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Role of CD4$^+$ T cells in cancer immunity: a single-cell sequencing exploration of tumor microenvironment

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Abstract
Recent oncological research has intensely focused on the tumor immune microenvironment (TME), particularly the functions of CD4 + T lymphocytes. CD4$^+$ T lymphocytes have been implicated in antigen presentation, cytokine release, and cytotoxicity, suggesting their contribution to the dynamics of the TME. Furthermore, the application of single-cell sequencing has yielded profound insights into the phenotypic diversity and functional specificity of CD4$^+$ T cells in the TME. In this review, we discuss the current findings from single-cell analyses, emphasizing the heterogeneity of CD4$^+$ T cell subsets and their implications in tumor immunology. In addition, we review the critical signaling pathways and molecular networks underpinning CD4$^+$ T cell activities, thereby offering novel perspectives on therapeutic targets and strategies for cancer treatment and prognosis.

Keywords Single-cell sequencing, CD4$^+$ T cells, Tumor microenvironment, Immunotherapy

Introduction

The tumor microenvironment (TME) is a complex network of stromal cells, immune cells, and extracellular components that sustain tumor cells through interactions with signaling molecules and physical factors [1, 2]. The interaction between TME and tumor cells has been demonstrated to affect tumor invasion and drug resistance [1, 3]. Therefore, it is essential to study the TME, especially the associated immune cells [2, 4]. Previous studies have demonstrated that CD4$^+$ T cells majorly contribute to tumorigenesis, metastasis, clinical diagnosis, and treatment prognosis. The functions of CD4$^+$ T cells are potentially diverse and paradoxical due to the complexity of the TME [5]. CD4$^+$ T cells exhibit different functional states and phenotypes and regulate the immune response, thereby coordinating the activities of other immune cells and affecting the behavior of tumors and response to therapy, and consequently exerting varying effects on tumor progression [5, 6]. Moreover, different CD4$^+$ T cell subsets have varying functions in the anti-tumor process, including regulating humoral immunity, activating B cells and CD8$^+$ T cells to exert anti-tumor effects, and killing tumor cells by cytotoxic CD4$^+$ T cells [7–9].

The recent and rapid development of single-cell sequencing technologies and their wide application in cancer research has accelerated the elucidation of CD4$^+$ T cell functions in TME. Single-cell sequencing can

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overcome the shortcomings of traditional bulk sequencing methods in studying $CD4^+$ T cells, such as the masking of cell heterogeneity, loss of cell-to-cell information, inability to differentiate cell subsets, and limited spatial information [2, 10, 11]. Single-cell RNA sequencing (scRNA-seq) can be applied to identify the accurate molecular information about each cell within tumors [4, 12]. In addition, scRNA-seq can investigate tumor heterogeneity, microenvironmental cell type composition, and cell state transitions [10, 13, 14]. It can comprehensively demonstrate the unique characteristics of different components in the TME and clarify the interactions between tumor cells and several components in the microenvironment, especially the relationship between tumors and immune cells [6, 12]. This allows us to better understand the functions of $CD4^+$ T cells in immune response, immune regulation, and tumor immunity, providing significant information to develop precise immunotherapeutic strategies [13, 15, 16].

Here, we review the recent advances in single-cell sequencing studies performed to study $CD4^+$ T cells in TME. Specifically, we provide an overview of the classification of $CD4^+$ T cells using single-cell sequencing. Surface markers of $CD4^+$ T cells, their spatial distribution characteristics in the TME, temporal distribution characteristics in tumor evolution, and their functions and mechanisms in tumor immunity are summarized.

Application of single-cell sequencing in research on CD4 + T cells in TME

Unique advantage of single-cell sequencing dissecting CD4+ T cells in TME

Single-cell sequencing technology has rapidly advanced over the past decade. The initial sequencing studies predominantly focused on the transcriptomic analysis of numerous mixed cells, known as bulk transcriptome sequencing. This method allowed researchers to analyze the average gene expression within a sample. However, it masked specific signals exhibited by individual cell populations or cell states. The development of sequencing technology led to single-cell sequencing, which enabled a comprehensive study of gene expression at the single-cell level. Examining individual cells at the genomic, transcriptomic, epigenomic, and proteomic levels can overcome the limitations of traditional bulk sequencing [14, 17].

