also defined high *TCF7* and *IL7R* expression in $CD8^+$ T cells as positive predictors of response. Interestingly, scRNA-seq studies from Yost et al. (2019) in patient samples from advanced basal and squamous cell carcinomas of the skin also noted the importance of the pretreatment $TCF7^+$ T cell signature in expansion and successful response to anti–PD-1 therapy. This positive correlation between *TCF7* expression and response to ICB may be due to the role of *TCF7* in the self-renewal and maintenance of memory $CD8^+$ T cells (Kurtulus et al., 2019). The authors found that after ICB of PD-1, T cells highly expressed other exhaustion markers indicative of chronic activation, such as TIM-3 and TIGIT, T_{RM} markers, and CD39 (*ENTPD1*), a marker of tumor-reactive $CD8^+$ TILs (Yost et al., 2019).

Interestingly, Yost et al. (2019) also found that posttreatment exhausted T cells had undergone clonal replacement and had TCRs with new antigen specificities that were not found before PD-1 blockade. The origin and fate of these T cell clones were examined more meticulously by Wu et al. (2020). Here, the authors performed scRNA-seq on biopsies from other cancer types—lung, endometrial, colon, and renal—in patients undergoing anti–PD-L1 immunotherapy. They found that clonal expansion of effector-like T cells occurred not only within the tumor, as Yost et al. (2019) had observed, but also in normal adjacent tissue and peripheral blood. Importantly, their findings suggest that a portion of T cell clones that replenish exhausted clones originate in the surrounding tissue and peripheral blood and that heightened clonal expansion in the peripheral blood predicted good prognosis. Overall, these studies have expanded the knowledge of the exhaustion signatures in T cell infiltrates and methods to monitor T cell clonal expansion, giving new considerations for ICB therapy.

scRNA-seq has also revealed the heterogeneity of expression of common oncogenic targets in melanoma, which has general implications for the treatment of melanoma (Gerber et al., 2017; Fattore et al., 2019). In an early landmark study of resected human melanoma tumors from a range of therapeutic backgrounds, Tirosh et al. (2016) used a modified Smart-seq2 protocol and found two distinct, coexisting malignant cell states that expressed high levels of either transcriptional factor *MITF* or the *AXL* kinase. *AXL*-high cells were resistant to RAF and MEK inhibitor drugs, a mechanism that may prompt the use of ICB therapies for patients who fail conventional treatment (Müller et al., 2014; Ribas et al., 2019). Using both the Fluidigm C1 and 10X Genomics platforms, Ho et al. (2018) identified the upregulation of the dopachrome tautomerase gene (*DCT*) in a rare subpopulation of *BRAF* inhibition–resistant cells. This novel resistance marker has evaded bulk RNA-seq studies. High expression of *AXL*, *JUN*, and *NRG1* also associated with a rare cancer cell population that was in a "pre-resistant" state, supporting the results of Shaffer et al. (2017). Similar single-cell investigations should be performed for melanoma patients undergoing immunotherapy in order to identify new mechanisms of resistance.

BC

As reported by the National Cancer Institute's Surveillance, Epidemiology, and End Results program, BC is the leading cause of cancer mortality in women worldwide, with metastatic disease being the underlying cause of death in most cases (Redig and McAllister, 2013). BC classifications include ductal carcinoma in situ, invasive ductal carcinoma, inflammatory BC, and triple-negative BC (TNBC). Each subtype has a distinct gene expression profile that correlates with a different clinical outcome (Koo et al., 2017; Martelotto et al., 2014; Jang et al., 2020). The relationship between TILs and progression has been controversial in BC. Some studies claim that high TIL numbers are a strong indicator of survival (particularly in triple-negative and $HER2^-$ overexpressing subtypes), while others disagree (Denkert et al., 2018; Lee et al., 2018). High-resolution mapping of the BC composition with scRNA-seq is therefore crucial to decipher the complex relationship between TILs and the TME.

