Trends in Genetics

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Review

Single-cell dissection of tumor microenvironmental response and resistance to cancer therapy

Yikai Luo[1,2] and Han Liang[2,3,1,*]

Cancer treatment strategies have evolved significantly over the years, with chemotherapy, targeted therapy, and immunotherapy as major pillars. Each modality leads to unique treatment outcomes by interacting with the tumor microenvironment (TME), which imposes a fundamental selective pressure on cancer progression. The advent of single-cell profiling technologies has revolutionized our understanding of the intricate and heterogeneous nature of the TME at an unprecedented resolution. This review delves into the commonalities and differential manifestations of how cancer therapies reshape the microenvironment in diverse cancer types. We highlight how groundbreaking immune checkpoint blockade (ICB) strategies alone or in combination with tumor-targeting treatments are endowed with comprehensive mechanistic insights when decoded at the single-cell level, aiming to drive forward future research directions on personalized treatments.

Highlights

Single-cell profiling technologies provide an unprecedented resolution in investigating the coordinated compositional and state alterations of immune and stromal components in response to cancer therapies on the tumor microenvironment, enabling a holistic and more nuanced understanding of treatment response and resistance.

Multimodal characterization of the heterogeneity of baseline and perturbed adaptive immune populations, especially T cells, offers a foundation for the discovery of the most actionable therapeutic targets.

Key players in the tumor microenvironment (TME) outside of the adaptive immune populations, including cancer-associated fibroblasts and macrophages, have emerged as effective mediators of cancer therapeutic response or resistance, frequently dependent on the crosstalk with T and B lymphocytes.

Tumor-targeting treatments demonstrate widespread TME-modulating effects, often indirectly through consequent immunostimulating signaling elicited by remodeled tumor cells, justifying a combinatorial administration with immunotherapies.

Distinct modes of action of established and emerging cancer therapies

Chemotherapy, targeted therapy, and immunotherapy are three major pillars but distinct modalities of cancer treatment that have different **modes of action (MoA)** (see Glossary). Chemotherapy exerts cytotoxic effects on cancer cells by interfering with their DNA synthesis, replication, or repair. It achieves this by using agents that can alkylate DNA, inhibit DNA or RNA synthesis, or disrupt microtubule function. Some examples of chemotherapy are alkylating agents (e.g., cyclophosphamide and cisplatin), antimetabolites (e.g., methotrexate and 5-fluorouracil), and plant alkaloids (e.g., vincristine and paclitaxel) [1][i]. Chemotherapy is a standard first-line treatment for many cancer types and can be used alone or in combination with other therapies. Targeted therapy inhibits oncogenic signaling pathways that drive cancer cell proliferation, survival, and invasion. It achieves this by using small-molecule inhibitors or monoclonal antibodies that can selectively bind to and interfere with the function of specific molecular targets. Some examples of targeted therapy are small-molecule drugs (e.g., imatinib and gefitinib) and monoclonal antibodies (e.g., sacituzumab govitecan, and cetuximab) [2][ii]. Targeted therapy has for more than two decades revolutionized the treatment of cancers with specific genetic alterations, with HER-2-targeting trastuzumab in breast cancer [3], multi-kinase-targeting sorafenib in hepatocellular carcinoma [4], and BRAF-targeting vemurafenib [5] being among the most successful pioneers. More recently, the discovery of new **cancer vulnerabilities** and breakthroughs in oncology pharmaceutical pipelines have led to the addition of more powerful weapons to the oncologist's arsenal, including PARP-inhibiting olaparib in ovarian cancer [6], TROP-2-targeting sacituzumab govitecan in triple-negative breast cancer [7], and KRAS-targeting sotorasib in non-small cell lung cancer (NSCLC) [8].

[1]Graduate Program in Quantitative and Computational Biosciences, Baylor College of Medicine, Houston, TX 77030, USA
[2]Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[3]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

*Correspondence:
hliang1@mdanderson.org (H. Liang).

Contrary to these tumor-targeting strategies relying on the understanding of **cancer-intrinsic factors**, a recent paradigm shift in cancer research toward forming a holistic view of the tumor ecosystem, with the cornerstone concept being the TME [9–12], has led to the focus on an emerging frontier in cancer therapeutics, which is immunotherapy [13,14]. Immunotherapy modulates the immune system to enhance its antitumor activity. It achieves this by using endogenous or exogenous agents that can stimulate or overcome **immune checkpoints**, induce immunogenic cell death, or elicit tumor-specific immune responses. Some examples of immunotherapy are chimeric antigen receptor (CAR) T cell therapy (e.g., axicabtagene ciloleucel and ciltacabtagene autoleucel), T cell engaging therapy (e.g., mosunetuzumab and teclistamab), and cancer vaccines (e.g., sipuleucel-T and talimogene laherparepvec) [15][iii]. ICB is probably the most successful immunotherapy class in solid tumors, which blocks the checkpoints that are normally involved in maintaining **immune tolerance** and preventing autoimmunity but inhibit the immune system from attacking cancer cells. ICB has shown remarkable clinical efficacy in various types of cancer. The nine FDA-approved checkpoint inhibitors targeting PD-1/L1, CTLA-4, and LAG-3 are now widely used to treat cancer patients in diverse clinical settings, including as a first-line treatment for advanced unresectable melanoma [16], as an **adjuvant treatment** for completely resected NSCLC [17], and as a first-line treatment but in combination with targeted therapy for advanced renal cell carcinoma [18]. Boundaries are now being pushed on the front of ICB therapeutics with exciting clinical data coming out of trials testing their efficacy in treating early-stage cancers at the **neoadjuvant**, preoperative stage [19–22]. This enthusiasm derives from a biologically sound theory that an intact tumor ecosystem encompasses a greater **tumor antigen load**, a better TME interaction infrastructure, and limited exposure to therapy-resistance-driving factors, thus bearing significantly more potential for optimal immune responses toward the therapy. The community is thus poised to witness the next wave of triumphs in ICB therapeutics.