Comparison of two single-cell sequencing platforms: 10X Genomics and Smart-seq2

Currently, sequencing research of $CD4^+$ T cells largely depends on two sequencing platforms, namely, 10X Genomics Chromium, and Smart-seq2. Significant differences exist between these two protocols in terms of sequencing principle, sequencing volume, and data presentation. Smart-seq2 is based on high-fidelity reverse transcriptase, template conversion, and pre-amplification to increase the yield of cDNAs and ultimately obtain full-length transcripts. This method has excellent coverage, which refers to the frequency or depth with which each position in the target genome or sample is sequenced. Higher coverage indicates that more reads(the short sequence fragments obtained from target DNA or RNA segments during the sequencing process) cover the target area, thus enhancing the accuracy and reliability of detection. It can also detect rare transcripts and does not require additional specialized equipment, thereby increasing its applicability [18]. The 10X Genomics single-cell sequencing is based on the 10X Genomics platform and can separate and label 5,000 to 10,000 single cells at once and detect them at the single-cell level as well. The method is based on microfluidics and is based on molecular biology principles similar to that of Smart-seq2, applying template conversion techniques [19]. However, it differs from Smart-seq2 in the approach and throughput of cell capture. Figure 10X Genomics method involves wrapping individual cells in a small oil droplet (containing a barcode and reverse transcriptase primer) and reverse transcribed into cDNA, following which the oil droplet ruptures to release the cDNA for library construction. Although this approach increases the experiment flux, it requires specialized experiment equipment [20]. There exist certain differences in the principles of constructing the library between these two techniques. Each method has its own set of advantages and limitations, as illustrated in Table 1.

Recently, Zhang et al. conducted a comparative analysis of scRNA-seq data of immune cells using the two platforms [20]. Smart-seq2 detected more genes and more complex datasets in cells, especially transcripts of lower abundance and alternative splicing transcripts. Smart-seq2 data are more similar to the bulk RNA-seq data. Approximately 10–30% of all transcripts detected by both platforms belonged to non-coding genes, with a higher proportion of lncRNAs in the 10X Genomics Chromium. Smart-seq2 has a higher sensitivity and detects a considerably larger number of expressed genes than the number of expressed genes detected by 10X Genomics Chromium. The Smart-seq2 data demonstrated a single-peaked distribution for the genes detected, with a few low-expressed genes detected in all cells. In contrast, the 10X Genomics data demonstrated a double-peaked distribution due to numerous near-zero expressed genes, suggesting a high level of noise or random capture of mRNA at very low expression in the 10X Genomics data. The 10X Genomics Chromium data allow for better detection of rare cell types due to its ability to cover numerous cells. In addition, each platform detects

Table 1 Comparison of 10X Genomics and Smart-seq2 for single-cell RNA sequencing

Feature	10X Genomics	Smart-seq2
Sequencing principle	Based on droplet microfluidics.	Based on full-length cDNA synthesis and amplification.
Throughput	High: Processes thousands to tens of thousands of cells per run.	Low: Handles hundreds of cells per run.
Sequencing coverage	High: Capable of processing thousands to tens of thousands of cells per run.	Low: Handles hundreds of cells per run
Sequencing depth	Shallow: Focuses on gene expression levels but lacks depth for low-abundance genes or full-length transcript.	Deep: Generates comprehensive full-transcriptome data, capturing low-abundance genes and rare transcripts.
Sensitivity	Moderate: Suitable for detecting moderately to highly expressed genes.	High: Excellent for identifying low-abundance genes and rare transcripts.
Data complexity	High: Generates massive datasets with complex barcoding and UMI deduplication, requiring significant computational resources.	Low: Easier data processing, though full-length transcriptome data demands substantial storage and computation.
Sample barcoding	Dual-end barcoding	Single-end barcoding
Cost	Low	High
Platform	Requires the 10X Genomics Chromium system with a high degree of automation.	Requires more manual steps, though certain automated equipment can be utilized.
Application	Suitable for large-scale cellular population analyses	Ideal for high-resolution single-cell analyses

different sets of genes expressed in clusters of cells, suggesting the complementarity of techniques [1, 20].