Profiling over 45,000 immune cells from eight breast carcinomas using the inDrop platform, Azizi et al. (2018) developed a human BC immune atlas, describing the incredible heterogeneity of T, B, NK, and myeloid cells in the BC TME. Immunosuppressive T reg cells expressed broad patterns of anti-inflammatory, exhaustion, hypoxia, and metabolism gene sets, suggesting a diverse range of functions among T reg cells. As observed in scRNA-seq experiments of NSCLC and melanoma, the authors described a plastic state of TAMs in between coexisting M1 and M2 gene signatures, often in the same macrophage. Chung et al. (2017) also noted that M2 TAMs in BC had an up-regulated immunosuppressive signature, including expression of *CD163*, *MS4A6A*, *TGFBI*, and other genes known to promote tumor progression and angiogenesis, such as *PLAUR* and *IL8*. To better study the origin of MDSCs and determine their capacity to suppress the adaptive immune response in cancer, Alshetaiwi et al. (2020) used transgenic mice models that developed human-like breast tumors. They found that aberrant neutrophil maturation in the spleen gave rise to MDSCs with an immunosuppressive

gene signature that included *IL1B*, *ARG2*, *CD84*, and *WFDC17*. In particular, CD84^{hi} MDSCs exhibited substantial T cell immunosuppression and increased ROS production, validating the key role of MDSCs in the BC TME.

In TNBC, Qui et al. reported several unique immune infiltrate subsets, including one of CD8$^+$ CXCL8$^+$ naive T cells that mediated neutrophil migration to the tumor site, activated MAPK/ERK pathways to promote tumor progression, and were associated with poor survival (Qiu et al., 2019 *Preprint*). Importantly, they discovered a novel subset of TCR$^+$ macrophages enriched in TCR signaling genes; this subset expressed productive TCRs capable of recognizing antigen and initiating downstream T cell–like signaling. In the same tumors, CD3$^+$ CD4$^-$ CD8$^-$ double-negative (DN) T cells were found split among effector, naive, and T reg DN clusters, the last of which has been associated with inflammation and autoimmunity (Juvet and Zhang, 2012; D'Acquisto and Crompton, 2011). Comprising only 1–5% of T cells in healthy adults, DN T cells made up a staggering 31% of the total infiltrated T cell population and thus may have major functional implications in TNBC. scRNA-seq of the BC TME has thus revealed unique cellular compartments, such as TCR$^+$ macrophages or the DN T cells, that were not described by bulk RNA-seq and yet may represent key players in the BC TME.

Overall response rates to checkpoint blockade in BC are low, although the limited responses observed are remarkably durable (Swoboda and Nanda, 2018). Current scRNA-seq studies have primarily focused on mechanisms that enhance checkpoint blockade. In primary and metastatic BC with high levels of TIL, Savas et al. (2018) used the 10X Genomics platform and defined a small cluster of CD8$^+$ CD103$^+$ T cells with proliferative features like that of T_{RM}. This T_{RM}-like cluster expressed high levels of both exhaustion markers (PD-1/CTLA-4) and effector markers (granzyme B). The single-cell–derived gene signature from this cluster was also significantly associated with a positive outcome in TNBC patients under both chemotherapy and nivolumab, thus suggesting CD8$^+$ CD103$^+$ T cells as a crucial target of checkpoint inhibition in BC. Jerby-Arnon et al. (2018) used scRNA-seq to reveal an intrinsic resistance program to ICB, commanded by cyclin-dependent kinases (CDKs) 4 and 6, expressed in melanoma cells before anti–PD-1 treatment. Later, Wang et al. (2019) performed scRNA-seq on mice with mammary tumors that acquired resistance during combination therapy, including the CDK4/6 inhibitor palbociclib. Interestingly, in CDK4/6-resistant tumors, scRNA-seq revealed high levels of immunosuppressive immature myeloid cells, which up-regulated the oncogenic drivers *Kit* and *Met*. Targeting *Kit* and *Met* using cabozantinib in combination with ICB led to significant mammary tumor shrinkage and greatly extended survival time in mice. These studies have demonstrated the practical guidance granted by scRNA-seq in the design of new immunotherapeutic regimens.