Single-cell dissection of TME as a cornerstone of elucidating MoA of cancer therapies

Once dismissed as bystanders in tumor progression, host stromal and immune cells surrounding tumor cells have taken center stage as essential components of a highly structured and interconnected ecosystem that is the TME, owing to their extensive communications with the malignancy. An emerging and fast-evolving concept in cancer research, the term TME now bears a context beyond the tumor *per se* that encompasses systemic immune responses materializing throughout the body, involving mostly lymph nodes and blood vessels [9]. Such complexity in the composition and dynamics of the TME calls for high-throughput, unbiased, and high-resolution methodologies for a better understanding. Traditional TME characterization approaches, such as flow cytometry, immunofluorescence imaging, or bulk RNA sequencing (RNA-seq), rely on pre-existing knowledge and cell-type defining marker panels, and dilute the contributions of small immune cell subsets in the overall signal. In contrast, single-cell profiling technologies allow detailed identification of diverse immune subsets in the TME at a higher resolution, thereby providing a better opportunity to understand the contribution of immune cells to tumor progression and response [23–27]. For example, single-cell (sc)RNA-seq measures the transcriptome of individual cells in a tissue sample. It can also interrogate multiple modalities simultaneously, characterizing different genetic and epigenetic sequence information in a cell, such as DNA, gene expression (GEX), chromatin accessibility, histone modification, protein abundance, T/B cell receptor (TCR/BCR) sequence, and DNA methylation status [28–31]. Furthermore, spatial transcriptomics enables the identification of spatially variable genes, prediction of tissue architecture, cell-type localization, and the inference of cell–cell communication in a TME [32–35]. These multimodal efforts in dissecting treatment-naive tumors have created comprehensive baseline references for key TME populations, including T cells [36–39], fibroblasts [40–42], and myeloid cells [43–45].

Box 1. Special considerations in single-cell TME data analysis

With the establishment of numerous methodological benchmarks and workflows, single-cell genomics, especially scRNA-seq, has seen the maturation of a data analytical paradigm [28,106], for example, implemented by Seurat [107] and scverse [108]. However, owing to the extraordinary heterogeneity of the TME and unique sampling conditions of tumor samples, there is a myriad of special considerations beyond the routine practices an analyst needs to keep in mind. For example, the coexistence of cell types with a wide range of total RNA content in the TME [109] requires extra effort on distinguishing empty droplets from those containing real cells. Specifically, a hard cutoff on per-droplet UMI or a single FDR threshold on the output of sophisticated algorithms such as EmptyDrops [110] may not be sufficient. Depending on the stage of tumor progression and the sampling, storage, and transportation conditions, single-cell/nucleus TME profiles may face regular to serious levels of ambient RNA contamination that could hinder a clear separation of cell states especially with regard to intra-tumor-cell heterogeneity, the mitigation of which is already a challenge in normal tissues [111–113]. On a similar note, a perhaps more inconspicuous issue is the enrichment of stress-related gene signatures in TME cells, which may often be caused by harsh dissection processes or simply heightened necrotic activities within the tumor core [114–116] but could lead to false interpretations as biologically authentic signals.

Even with a clean and balanced post-quality-control single-cell TME dataset, the analysis and interpretation of it need to be conducted prudently with TME biology taken *a priori*. For instance, how to strike a balance between complying with community knowledge base and highlighting dataset specificity by choosing different clustering resolutions and following different classification paradigms, when annotating cell population identities, is a delicate job. The consensus that cancer cells retain unique transcriptomes shaped by patient-specific driver genetic aberrations while nonmalignant cells show cross-patient unified cell states would warrant the universal application of integration algorithms such as Harmony [117]. However, the latest research has demonstrated a potential overcorrection effect of such methods where context-dependent immune cell states failed to be preserved by single-cell data integration [118,119]. Cell state continuity is an intriguing and important concept in understanding the TME dynamics, especially under therapeutic interventions. Methods such as pseudotime estimation and RNA velocity, as well as the combination thereof, which saw their initial success in delineating organogenesis or induced cellular differentiation, are now often applied to modeling a variety of cell state transitioning events in the TME, including T cell exhaustion progression. However, whether the underlying mathematic assumptions hold true in such diverse settings has rarely been deliberated, as called out by relevant comments [38,120]. Similarly, the statistical assumption of independent and identical distributions is often violated in cell-population-level differential gene expression analyses between conditions when it is not done in a pseudobulk manner but using single cells as subjects [121].

When paired with the longitudinal sampling of patient tissue samples under specific treatment regimens, single-cell profiling can also capture the dynamic changes of the TME in response to different therapeutics, such as chemotherapy, targeted therapy, and immunotherapy (Box 1). Alternatively, single-nucleus profiling can access a larger and more diverse collection of archival tumor samples due to its capability of profiling single nuclei isolated from frozen tissues that have been stored for long periods of time and are difficult to dissociate [46–48]. Even with only single-time-point sampling; for example, either pre- or post-treatment, these efforts can be useful in building a knowledge base of baseline or treatment-perturbed TME composition that has predictive values given longitudinally collected patient response data. Beyond cell composition alterations, single-cell TME characterization can also reveal how these treatments affect the expression of genes and pathways that are involved in tumor growth, invasion, angiogenesis, inflammation, and immunity at a cell-population or subpopulation resolution. These cellular and molecular correlates with real-world clinical implications help uncover the mechanisms of treatment resistance and sensitivity, as well as potential biomarkers and therapeutic targets [49–54]. Notably, many of these findings can be quickly visualized and analyzed through web portals such as TISCH [55][iv], SCRP-TCM [56][v], and metaICB [57][vi]. The ultimate utility of these insights is to help predict tumor response, as well as to discover new targets and biomarkers for personalized cancer treatment (Figure 1).

TME-perturbing effects of ICB

Single-agent ICB

The initial findings of characterizing the TME of ICB-treated cancer patients were limited to changes in T cell states induced by single-agent anti-PD-1/L1 or anti-CTLA-4 treatments. Specifically, the therapeutic responses to ICB were thought to be driven by the reversal of the

Glossary

Adjuvant treatment: a cancer treatment given after the primary treatment, such as surgery, to reduce the risk of cancer recurrence.
Cancer vulnerabilities: weaknesses or susceptibilities of cancer cells that can be exploited by drugs or other treatments to kill or inhibit them.
Cancer-intrinsic factors: factors that originate from the cancer cells themselves and that affect their growth, survival, invasion, metastasis, or response to therapy.
Clonal expansion: a process that occurs when a specific subset of T cells undergoes rapid expansion in response to foreign antigen stimulation through specific TCR recognition.
Homing: the process of recruitment and migration of immune cells to specific organs or tissues, including cancer, governed by interactions between homing receptors on immune cells and their ligands in tissues.
Immune checkpoints: molecules that are expressed on the surface of immune cells and interact with their ligands on other cells to trigger either stimulatory or inhibitory immune response signals.
Immune tolerance: a state of unresponsiveness or nonreactivity of the immune system to substances or tissues that would normally elicit an immune response.
Major histocompatibility complex (MHC): a group of genes that code for cell surface proteins that help the immune system recognize foreign antigens.
Major pathological response (MPR): a strong degree of tumor reduction after neoadjuvant therapy, usually defined as having less than or equal to 10% of residual viable tumor cells in the resected specimen.
Minimal residual disease (MRD): the small number of cancer cells that remain in the body after treatment, a major cause of relapse for patients with blood cancer.
Mode of action (MoA): changes in cell-level biochemical pathways or processes induced by a drug or substance, without specifying the exact molecular targets or interactions.
Mutation-associated neoantigen (MANA): altered peptides derived from somatic mutations and presented by MHC to be recognized by the immune system to elicit an antitumor T cell response.