Application and development of spatial transcriptomics to dissect TME

Emerging spatial transcriptomics is increasingly being applied to study tumor CD4 + T cells. Currently, 10X Genomics Visium and GeoMx DSP are the most representative technologies in this field [21]. 10X Genomics Visium uses spatial barcoding and high-throughput sequencing to provide unbiased whole-transcriptome data, making it ideal for large-scale tissue analysis. However, its spatial resolution is limited (about 55 μm), preventing single-cell precision. In contrast, GeoMx DSP combines targeted probe hybridization with optical selection to achieve high-resolution multi-omics analysis of RNA and proteins, with near single-cell resolution [22]. It is well-suited for precise molecular detection in specific regions of interest but requires preselected targets and has lower throughput. Temporal specificity can be studied by obtaining samples from different time points and using single-cell transcriptome sequencing techniques to differentiate cell types and gene expression patterns in the temporal dimension [23]. However, spatially specific information is relatively difficult to obtain because of losing cellular spatial relationships during tissue dissociation [1]. Spatial transcriptomics sequencing can identify different cell populations while preserving their spatial location information. Thus, it provides crucial data on the relationship between cell function, phenotype, and location in the tissue microenvironment [24]. The location of gene expression can be used to map the complex and complete expression of disease-causing genes [18]. This approach allows the researchers to reveal the biological structure of normal and abnormal tissues and discover novel tissue biomarkers [25, 26].

Spatial transcriptomics has been applied to study primary pancreatic tumors, and subpopulations of ductal cells, macrophages, dendritic cells (DCs), and cancer cells are spatially restricted with significant co-enrichment with other cell types [27]. A study provided a spatially resolved fine map of the immune landscape in pancreatic ductal adenocarcinoma. The researchers highlighted immune subsets with potentially underappreciated functions in pancreatic ductal adenocarcinoma, particularly $CD4^+$ T cell subsets and natural killer (NK) T cells that are terminally exhausted and acquire a regulatory phenotype [28]. Another study provided the whole transcriptome profile of metastasis by analyzing the spatial RNA sequencing of primary and metastatic tumor specimens obtained from 44 patients with non-small cell lung cancer (NSCLC). The results suggested that TME creates an immunosuppressive and fibrotic ecology in the brain for metastatic brain tumors [29]. Similarly, other researchers have applied spatial transcriptomics to melanoma lymph node biopsies, thereby successfully sequencing the transcriptomes of over 2,200 tissue domains. They used this approach to generate gene expression profiles, revealing details of melanoma metastases [30]. Furthermore, a combination of scRNA-seq and spatial transcriptomics has been applied to human squamous cell carcinomas. For example, researchers found that cancer cells localize to fibrovascular ecotopes, whereas immune cells are spatially confined to different compartments, suggesting that cancer cells could employ a spatial mechanism to evade immune surveillance [31]. These studies demonstrate that spatial transcriptomics approaches can be used to study the subpopulation structure and reveal the interactions inherent in complex tissues.

Reclassification of CD4 + T cells in single-cell studies

In the previous section, we discussed how scRNA-seq and spatial transcriptomics techniques help identify and localize different cell populations within the TME. These technologies not only provide molecular characteristics of cells but also preserve their spatial information. Specifically, recent pancreatic cancer studies combining these two techniques have revealed differential distribution of CD4 + T cells at the tumor periphery and core, which is crucial for understanding their role in tumor immunity [27, 32]. In head and neck cancers, spatial profiling of the immune microenvironment in PD-L1 + CTLA4 combination therapy patients identified CD4 + T cell activation and T cell recruitment to tumor-draining lymph nodes as early indicators of immune response to combination therapy [33]. This multi-omics approach is gradually being applied to study CD4 + T cells in the tumor microenvironment of various cancers, including breast cancer [34], gastric cancer [35], hepatocellular carcinoma [36], lung cancer [37], nasopharyngeal carcinoma [38], cervical squamous cell carcinoma [39] and glioblastoma [40].

Exhausted CD4+ T cells

Initially, in the context of chronic infections and autoimmune diseases, CD4 + T cells characterized by high expression of exhaustion markers, including TIGIT, PD-1, CTLA-4, and CD39, were identified as exhausted [41, 42]. Subsequent scRNA-seq studies in pan-cancer research have provided further evidence for the existence of this distinct population of exhausted CD4 + T cells [43–46].