Hepatocellular carcinoma (HCC)

HCC is the most common primary tumor of the liver (75–85%) and the fourth leading cause of cancer mortality (Rawla et al., 2018). Immunotherapies for HCC still remain in their infancy, partly due to our limited knowledge of the function of specialized immune cells in the liver such as Kupffer cells (macrophages), NK, and various innate T cell subsets (Johnston and Khakoo, 2019; Chew et al., 2012). These immune cells and their distinct subsets form important components in HCC and have been recently scrutinized through scRNA-seq.

Zemin Zhang's group applied both Smart-seq2 and 10X Genomics to patient biopsies and found that HCC TAMs highly expressed the iron exporter ferroportin (*SLC40A1*), suggesting that iron metabolism shapes innate cancer immunity (Zhang et al., 2019b). In the HCC TME, DCs expressing the maturation marker *LAMP3* also expressed the greatest number of ligands for T and NK cell receptors and were suggested to be the most active regulators of these lymphocytes. Using a transwell migration assay, the authors suggested that *LAMP3*$^+$ DCs may migrate from tumors back to lymph nodes to prime T cells. However, the *LAMP3*$^+$ DC signature had a strong correlation with a T reg cell–like signature, implying that DCs could actually suppress T cells in the TME and contribute to T cell dysfunction (Zhang et al., 2019b). Within primary samples, scRNA-seq has also revealed two distinct intrahepatic CD68$^+$ macrophage subsets, one with an enriched expression of inflammatory markers (*LYZ*, *CSTA*, *CD74*) and the other with expression of tolerogenic genes like *VSIG4* and hemoxygenase (MacParland et al., 2018). Studies have also identified new subsets of Kupffer cells (specialized macrophages found only in the liver) that present antigens (*CD1C*$^+$) or may regulate the complement cascade (*LILRB5*$^+$; Aizarani et al., 2019). These specialized and unique macrophage subsets showcase the immense diversity of the immunoregulatory network in the HCC TME.

Furthermore, Ma et al. (2019b) found that T cell infiltrates of highly heterogeneous tumors had up-regulated genes for the epithelial-to-mesenchymal transition (EMT)—a process that may confer highly motile properties to T cells. Conversely, EMT in tumor cells promotes immune exclusion and immunotherapy resistance through several mechanisms, including enhanced resistance to T cell–mediated killing and reduced expression of surface antigens (Romeo et al., 2019; Chae et al., 2018). Ma's studies (Ma et al. 2019b) also found that highly diverse HCC tumors include a high fraction of immunosuppressive T reg cells. Tumors with a lower diversity score contained more proliferative T cells enriched in markers for activation (*IFNα*/*IFNγ*, *MYC*), cytotoxicity markers (*GZMA*, *GZMB*, *GZMH*, *PRF1*), and immune checkpoints (PD-1). These new immune subsets and their respective transcriptional states should, as in other cancers, be considered carefully upon designing new immunotherapies, as specific immunosuppressive populations of the tumor niche such as regulatory macrophage subsets may blunt the response to targeted therapies.

Colorectal cancer (CRC)

Although CRC is the third-most common cancer globally, single-cell transcriptomic studies are relatively limited, as are effective immunotherapies (Dai et al., 2019). In sharp contrast to the previously discussed cancer types, immunotherapy has failed to become a standard treatment for CRC patients, whose tumors very often have low neoantigen load and respond poorly to PD-1 or CTLA-4 blockade (Tintelnot and Stein, 2019). However, around 15% of patients have a microsatellite instable (MSI)

tumor akin to a "hot" tumor niche and thus have good responses to immunotherapy (Xiao and Freeman, 2015). Thus, more scRNA-seq studies can lead to improved understanding of the mechanisms that underlie immunotherapeutic resistance for difficult-to-treat cancers such as CRC.