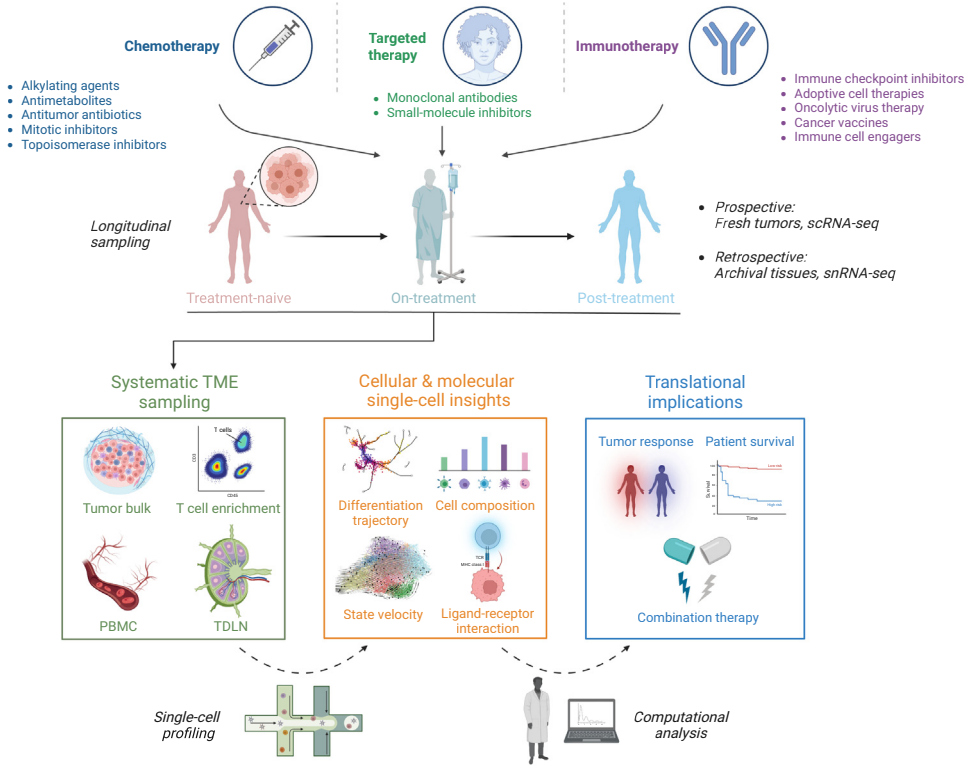

Figure 1. Elucidating TME dynamics under cancer therapies through longitudinal single-cell profiling. Digital portrayals of therapy-experienced TME can be obtained from longitudinal multi-context single-cell sampling and will inform research and clinical decisions based on therapy-associated biological signals. Abbreviations: PBMC, peripheral blood mononuclear cell; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing; TDLN, tumor-draining lymph node; TME, tumor microenvironment. The image was created using BioRender (https://biorender.com/).

Neoadjuvant treatment: a cancer treatment given before the main or primary treatment, usually to shrink the tumor and make it easier to remove or destroy by the main treatment.
Tertiary lymphoid structure (TLS): an ectopic lymphoid tissue that forms in nonlymphoid organs or tissues in response to chronic inflammation including cancer.
Tissue-resident memory (TRM): TRM T cells are a group of long-lived memory T cells that reside and function within specific tissues without recirculating in the blood or lymph.
Tumor antigen load: the total amount of tumor antigens that are present in a tumor, including tumor-specific antigens and tumor-associated antigens.
Tumor-draining lymph node (TDLN): a lymph node that is connected to a tumor by a network of lymphatic vessels and receives lymph fluid, soluble factors, and cells from the tumor site and is often the first site of solid tumor metastasis.

exhaustion program in tumor-reactive $CD8^+$ T cells (Tex) within the TME (Box 2) [58–60]. Thus, gene expression programs linked to cytotoxicity and acute activation have been recurrently reported to be associated with a favorable response toward ICB (Figure 2 and Table 1). For example, in basal cell carcinoma, PD-1 blockade induced **clonal expansion** of $CD8^+CD39^+$ T cells expressing markers of chronic T cell activation and exhaustion [61]. Prostate cancer-specific $CD8^+$ T cells had increased IFNγ expression after androgen receptor blockade, leading to enhanced function and sensitization to anti-PD-1 immunotherapy [62]. Similarly, the expansion of PD-1^+ $CD8^+$ T cells expressing cytotoxic activity, immune-cell **homing**, and exhaustion markers, as well as $CD4^+$ T cells showing T helper-1, and follicular-helper signatures, was observed in breast cancer after anti-PD-1 administration [63]. In endometrial cancer, tumor regression after anti-PD-1 therapy was associated with effector $CD8^+$ T cells and activated $CD16^+$ NK cells in the circulation [64]. Additionally, **tissue-resident memory (TRM)** seems to be an indicator of fast-responding antitumor $CD8^+$ T cells. In a melanoma patient responding to anti-PD-1, a subset of $CD8^+$ TRM cells that are positive for both PD-1 and TIM-3 was significantly enriched after treatment [65]. A study in NSCLC offered a more definitive connection between $CD8^+$ T cell TRM and tumor reactivity because it, along with an incompletely activated cytolytic program and less IL-7R activity, marked a unique $CD8^+$ T cell population that reacted specifically to **mutation-associated neoantigens (MANAs)**. However, in tumors without a **major pathological response (MPR)** to anti-PD-1, these cells not only showed heightened expressions of T cell

Box 2. Emerging cancer therapeutic implications of T cell exhaustion

T cell exhaustion, initially discovered in the study of immune responses against chronic viral infections [122,123], has emerged as a central theme in understanding the TME mechanisms of response and resistance towards immunotherapy and perhaps tumor-targeting therapies as well [37,124,125]. A general consensus of its definition includes impaired effector functions such as decreased production of cytotoxic markers (e.g., granzyme B and perforin) and inflammatory cytokines (e.g., IFN and TNF), heightened expression of inhibitory receptors (e.g., PD-1 and TIM-3), and a hyporesponsive proliferation capacity to antigen stimulation [126]. Beyond that, there is little to agree upon when it comes to the multifaceted nature of T cell exhaustion. Perhaps most frustratingly, we know these unique cell states are induced by chronic antigen encounter, but we do not know for a fact whether the aforementioned alterations represent a failed T cell state in the face of unresolved tumor burden or an adapted new identity in the pursuit of a balance between tumor control and immunopathology. We are also only beginning to understand the level of heterogeneity in this seemingly unified population, dependent on disease progression and tissue context, and how a wide range of microenvironmental factors may shape the trajectory of T cell exhaustion.