A lung cancer study using single-cell sequencing has discovered certain exhausted $CD4^+$ T cell subsets in TME. Certain evidence suggested that the enrichment of exhausted $CD4^+$ T cells is associated with increased $CD1C^+$ DC population [47], and in some subsets, the expression of *Lag3* and *Tim3* increased with a decrease in the expression of *CD45RO* [48]. Recent research constructed a $CD4^+$ T cell atlas using scRNA-seq data from patients with multiple cancer types and identified two exhausted $CD4^+$ T cell subgroups expressing PD-1 and CD200 in bladder cancer [49]. One of them highly expresses PD-1 and CD200 ($PD1^{hi}CD200^{hi}CD4^+$ T cells), and the other one highly expresses PD-1 with low expression of CD200 ($PD1^{hi}CD200^{low}CD4^+$ T cells). Researchers found that patients with high proportions of $PD1^{hi}CD200^{hi}CD4^+$ T cells displayed a worse response to immunotherapy than those with $PD1^{hi}CD200^{low}CD4^+$ T cells. They later detected that $PD1^{hi}CD200^{hi}CD4^+$ T cells recruit tip endothelial cells and initiate angiogenesis through UTP and its transporter *P2RY6*. Furthermore, they found that tumor cells recruit $PD1^{hi}CD200^{hi}CD4^+$ T cells to promote epithelial–mesenchymal transition by enhancing m6A-mediated GAS6. These findings indicate that a combination of immunotherapy and anti-CD200 drugs could be a promising treatment strategy for patients with bladder cancer [49]. In a recent single-cell study of endometrial cancer, researchers used identified exhausted $CD4^+$ T cells with scRNA-seq, and malignant epithelial cells induce immune suppression through ligand receptor pairs (such as TNF-ICOS) interacting with depleted $CD4^+$ T cells. Blocking the binding of TNF-ICOS may inhibit the induction of immunosuppressive microenvironment and may become an effective therapeutic target for endometrial cancer in the future [50]. Appling single-cell sequencing in PD-1 blockade tumor sample, some researchers found that exhausted T cell clonotypes directly attack tumor cells, and PD-1 blockade can promote infiltration of these exhausted T clonotypes [51]. We believe that gaining insights into these unexplored mechanisms could help us identify approaches to reactivate exhausted T cells and enhance the effectiveness of the existing immunotherapy.

CD4+ cytotoxic T lymphocytes

$CD4^+$ cytotoxic T lymphocytes are crucial effectors of anti-tumor immunity. Previous studies have reported that T lymphocytes with cytotoxic functions are largely limited to the conventional major histocompatibility complex (MHC) class I-restricted $CD8^+$ T lymphocytes. However, recent preclinical and clinical studies have demonstrated that MHC class II-restricted T helper cells with cytotoxic potential exist in tumors and that these CTLs can directly kill cancer cells [52, 53]. These cells can secrete GZMB and perforin, and kill target cells in an MHC class II-restricted manner, thus exerting antiviral and antitumor immunity and inflammation. Previously, certain scholars believed that $CD4^+$ cytotoxic T lymphocytes can be differentiated from other Th, most of which are related to Th1 [7]. The latest research demonstrated that the development of thymocytes to $CD4^+$ or $CD8^+$ lineages and the acquisition of helper or cytotoxic activities are controlled by antagonistic transcription factors, namely, *ThPOK* and *Runx3*. *ThPOK* inhibits the cytotoxic program of $CD4^+$ thymocytes and promotes T cell maturation, whereas *Runx3* promotes this program of $CD8^+$ T cells [54]. A few other studies reported that the competition for IL-2 Tregs can regulate the acquisition of cytotoxic activity by $CD4^+$ T cells. Moreover, Blimp-1 and T-bet independently control the cytotoxic and helper activities of $CD4^+$ T cells in TME [55]. However, the specific differentiation mechanism of these cells has not been systematically elucidated, with ongoing research on $CD4^+$ cytotoxic T lymphocytes.