Although MSI tumors have been observed to conduce good responses to immunotherapy, this mechanism is unclear. Zemin Zhang's group used scRNA-seq on CRC biopsies to identify 20 unique T cell subsets (Zhang et al., 2018). A Th1-like cluster expressing *BHLHE40*, a regulator of IFN-γ secretion and a repressor of IL-10, was enriched in patients with MSI tumors. This cluster may partly explain the favorable responses of patients with MSI tumors in PD-1 blockade, an observation documented across multiple cancers (Le et al., 2017). The same group also identified two distinct TAM populations (Zhang et al., 2020): one expressing *C1QC*—a member of the serum complement subcomponent C1q—and the other expressing *SPP1* (osteopontin), a marker of early T cell activation. *C1QC*$^+$ TAMs had significant enrichment of the complement activation, antigen processing, and presentation pathways, implicating its proinflammatory role through T cell recruitment. *SPP1*$^+$ TAMs, however, were more prevalent and enriched for regulators of angiogenesis and metastatic pathways, suggesting protumorigenic and pro-metastatic roles. Importantly, the authors found poor prognosis of patients with high *SPP1*$^+$ and low *C1QC*$^+$ TAM signatures in a Cancer Genome Atlas validation cohort, suggesting that specific depletion of *SPP1*$^+$ TAMs may help improve myeloid-targeted immunotherapy or ICB combination therapies. Another study profiling resected primary CRC tumors found that cancer-associated fibroblasts (CAFs) had enhanced expression of EMT-related genes and of the immunosuppressive cytokine TGF-β, suggesting the role of CAFs in TGF-β pathway activation of tumor epithelia (Li et al., 2017).

scRNA-seq studies of CRC have shed more light on the numerous roles of helper and cytotoxic T cells. In a mouse model of colon adenocarcinoma, Magen et al. (2019) studied CD4$^+$ cells that were found either in the tumor or in draining lymph nodes. Importantly, the authors discovered a type I IFN–responsive signature in Th1-like TILs that also associated negatively with patient responses to ICB in liver cancer and melanoma lesions. This signature includes expression of *STAT1*, *IRF7*, and *IRF9*. Interestingly, relative to Th1 cells, these unique Th1-like TILs expressed lower levels of *BHLHE40*, the IFN-γ regulator described earlier in Zheng's work (Zhang et al., 2018) that associated with good prognosis in CRC patients with MSI. Although T helper cells contribute heavily to antitumor responses, recent scRNA-seq studies have demonstrated the large and confusing functional spectrum of T helper subsets (Szabo et al., 2019; Cano-Gamez et al., 2020); this delays any straightforward method to probe these T helper populations for cancer treatment. Lastly, Kurtulus et al. (2019) revealed in mice that dual inhibition of TIM-3 and PD-1 checkpoints recruited T cells that lack expression of PD-1 and other checkpoint receptors (TIM-3, LAG-3, TIGIT). In particular, a memory-like, precursor-like CD8$^+$ PD1$^-$ T cell subset was similar to T cells in NSCLC/melanoma patients who achieved long-term responses to ICB (Das et al., 2015). This observation may be the result of earlier T cell precursors in the CD8$^+$ PD1$^-$ subset, which have better persistence and also steadily seed the effector T cell pool to achieve sustained responses.

These studies have uncovered new, clinically relevant subsets in the CRC tumor niche—such as *SPP1*$^+$ macrophages, PD1$^-$CD8$^+$ T cells, *BHLHE40*$^+$ Th1-like cells, and CAFs—that may be the focus of future immunotherapeutic strategies in CRC.