Despite the enigmatic biology of T cell exhaustion, we have now developed a set of operational principles on where it is positioned in cancer therapeutics, owing to extensive research on T cell responses to ICB in both mouse and human tumors, often using single-cell technologies. For example, the abundance of pretreatment Tex cells often portends favorable patient survival in multiple cancer types [127–129] but serves as a negative indicator of therapeutic efficacy when examined at a post-treatment time point [66,70]. These observations point to the other side of exhaustion being a measurement of tumor reactivity. Longitudinally, single-cell profiling studies have consistently identified that a common consequence of ICB on the TME is a shift in Tex cells to regain effector and even memory functions. Further, studies utilizing bulk or single-cell T cell receptor sequencing (scTCR-seq) to coanalyze ICB-experienced TME, blood, and TDLNs have uncovered the origin of these newly generated effector populations, a major contributor being the TCF1-marked pre-existing progenitor exhaustion population [130–132]. Whether they are sourced from TME-infiltrated Tex reinvigoration [66,97,133] or replenished Tpex from the periphery, particularly the TDLN [63,71–73,134,135], however, show considerable variations among cancer types. Thus, instead of solely focusing on reinvigorating intratumoral Tex cells, a more viable approach in many cases may be to elicit systemic responses where Tpex can be recruited and clonally expanded. Indeed, mechanistic studies in relevant mouse models have demonstrated that the key mediator of anti-PD-1/PD-L1 treatment is a group of PD-L1$^+$ conventional dendritic cells residing in the TDLN [136–138].

dysfunction genes and reduced memory or effector activities but also stronger presence of TRM signatures, suggesting greater plasticity and a more complex role of TRM cells in immunotherapy than previously appreciated [66].

Another perspective to look at the Tex rejuvenation phenotype in terms of CD8$^+$ T cell state transformation involves the emergence of a progenitor-exhaustion CD8$^+$ T cells (Tpex) program (Figure 2 and Box 2) [57,67–69]. For example, anti-PD-1 treatment in advanced melanoma led to two distinct states in CD8$^+$ T cells, with one defined by the activation of Tpex transcription factor TCF1 and associated with positive clinical outcomes [70]. Findings from clear cell renal cell carcinoma (ccRCC) identified the increased composition of Tpex, which is characterized by low expression of coinhibitory molecules and high expression of GZMK as a major mediator of ICB efficacy [71]. Through longitudinally profiling blood samples from head and neck squamous cell carcinoma (HNSCC) patients undergoing anti-PD-L1 treatment, a recent study revealed that CD8$^+$ T cells with an intermediate strength of the progenitor exhaustion program were the most enriched population in post-treatment circulation, offering a higher-resolution view of the Tpex–Tex dichotomy in ICB response [72]. Adding a spatial context to Tpex emergence, a recent study in NSCLC provided strong evidence of the coordination between CD4$^+$ and CD8$^+$ T cells with common exhaustion programs being a predominant reactive force against tumor antigens and their common supply being progenitors in **tumor-draining lymph nodes (TDLNs)**. These key findings were made through a direct comparison of tumor-infiltrating T cells between tumor regions with or without viable cancer cells and those in the lymph nodes [73]. Finally, although functioning through distinct mechanisms, the efficacy of an antibody-based T cell engager (TCE) was also reported to be driven by the expansion of an effector CD8$^+$ T cell population. However, contrary to most ICB-based studies, the baseline abundance of terminally exhausted CD8$^+$ T cells in multiple myeloma was predictive of therapeutic failure [74], putting a

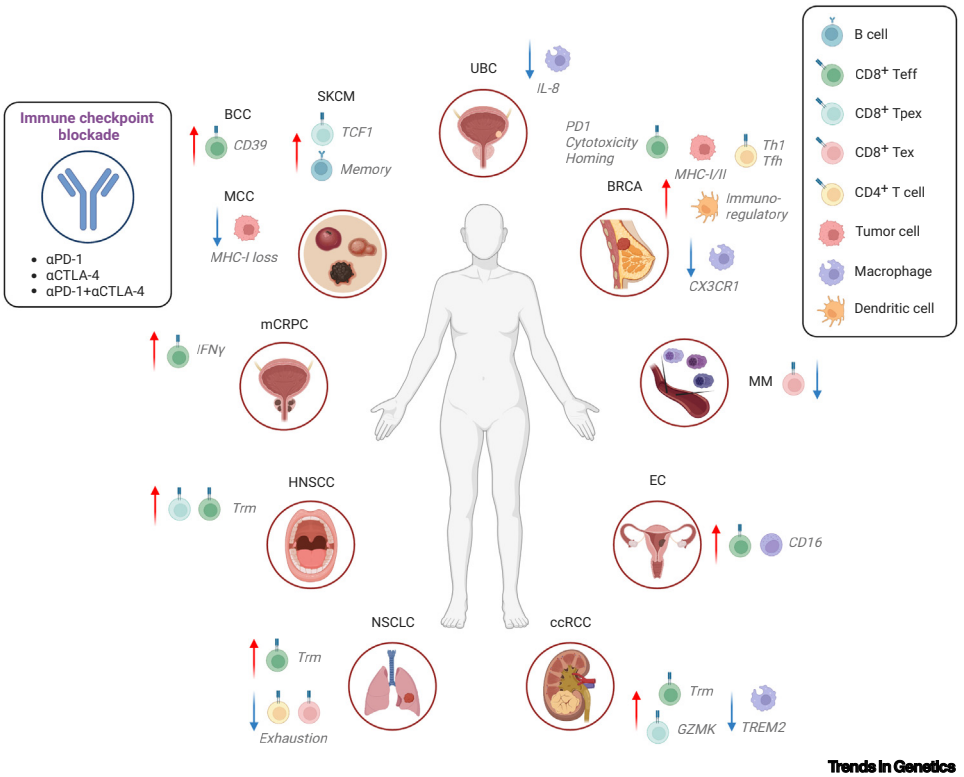

Figure 2. ICB-perturbed TME at the single-cell level. Alterations by ICB in the composition and cell state of specific TME populations are highlighted for various cancer types. Red upward arrows indicate cell groups that are enriched after treatment or positively correlated with the therapeutic response or described as a favorable TME factor. Blue downward arrows indicate the opposite cases. Grey italicized texts indicate genes or gene signatures highlighted in original studies. Abbreviations: BCC, basal cell carcinoma; BRCA, breast invasive carcinoma; ccRCC, clear-cell renal cell carcinoma; EC, endometrial carcinoma; HNSCC, head, and neck squamous cell carcinoma; ICB, immune checkpoint blockade; mCRPC, metastatic castration-resistant prostate cancer; MCC, Merkel cell carcinoma; MM, multiple myeloma; NSCLC, non-small cell lung cancer; SKCM, skin cutaneous melanoma; TME, tumor microenvironment; UBC, urothelial bladder cancer. The image was created using BioRender (https://biorender.com/).

heavier emphasis on a favorable pretreatment TME rather than the rejuvenation potential thereof for the success of T cell engagement and activation (Box 2) [74].