Recently, some researchers used scRNA-seq and paired TCR sequencing to study the characterization of TME in seven types of bladder cancer resections [8]. They

identified multiple $CD8^+$ and $CD4^+$ T cell populations in the immune microenvironment. Two cytotoxic $CD4^+$ T cell populations in these tumors were identified, expressing *GZMB* and *GZMK*. These two cell subpopulations were labeled as $GZMB^+CD4^+$ T cells and $GZMK^+CD4^+$ T cells, respectively. The $GZMB^+CD4^+$ T cells express high levels of cytotoxic molecules GZMB, perforin, GNLY, and natural killer cell granule protein 7 (NKG7). The $GZMK^+$ $CD4^+$ T cells express high levels of GZMK and lower levels of NKG7. Both these subsets produce high levels of antitumor IFN-γ and TNF-α. These cytotoxic $CD4^+$ T cells can recognize MHC-II on tumor cells and directly kill them. In addition, the researchers reported that the antitumor effects of these cytotoxic $CD4^+$ T cells can be inhibited by $CD4^+$ Tregs within the TME [56]. Therefore, cytotoxic $CD4^+$ T cells could affect the MHC-II antigen presentation mechanism in tumor cells. The cytotoxic $CD4^+$ T cell population in bladder cancer is characterized by directly killing autologous tumor cells in an MHC-II dependent manner and is related to improved response to immune checkpoint blockade (ICB). However, the existence, function, and clinical significance of cytotoxic $CD4^+$ T cells in NSCLC remain elusive. The unique expression of MHC-II on NSCLC cells is speculated to provide a feasible method for cytotoxic $CD4^+$ T cells to drive the ICB response of patients with MHC-I damage [57]. In addition, CD4 + cytotoxic T cells have been found to exert anti-tumor effects directly or indirectly in liver cancer, colorectal cancer, breast cancer, melanoma, osteosarcoma, and head and neck tumors [53, 58–63]. Further studies on the characteristics of cytotoxic $CD4^+$ T cells will help develop the next-generation ICB.

Altogether, these findings highlight the extensive and multifunctional roles of $CD4^+$ T cells in antitumor immunity. The role of cytotoxic $CD4^+$ T cells in inducing an antitumor immune response is still not completely elucidated, including the exact mechanism supporting its development, and the reason behind the significantly limited response of the influence on PD-L1 inhibitors in certain tumor responses [64]. The ongoing research on cytotoxic $CD4^+$ T cells could help better understand the reactivity and resistance to existing immunosuppressive checkpoint inhibitors and determine novel targets for immunotherapy.

Regulatory T cells

Regulatory T cells (Tregs) are a subset of T cells with significant immunosuppressive properties. They are characterized by the expression of *Foxp3*, *IL2RA*, *CD25*, and *CD4* [1, 45, 46]. *IKZF2*, *TNFRSF9*, *TNFRSF14*, *TIGIT*, and *CTLA4* are well-defined Treg-associated genes [65, 66]. Tregs regulate the body's immune homeostasis and block autoimmune diseases, exert a suppressive effect on the immune system, and are the primary controllers of self-tolerance. Tregs suppress excessive immune responses by expressing *CTLA4*, *IL-10*, and *TGF-β* [1]. This immunosuppressive effect suppresses anti-cancer immunity, such that Tregs promote the immune escape of tumor cells and promote tumorigenesis and progression [67, 68]. Specifically, the effect is multifaceted. For example, Tregs participate in the epithelial–mesenchymal transition of tumor cells and angiogenesis by secreting growth factors and simultaneously supporting the expansion of stromal cells such as fibroblasts and endothelial cells [68–70]. In conclusion, the function of Tregs in the TME is highly complex [3, 71].

Recently, several researchers have used scRNA-seq to study Tregs in TME. Numerous studies have confirmed significantly elevated numbers of Tregs in the peripheral blood or intratumor of patients with liver cancer [72], lung cancer [3], gastrointestinal tumors [73], breast cancer [74], and colorectal cancer [63]. Tregs are currently believed to originate from $CD4^+$ naïve T (Tn) cells that have been stimulated by relevant cytokines. ScRNA-seq deepens our understanding of the origin of Tregs. For example, a study revealed that Tregs in cancer are largely derived from $CD4^+$ Tn cells recruited by tumor-associated macrophage (TAM)-derived CCL18 [75, 76]. Two recent breast cancer studies reported that although Tn and Tregs in breast cancer have overlapping TCR repertoires, they rarely overlap with circulating Tregs, suggesting that intratumoral Tregs develop largely from Tn cells in situ rather than from recruited Tregs. In addition, patients with breast cancer with high levels of Tn infiltration have higher levels of Treg infiltration and poor prognosis. Blocking the recruitment of Tn reduced the number of Tregs and inhibited tumor progression [77].