Other cancers

Additional cancer types have been studied using scRNA-seq, including ovarian (Izar et al., 2020; Shih et al., 2018; Lawrenson et al., 2019), head and neck (Puram et al., 2017), pancreatic (Hosein et al., 2019; Qadir et al., 2020; Peng et al., 2019; Schlesinger et al., 2020; Dominguez et al., 2020; Lin et al., 2020), prostate (Karthaus et al., 2020; Berglund et al., 2018; Horning et al., 2018), bladder (Chen et al., 2020; Sfakianos et al., 2020; Oh et al., 2020), and gastric cancer (Sathe et al., 2019 *Preprint*; Zhang et al., 2019a).

Ovarian cancer has a low overall survival rate (30–40%) with limited increase in the last several decades (Reid et al., 2017). Immunotherapy trials for ovarian cancer patients have included checkpoint blockades and adoptive T cell therapy using anti-mesothelin CAR T cells or anti–NY-ESO-1 T cells (Palaia et al., 2020). Disappointingly, only a limited subset of patients benefitted from these immunotherapies. Despite the barriers in our understanding of the ovarian cancer TME, scRNA-seq studies remain limited. Recently, Izar et al. (2020) used scRNA-seq to characterize the landscape of high-grade ovarian cancer in 11 patients. Specifically, they drained patients' ascites fluid, which is composed of a diverse collection of cell types and is frequently associated with poor response to chemotherapy. Of note, nonmalignant CAFs strongly expressed pro-inflammatory IL-6 and tumor-promoting cytokines. Increased infiltration of these CAFs may correspond to decreased response to ICB. Other studies have also noted pro-tumorigenic roles of CAF (Shih et al., 2018) and *SOX18* expression in fallopian tube epithelia that drive EMT (Lawrenson et al., 2019).

scRNA-seq studies are also limited in head and neck cancer, an aggressive disease with a complex ecosystem, a strong association with alcohol and tobacco use (Alsahafi et al., 2019), and a dire need for better immunotherapies (Economopoulou et al., 2016). In a key scRNA-seq study of primary tumors from the oral cavity and lymph node metastases, Puram et al. (2017) notably discovered a "partial" EMT expression program in malignant cells that appeared to be proximal to CAF under immunohistochemistry staining (Puram et al., 2017). This program lacked the classic regulatory markers of EMT but still appeared to promote local invasion and lymph node infiltration and was associated with poor patient outcome.

Pancreatic cancer typically presents at an advanced stage and has dismal rates of survival (2–9%); unfortunately, current immunotherapies are of no substantial benefit to these patients (McGuigan et al., 2018; Balachandran et al., 2019). It is therefore crucial to better understand pancreatic tumor biology to develop new therapeutic options. Importantly, thus far scRNA-seq studies have shown that T cells in pancreatic tumors with low T cell infiltration up-regulate cell cycle regulators *E2F* and *MYC*

and that the transcription factors Onecut2 and Foxq1 in malignant cells may drive early-stage pancreatic cancer development (Schlesinger et al., 2020). One group discovered a CAF population during pancreatic cancer development that was programmed by TGF-β and highly expressed the leucine-rich repeat containing 15 (*LRRC15*) protein (Dominguez et al., 2020). Shared across several cancers, the *LRRC15*$^+$ CAF signature correlated with poor response to ICB. This finding, along with other recent studies (Lin et al., 2020), further underlines the overlooked immunoregulatory role of CAFs in pancreatic cancer.