Outside of the T cell compartment, single-agent ICB can also elicit a systemic immune response where the composition and state of numerous classes of non-lymphocytes can be modulated, including even tumor cells (Figure 2 and Table 1). An example is the aforementioned breast cancer study, where the enrichment of activated $CD8^+$ T cells was correlated with the expression of **major histocompatibility complex (MHC)** class I/II molecules in cancer cells and immunoregulatory dendritic cells, and inversely correlated with the presence of inhibitory macrophages [63]. In another case, peripheral-myeloid-cell-specific interleukin-8 expression level at the baseline stage was associated with loss of the myeloid antigen-presentation machinery in bladder cancer patients not responding to an anti-PD-L1 antibody [75].

Combination ICB

PD-1 and CTLA-4 are the two best-studied immune checkpoints that are vital for achieving self-tolerance and modulating immune responses. However, targeting only one of these checkpoints at a time in clinics often leads to cancer immune resistance [76]. This is likely because, while both

Table 1. Single-cell TME profiling studies of tumors treated with immunotherapy

Cancer type	Therapy class	Therapy	Target	Cell type	Data modality	Refs
BCC	ICB	Pembrolizumab, cemiplimab	PD-1	TME	GEX + TCR	[61]
ccRCC	ICB	Nivolumab, nivolumab + ipilimumab	PD-1, CTLA-4	TME	GEX + TCR	[78]
ccRCC	ICB	Nivolumab	PD-1	Tumor-infiltrating T cells	GEX + TCR	[71]
EC	ICB	Pembrolizumab	PD-1	PBMCs	GEX + TCR	[64]
HNSCC	ICB	Nivolumab, nivolumab + ipilimumab	PD-1, CTLA-4	Tumor-infiltrating immune cells, PBMCs	GEX + TCR	[79]
HNSCC	ICB	Atezolizumab	PD-L1	TME, TDLN	GEX + TCR, GEX + surface protein abundance	[72]
HR+ and triple-negative BRCA	ICB	Pembrolizumab	PD-1	TME	GEX + TCR, GEX + surface protein abundance	[63]
MCC	ACT + ICB	Autologous virus-specific $CD8^+$ T cells, followed by pembrolizumab+ ipilimumab	PD-1, CTLA-4	TME, PBMCs	GEX	[81]
mCRPC	ICB	Pembrolizumab	PD-1	Tumor-infiltrating immune cells	GEX	[62]
MM	TCE	Elranatamab	BCMA, CD3	Tumor-infiltrating immune cells, PBMCs	GEX + TCR + BCR	[74]
NSCLC	ICB	Nivolumab	PD-1	Tumor-infiltrating, lymph node, adjacent normal lung T cells	GEX + TCR	[66]
NSCLC	ICB	Pembrolizumab, nivolumab	PD-1	Tumor-infiltrating, lymph node, adjacent normal lung T cells	GEX + TCR	[73]
SKCM	ICB	αPD-1, αPD-1 + αCTLA-4	PD-1, CTLA-4	Tumor-infiltrating immune cells	GEX	[70]
SKCM	ICB	Nivolumab, nivolumab + ipilimumab	PD-1, CTLA-4	Tumor-infiltrating immune cells	GEX	[80]
SKCM	ICB	Pembrolizumab, nivolumab + ipilimumab	PD-1, CTLA-4	Tumor-infiltrating T cells	GEX	[65]
UBC	ICB	Atezolizumab	PD-L1	PBMCs	GEX	[75]

can inhibit T cell responses to tumors, they do so at different stages and locations of an immune response. PD-1 suppresses T cells later in the immune response, mainly in peripheral tissues, by binding to its ligands (PD-L1 and PD-L2) on tumor cells or other antigen-presenting cells (APCs) and inhibiting signaling through CD28 and other receptors that promote T cell survival and function. CTLA-4 controls T cell proliferation early in the immune response, mainly in the TDLNs, by competing with CD28 for binding to the same ligands (B7 molecules) on APCs [77]. The complementary therapeutic windows offered by diverse immunostimulatory effects were then exploited in clinical trials to achieve improved patient response towards combination ICB. The US FDA has now approved ipilimumab plus nivolumab and tremelimumab plus durvalumab, respectively, as anti-PD-1/anti-CTLA-4 combination strategies to treat various types of cancer[vii].

Mechanistically, these combinations often demonstrate similar TME-perturbing effects as single agents in single-cell studies (Figure 2 and Table 1). For example, the application of anti-PD-1 and anti-CTLA-4 in ccRCC revealed patient-dependent immune response heterogeneity, marked by either $CD8^+$ tissue-resident T cells in responsive patients or tumor-associated macrophages (TAMs) in resistant patients [78]. Likewise, in oral cancer patients, neoadjuvant anti-PD-1

and anti-CTLA-4 blockade induced the expansion of activated T cells that were further characterized by elevated TRM and cytotoxicity programs [79]. Switch from naive B cells to memory and plasma B cells appeared to be a favorable contributor to effective ICB response in melanoma and renal cell carcinoma, suggesting a pivotal role of B cells and **tertiary lymphoid structures (TLSs)** in ICB response [80]. Although proven more effective than single agents, combinations can still result in treatment resistance. A patient with metastatic Merkel cell carcinoma receiving autologous Merkel cell polyomavirus specific $CD8^+$ T cells, an adoptive cell therapy (ACT), followed by anti-PD-1 and anti-CTLA-4 resulted in the transcriptional suppression of MHC-I genes in the tumor cells and loss of activated tumor-infiltrating $CD8^+$ T cells at the relapsed stage, an indication of acquired resistance to the treatment [81].