Single-cell sequencing can be used to cluster Tregs to better study their heterogeneity. For instance, a recent single-cell study in NSCLC was conducted in which the researchers divided Tregs into activated and resting Tregs based on the Treg activation antigen-specific marker *TNFRSF9*. They sequenced 404 characteristic genes of Tregs and subsequently found that activated $TNFRSF9^+$ Tregs were highly expressive compared to the *TNFRSF9* population of a set of 260 characteristic genes. In addition, the activated $TNFRSF9^+$ Tregs were found in another two single-cell sequencing studies [78, 79]. This set of genes overlapped with the 404 genes described above and included genes associated with immunosuppressive functions such as *REL27* and *LAYN20*. Their subsequent survival analysis using the TCGA-LUAD dataset demonstrated that these 260 genes were associated with a poorer prognosis in patients, particularly *IL1R2* expression, whereas 404 genes originating from all Tregs of tumor sites were insignificantly related to survival. The clinical significance of activated Tregs in tumors warrants further investigation by subsequent

single-cell sequencing [80]. Zhang et al. conducted deep scRNA-seq on 12,346 T cells obtained from 14 patients with NSCLC in the early stages of treatment [80]. They found heterogeneity within several different groups of Tregs. Tregs clonal amplification is largely cluster-specific, occurs within the tumor, and is associated with the function of Tregs. These studies provide a novel approach to classify patients and will contribute to further understanding of the functional status and differentiation of Tregs in lung cancer [80]. Several years ago, certain researchers have identified a new Treg subset ($CD4^+CD69^+CD25^-$), which differs from the previously identified Tregs as it does not express *FOXP3* and does not secrete IL-10 [81]. However, it highly expresses *CD122* and *TGF-β* [82]. Recently, researchers have used single-cell sequencing studies this new subpopulation of Tregs in more depth and found that $CD4^+CD69^+CD25^-$ T cell expansion significantly correlates with tumor size, vascular invasion, and TNM staging in hepatocellular carcinoma (HCC) [83]. Therefore, subsequent sequencing of this new subpopulation of Tregs could provide new insights for tumor immunotherapy.

Single-cell sequencing can identify the relevant signature genes of Treg, which are significant for constructing prognostic models for different tumors. In a single-cell study of pancreatic cancer, researchers identified the genes *CASP4*, *TOB1*, and *CLEC2B* as associated with poorer prognosis in patients, whereas the gene *FYN* was associated with better prognosis [84]. The Tregs can be roughly divided into two subgroups depending on the variability of these four genes within the Treg population. The two subgroups have significantly different HIF-1 signaling pathways. A breast cancer single-cell research constructed a prognostic predictive model using Treg genes, namely, *TK1*, *LOX*, *KDM5B*, *PSMD4*, and *NFE2L3*, in which *TK1* demonstrated the most significant prognostic value [85]. Knockdown of *TK1* impaired proliferation, migration, and invasion of cancer cells. Pan-cancer analyses demonstrated that *TK1* was associated with poor prognosis and activation of proliferative pathways in different cancers. Similarly, another single-cell study in esophageal cancer constructed a Treg-associated prognostic prediction model using the *SH2D2A* and *TERF2IP* genes, which can accurately predict patient prognosis [86]. The single-cell data in osteosarcoma demonstrated high activation of oxidative phosphorylation, angiogenesis, and mTORC1 in Tregs [87]. Cellular communication analyses demonstrated that Tregs interact with cells such as osteoblasts, endothelial cells, and myeloid cells via CXCR4 and CXCL12/TGFB1, and they can collaborate to promote tumor progression. Treg genes *CD320* and *MAF* were subsequently selected to construct a prognostic model and achieve good prognostic accuracy [87]. The complexity and importance of interactions between Treg and tumor-associated cells was confirmed by another osteosarcoma single-cell study, which revealed that the interaction gene set between osteoclasts and Tregs can accurately predict the prognosis of patients with osteosarcoma [88]. In addition to constructing prognostic models, these Treg-related signature genes can be used to develop tumor immunotherapies, including efficacy prediction and the development of new targets, which we will discuss in detail later in this review.