Conclusion and future perspectives

Single-cell transcriptomics has uncovered a plethora of diverse transcriptional programs that lie within the individual cells of the TME. The fine resolution of scRNA-seq technologies has granted cancer immunotherapy the ability to observe Darwinian evolution of a population at the level of a single cell under selective pressures. scRNA-seq has refined our understanding of the negative influences of dysfunctional or exhausted T cell infiltrates, T reg cells, MDSCs, monocytes, TAMs, and CAFs in several cancers. The prognostic value of functional, potent cytotoxic T cell states has even been ascribed to particular genes such as *TCF7* and *IL7R* in melanoma or a tissue-resident memory-like state in BC (Sade-Feldman et al., 2018; Savas et al., 2018). Importantly, scRNA-seq has unveiled new subsets of immune cells, such as unconventional "activated" DCs (Zilionis et al., 2019), TCR$^+$ macrophages (Qiu et al., 2019 *Preprint*), or tumor-promoting CD8$^+$ CXCL8$^+$ naive T cells (Qiu et al., 2019 *Preprint*) and has already guided the design of new immunotherapy combination regimens such as that of anti–HER2/neu antibody and CDK4/6 inhibitor in a murine BC model (Jerby-Arnon et al., 2018; Wang et al., 2019). In profiling malignant cells, scRNA-seq has discovered rare subpopulations (Zilionis et al., 2019; Ho et al., 2018; Aizarani et al., 2019), including those that emerge in response to treatment and those present at diagnosis that later confer resistance during treatment (van Galen et al., 2019). In the future, scRNA-seq of cancer will help to clarify controversies such as that surrounding EMT in cancer metastasis (Lourenco et al., 2020; Ono et al., 2019 *Preprint*; Puram et al., 2017). In several cancer types, we discussed how scRNA-seq has uncovered the genotypic and phenotypic alterations in individual cells, which affect population dynamics and ultimately underlie cancer pathogenesis or resistance to immunotherapy.

Our understanding of the TME complexity and cancer cell heterogeneity, however, remains incomplete. Harvesting the information granted by scRNA-seq to guide future drug development will require further studies to identify broad-scoped, shared markers between tumors vulnerable to ICB or adoptive cell therapy. Although many studies employ scRNA-seq on treatment-naive samples that are indirectly validated through external datasets, more studies on treatment-matched samples will reveal important resistance mechanisms under direct immunotherapeutic pressure. Furthermore, scRNA-seq platforms have much room for improvement in their transcript coverage or bias, sequencing depth, and overall cost. Making these technologies more readily accessible will significantly augment the fields of both cancer and cancer immunotherapy. In addition, the high dimensionality of scRNA-seq results may prevent user-friendly or easily interactive analysis of the data (Cakir et al., 2020). Although novel visualization tools, including some that are web-based, have been developed to enable experimentalists to use scRNA-seq data without the need to hard-code its analysis, more publicly available, relevant datasets are required to permit a more global adoption of scRNA-seq techniques. Lastly, scRNA-seq results, like any transcriptomic finding, will require extensive experimental validation to confirm their actual role; reducing this in vitro burden will also facilitate the wider use of scRNA-seq.

Newer technologies such as single-cell transposase-accessible chromatin sequencing (scATAC-seq) can be paired with scRNA-seq to map altered nucleosomes and transcription factors that lie at the core of disease progression. In particular, scATAC-seq can identify chromatin regulators of therapy-responsive T cell subsets and discover gene regulatory programs that govern CD8$^+$ T cell exhaustion in the TME (Satpathy et al., 2019). scATAC-seq may be useful to validate claims that specific DNA methylation programs restrict T cell expansion during ICB (Ghoneim et al., 2017). Spatial transcriptomics such as GeoMx (NanoString) or Visium (10X Genomics) can further enhance scRNA-seq by validating the inferred interactions of cells based on their spatial positioning within the tumor. Of note, spatial transcriptomics can add to the gene expression profile of immune cell information on their spatial positioning. For example, this can lead to a better understanding of the intrinsic causes of "hot" versus "cold" tumors and may also help us better associate the role of tertiary lymphoid structures with prognosis (Ji et al., 2020; Dieu-Nosjean et al., 2014). Another single-cell technology, CyTOF (time-of-flight mass cytometry), enables the profiling of over 40 protein parameters simultaneously from a limited cell sample (Gadalla et al., 2019). For example, CyTOF has been used for characterization of intra- and intertumor heterogeneity and simultaneous identification of the specific T cell subsets that are targeted by anti–CTLA-4 and PD-1 blockade (Wei et al., 2017). Observing evolving populations in the TME in response to immunotherapy, including infiltrates of engineered cellular therapies, has and will continue to provide clairvoyance into the barriers to remission and clinical success.

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