TME-targeting potentials of cancer-intrinsic treatments

Despite strides in translational research and preliminary clinical trials, the approval rate for advanced immune checkpoint inhibitors or immunomodulators remains somewhat low[viii]. However, increasing evidence shows that traditional anticancer treatments can boost anticancer immunity, either by directly affecting tumor cells or by producing off-target effects on immune cells [82–84].

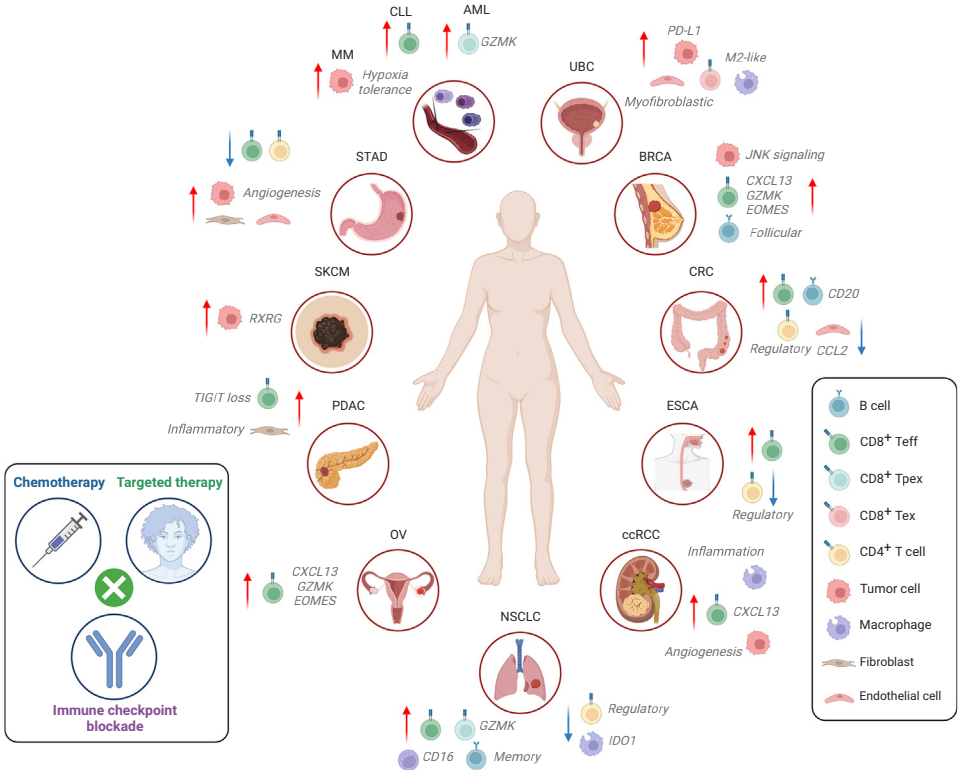

Figure 3. Chemotherapy- and targeted-therapy-perturbed TME at the single-cell level. Alterations by tumor-targeting therapies or their combination with ICB in the composition and cell state of specific TME populations are highlighted for various cancer types. Red upward arrows indicate cell groups that are enriched after treatment or positively correlated with the therapeutic response or described as a favorable TME factor. Blue downward arrows indicate the opposite cases. Grey italicized texts indicate genes or gene signatures highlighted in original studies. Abbreviations: AML, acute myeloid leukemia; BRCA, breast invasive carcinoma; ccRCC, clear-cell renal cell carcinoma; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; ESCA, esophageal cancer; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OV, ovarian cancer; PDAC, pancreatic adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TME, tumor microenvironment; UBC, urothelial bladder cancer. The image was created using BioRender (https://biorender.com/).

Table 2. Single-cell TME profiling studies of tumors treated with chemo-/targeted therapy and in combination with ICB

Cancer type	Therapy class	Therapy	Target	Cell type	Data modality	Refs
AML	Chemotherapy, ICB	Azacitidine + nivolumab	PD-1	TME, normal bone marrow	GEX + TCR	[96]
ccRCC	Targeted therapy, ICB	Unspecified αPD-1, αPD-1 + αCTLA-4, αPD-1 + VEGFi	PD-1, CTLA-4, VEGF	TME	GEX	[101]
CLL	Targeted therapy	Ibrutinib	BTK	PBMCs	GEX + surface protein abundance	[95]
CRC	Targeted therapy, ICB	Dabrafenib + trametinib + spartalizumab	PD-1, BRAF, MEK1/2	TME	GEX	[102]
CRC	Targeted therapy, ICB	Celecoxib + toripalimab	PD-1, COX-2	TME, normal tissue	GEX	[103]
ER-positive BRCA	Targeted therapy	Letrozole, letrozole + ribociclib	Estrogen, CDK4/6	TME	GEX	[92]
Triple-negative BRCA	Chemotherapy, ICB	Paclitaxel, paclitaxel + atezolizumab	PD-L1	Tumor-infiltrating immune cells, PBMCs	GEX + TCR + chromatin accessibility	[100]
ESCA	Chemotherapy	FLOT		TME, normal tissue	GEX	[88]
Metastatic BRCA or OV	Targeted therapy, ICB	Ribociclib + spartalizumab	PD-1, CDK4/6	Peripheral T cells, tumor-infiltrating immune cells	GEX + TCR	[104]
MM	Targeted therapy	Daratumumab, carfilzomib, lenalidomide, dexamethasone	CD38, proteosome, cereblon	Bone marrow plasma cells	GEX	[90]
NSCLC	Chemotherapy, ICB	Pembrolizumab + carboplatin + pemetrexed, pembrolizumab + carboplatin + albumin-bound paclitaxel	PD-1	Tumor-infiltrating T cells	GEX + TCR	[97]
NSCLC	Chemotherapy, ICB	Carboplatin + toripalimab/camrelizumab/sintilimab + docetaxel/pemetrexed/gemcitabine	PD-1	TME	GEX	[98]
NSCLC	Targeted therapy	Multiple TKIs	Tyrosine kinases	TME	GEX	[94]
PDAC	Chemotherapy	FOLFIRINOX, gemcitabine/abraxane-based		TME	GEX	[86]
PDAC	Chemotherapy	FOLFIRINOX, gemcitabine + nab-paclitaxel		TME	GEX	[87]
SKCM	Chemotherapy, targeted therapy, ICB	Unspecified αPD-1 + αCTLA-4 + chemotherapy/multiple targeted therapies	PD-1, CTLA-4, multiple tumor targets	TME	GEX	[99]
SKCM	Targeted therapy	Dabrafenib, dabrafenib + trametinib	BRAF, MEK1/2	PDX	GEX	[91]
STAD	Chemotherapy	Paclitaxel + oxaliplatin, oxaliplatin + S-1, oxaliplatin + calcium folinate + 5-FU		TME	GEX	[89]
UBC	Targeted therapy	Tipifarnib	HRAS	TME	GEX	[93]

These observations came as no surprise as chemotherapy and targeted therapy, for instance, have long been known to induce immunogenic cell death, which releases damage-associated molecular patterns and tumor antigens that stimulate antitumor immunity. For example, anthracyclines can induce the exposure of calreticulin on the surface of dying tumor cells, which can then facilitate their phagocytosis by dendritic cells [85]

However, these implications are largely confined to preclinical models or clinical histological observations, limited in comprehensiveness and resolution. Thus, single-cell profiling of the TME

perturbed by chemotherapy or targeted therapy as a large-scale unbiased approach has been able to offer a more precise and definitive view of the specific cellular mediators underlying their immunostimulatory effects (Figure 3 and Table 2). These studies commonly adopted a whole-tissue sample preparation strategy where the *bona fide* composition of a complete TME is retained, thus offering vital insights into how a systemic immune response is elicited by cancer treatments. This is a unique advantage in studies involving tumor-targeting strategies because such information is often masked in ICB-related studies where a prior bias toward enriching the immune component is applied to increase the immune cell capture rate.

Chemotherapy

A case study in pancreatic ductal adenocarcinoma showed that chemotherapy suppressed immunosuppressive TME reprogramming by downregulating TIGIT on $CD8^+$ T cells and inhibiting their interactions with cancer cells [86]. A second study in the same disease context revealed more cellular mediators of chemotherapy-induced immunostimulation, including significant enrichment for inflammatory cancer-associated fibroblasts (CAFs) and an increase in $CD8^+$ T cells [87]. Similarly, in the TME of esophageal adenocarcinoma, an increase in the ratio of effector $CD8^+$ T cells to regulatory $CD4^+$ T cells (Tregs) appeared to be a major consequence of chemotherapy [88]. However, immunosuppressive effects were also observed for chemotherapy in other cancer types, including in stomach adenocarcinoma, where it decreased $CD4^+$ and $CD8^+$ T cells, increased endothelial cells and fibroblasts, and activated proangiogenic pathways in cancer cells [89]. These distinct chemotherapy outcomes in post-treatment T cell abundance and fitness suggest that while conventional chemotherapy is widely recognized for its expansive cytotoxic impact on rapidly dividing cells, particularly those in the immune system, other agents may bring in favorable net effect to the TME, thus being synergistic with ICB (which is discussed later).

Targeted therapy

Similar to chemotherapy, several single-cell TME profiles have been generated for samples from patients treated with targeted therapy (Figure 3 and Table 2). However, a large proportion of these efforts were concentrated on the perturbation effect on cancer cells, presumably due to their perceived highly cancer-specific MoA. For instance, in relapsed multiple myeloma patients, a combination of chemotherapy-induced changes in malignant plasma cells was characterized by hypoxia tolerance, protein folding, and mitochondrial respiration signatures [90]. Concurrent RAF/MEK inhibition in melanoma resulted in a shift in the composition of **minimal residual disease (MRD)** cells, marked by the expression of a neural crest stem cell transcriptional program primarily driven by the nuclear receptor RXRG [91]. Combination therapy of endocrine treatment with a CDK inhibitor in early-stage estrogen-receptor-positive breast cancer led to cancer-cell-specific loss of estrogen signaling and upregulation of JNK signaling in resistant tumors [92].

Several studies also support the TME-perturbing role of targeted therapy while connecting them to cancer-intrinsic alterations (Figure 3 and Table 2). In chemorefractory muscle-invasive urothelial bladder cancer, treatment with an HRAS-targeting agent boosted PD-L1 expression in surviving tumor cells and accumulated multiple immunosuppressive immune subsets, including M2-like macrophages, exhausted $CD8^+$ T cells, and myofibroblastic CAFs, leading to acquired resistance to this treatment but a favorable response to subsequent treatment with a PD-L1 inhibitor [93]. In metastatic lung cancer patients, targeted therapy, particularly with tyrosine kinase inhibitors (TKIs), enabled an antitumor TME in the residual disease stage by enriching for T cells and depleting macrophages (especially an $IDO1^+$ subset) but later enhanced an opposite trend when the disease progressed. Such retreat from a transitory immunostimulatory TME was linked to a series of progression-friendly behaviors retained in surviving cancer cells in the residual disease,

including an alveolar-regenerative cell signature and upregulation of kynurenine, plasminogen, and gap-junction pathways [94]. The application of ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor, resulted in a marked transformation in the chronic lymphocytic leukemia (CLL) microenvironment, highlighted by the increased percentage of $CD8^+$ T cells exhibiting a cytotoxic and/or exhaustion program [95].

Chemotherapy or targeted therapy in combination with ICB as superior TME immunostimulants

ICB with chemotherapy

The newly developed TME-focused perspectives for tumor-targeting therapies have opened new avenues to explore their possible synergies with ICB (Figure 3 and Table 2). Consequently, many recent FDA drug approvals that widen treatment choices for patients with solid tumors primarily involved antibodies that target PD-1/L1 in combination with chemotherapy and TKI. In acute myeloid leukemia (AML), tumors responding to a combination of azacytidine, a cytidine nucleoside analog, and anti-PD-1 treatment were enriched for a clonally expanded $GZMK^+$ $CD8^+$ T cell population with stem-like features, while nonresponding tumors were characterized by cancer-intrinsic chromosome 7/7q loss [96]. Similarly, integrating anti-PD-1 with multiple chemotherapies to treat NSCLC patients drove the emergence of clonally expanded $GZMK^+$ Tpex cells [97]. Another study in NSCLC, where PD-1 blockade was combined with chemotherapy in a neoadjuvant setting, induced expansion and activation of cytotoxic $CD8^+$ T cells and $CD16^+$ NK cells, reduction of immunosuppressive Tregs, and remodeling of TAMs into a neutral phenotype. Accordingly, tumors showing MPR were specifically characterized by activated antigen presentation in cancer cells and transcriptional signatures of $FCRL4^+FCRL5^+$ memory B cells and $CD16^+CX3CR1^+$ monocytes, while those with no major responses exhibited overexpression of estrogen metabolism enzymes and elevated serum estradiol in cancer cells [98]. Efforts were also made to search for cancer-intrinsic factors in connection with a favorable TME state shift. For example, in melanoma patients treated with a variety of therapeutics, including anti-PD-1, anti-CTLA-4, chemotherapy, and their combinations, the expression of a resistance program was induced in cancer cells, which was associated with T cell exclusion and immune evasion, thus leading to treatment resistance. Interestingly, the expression of this program could be repressed through CDK4/6-inhibition in a mouse model [99].

Given the shared TME phenotypic outcomes in terms of T cell rejuvenation, it would be meaningful to disentangle the effects independently contributed by chemotherapy and ICB. This would not only help gain mechanistic insights into immunostimulatory mediators of vastly different natures, but also facilitate better-informed clinical decisions to reduce treatment burden. A study involving advanced triple-negative breast cancer patients offered valuable data to address this issue by directly comparing the TME alteration effects of the two treatment arms where either paclitaxel or paclitaxel plus anti-PD-1 was applied. While the combination therapy boosted the composition of multiple immunostimulatory populations, such as $CXCL13^+$ T cells, lymphoid tissue inducer cells, follicular B cells, and conventional type 1 dendritic cells, the monotherapy significantly decreased their abundance and instead led to the activation of suppressive TAMs. Paired single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) profiling of combination-treated tumors also revealed enhanced chromatin accessibilities in genes of effector properties, especially in the $CXCL13^+$ $CD8^+$ T cell population [100].

ICB with targeted therapy

Concern about ICB–chemotherapy combination causing severe adverse effects is certainly justified, given the extensive damage to normal tissue cells, despite largely positive outcomes in clinical trials. Targeted therapy generally causes fewer and less severe side effects compared

with chemotherapy. It also induces swift disease control and high response rates but limited durability when used as monotherapy, thereby offering an opportunity for ICB synergy (Figure 3 and Table 2). For instance, patients with advanced ccRCC who responded to combined PD-1 blockade and VEGF inhibition had augmented effector T cell response, while all patients including nonresponding individuals had increased inflammatory and immunosuppressive macrophages as well as angiogenic cancer cells [101]. In colorectal cancer patients, combined PD-1, BRAF, and MEK inhibition had greater induction of tumor cell-intrinsic interferon signaling response, stronger suppression of MAPK signaling activity, and significant enrichment of $CD8^+$ T cells in those who had longer progression-free survival [102]. Again, in colorectal cancer, a coordinated reduction of proliferative $CD8^+$ TRM cells, $CD4^+$ Tregs, proinflammatory $IL1B^+$ monocytes, and $CCL2^+$ fibroblasts, and an increase in the proportions of $CD8^+$ Tem cells, $CD4^+$ T helper cells, $CD20^+$ B cells, and HLA-DRA^+ endothelial cells were observed following neoadjuvant PD-1 blockade and COX-2 inhibition [103]. Even a chemotherapy-like combination strategy that would theoretically deplete fast-expanding lymphocytes, such as tumor-reactive $CD8^+$ T cells, was successful, echoing the TME-reinvigorating synergy observed for ICB–chemotherapy combinations. This study in metastatic breast and ovarian cancer found that CDK4/6 inhibition along with PD-1 targeting not only allowed highly expanded cytotoxic effector $CD8^+$ T cell clones to emerge in the circulation but skewed them toward a memory program with high GZMK and EOMES expression, reinforcing a stem-like antitumor T cell pool [104]. However, this is not to postulate that targeted therapy will always be a positive force to improve the immunoactivity of TME and a better choice in combination with ICB than chemotherapy. Instead, the efficacy of such a strategy would still depend on (i) utilizing appropriate biomarkers to monitor and predict therapeutic response and toxicity, and (ii) establishing optimal timing, sequence, and dosage for a specific combination. For example, head and neck cancer patients benefited from longer intervals between PI3Kδ inhibitor administration, which caused less depletion of Treg cells and less severe colitis. Follow-up single-cell TME profiling of mouse models treated with PI3Kδ validated intermittent dosing regimens to be superior in mitigating Treg-related adverse effects [105].

Concluding remarks

The integration of traditional cancer treatments with immunotherapy, such as ICB, represents one of the most exciting frontiers in cancer research and treatment. By modulating the TME, these treatments have the potential to significantly improve patient outcomes. However, a deeper understanding of the interactions between cancer cells, immune cells, and other components of the TME is needed to fully realize the potential of these treatment strategies. To this end, we anticipate that breakthroughs in this field will derive from three aspects of technological advancements. First, integrating multimodal data from various sources, including genomics, transcriptomics, proteomics, and metabolomics, along with cutting-edge computational and machine learning techniques, will help create more comprehensive models of the TME. Second, longitudinal single-cell profiling of patient samples at a finer time scale, rather than just before, during, and after treatment, could provide valuable insights into fine-grained dynamic changes in the TME and reveal highly transitory but key events mediating treatment resistance. Third, the development of new spatial profiling technologies, such as spatial transcriptomics and multiplex imaging, can help provide a systematic view and illuminate the complexity of the TME on an unprecedented scale, such as its cell organization, microenvironmental niches, nutrient gradients, and cell–cell interactions. These features, when extracted jointly, can pinpoint organizational immune hallmarks that are associated with patient outcomes. As we continue to unravel the complexity of the TME through single-cell profiling and other advanced techniques, we may uncover new treatment targets and strategies to overcome treatment resistance, ultimately bringing us closer to the goal of personalized cancer therapy (see Outstanding questions).

Outstanding questions

How can we exploit emerging single-cell sequencing modalities such as scATAC-seq, to establish new dimensions of treatment-induced T cell state shifting on top of transcriptional programs and surface protein expressions, especially those regarding upstream epigenetic regulatory circuitries?

What are the direct and indirect mechanisms underlying the immunostimulatory effects of chemotherapy and targeted therapies and how to disentangle their differential impacts on the TME than immunotherapy in patients treated with combination therapies?

While current studies provide valuable insights into the TME in response to various cancer treatments, most of them are based on endpoint analysis. How can we monitor the fine-grained TME dynamics over the course of treatments, especially in the context of treatment resistance?

Can we develop more sophisticated experimental and computational models that factor in the spatial contexts to capture TME perturbations under various treatments, predict patient response, and inform personalized therapeutic strategies?

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Declaration of interests

H.L. is a shareholder and scientific advisor of Precision Scientific Ltd.

Resources

[i]www.cancer.gov/about-cancer/treatment/types/chemotherapy

[ii]www.cancer.gov/about-cancer/treatment/types/targeted-therapies

[iii]www.cancer.gov/about-cancer/treatment/types/immunotherapy

[iv]http://tisch.comp-genomics.org/documentation/

[v]https://singlecell.mdanderson.org/TCM/

[vi]http://metaicb.cancer-pku.cn/

[vii]www.cancerresearch.org/blog/march-2022/fda-immunotherapy-approvals-in-2022

[viii]www.cancerresearch.org/regulatory-approval-timeline-of-active-immunotherapies

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