Several recent studies on Tregs in tumors demonstrated that Tregs are a hot spot in TME studies due to their highly immunosuppressive effects and the complexity of the effector mechanism. Targeted studies of certain cancers using single-cell sequencing have provided novel insights into the impact of Tregs on TME. The study on the interaction of Tregs with other tumor-associated immune cells as well as their impact on treatment and prognosis holds great potential value for clinical immunotherapy of tumors [76, 89].

Naive CD4+ T cells

Tn cells grow and mature in the thymus before migrating into the peripheral lymphoid organs and lymphoid tissues [90, 91]. They belong to a class of T cells that grow in the thymus and express surface-differentiated antigens such as CD4, TCR/CD3, CD28, CD62L, and CCR7 before being exposed to antigenic stimuli [92]. Previous studies on $CD4^+$ T cells have largely focused on central memory T cells (TCM), effector memory T cells (TEM), resident memory T cells (TRM), and Tregs. According to these studies, $CD4^+$ Tn cells constitute a static cell population; single-cell sequencing technology can provide us with more detailed molecular characteristics of cell subpopulations, thereby helping us to understand the function of CD4 subpopulations in tumors [93, 94].

Tn is initially maintained in a quiescent state, with a relatively low transcriptional activity. Two single-cell studies on melanomas demonstrated that Tn only expressed its characteristic genes (*CCR7*, *TCF7*, *LEF1*, *SELL*, and *IL7R*), these signature genes are not involved in tumor development, and the same result was reported in another breast cancer study [95]. The effect of related expressed genes on the TME was found from additional research; Tn displayed high levels of markers (*CCR7*, *LEF1*, and *TCF7*) and co-stimulatory genes *ICOS*, *CD226*, and *SLAMF1*, suggesting an immunosuppressive environment [96]. However, a pan-cancer analysis in which a group of melanoma patients were exposed to immune checkpoint inhibitors, followed by conducting scRNA-seq after biopsies were obtained before and after treatment found that Tn expressed the same PD-1 as $CD8^+$ T cells. Therefore, Tn cells serve as targets for immunotherapy as well [97]. Another research reported that Tn can influence tumor development by interacting with TAMs

and tumor-associated fibroblasts in the immune microenvironment. The study showed that Tn populations exerted similar TCR sequences to Tregs using a database obtained by scRNA-seq, with little overlap with circulating Tregs, suggesting that Tregs developed primarily from Tn cells in situ in the tumor [77]. Further studies demonstrated that the chemokine CCL18 produced by TAMs recruited Tn in circulation to the tumor site by binding to the PITPNM3 receptor. Finally, in vitro experiments in mice demonstrated that blocking CCL18 could be a promising strategy for cancer immunotherapy [76]. Similar results were obtained in HCC sample [75]. Studies on pancreatic ductal adenocarcinoma demonstrated that Tn progress to immunosuppressive Tregs by interacting with antigen-presenting cancer-associated fibroblasts (apCAF), indicating that apCAF could be a unique immunomodulatory CAF population that can induce the formation and expansion of Tregs through antigen-dependent TCR ligations [28, 98]. Interestingly, Tn not only take their effects through tumor antigen stimulation and neurometabolic regulation but also partially reverse their immunosuppressive trait with radioimmunotherapy and radiochemotherapy [99]. Our team demonstrated that patients undergoing radioimmunotherapy and radiochemotherapy displayed an increased proportion of Tn in the TME and showed extremely higher anti-immunosuppressive activity, improving the immunosuppressive microenvironment [77, 100].

Although the majority of the previous studies implied that Tn is mostly in a static state, newly emerging outcomes demonstrated that Tn are involved in steps after cloning expansion and differentiation. Single-cell sequencing provides us with unique insights, suggesting that Tn populations can reshape the TME to affect tumor progression and treatment. However, the existing research is still considerably limited.

Memory CD4+ T cells

Single-cell sequencing divided the $CD4^+$ T cell subset into TCM, TEM, and TRM cells [90, 101, 102]. In addition, Tn cells are activated by several cytokines when they differentiate into these populations [103]. Therefore, we will discuss the unique perspectives provided by the above-mentioned cell populations through single-cell techniques in the TME.

$CD4^+$ TEM are a subtype of $CD4^+$ T cells with memory functions. They play a crucial role in the TME. Firstly, TEM can recognize and identify antigens on the surface of tumor cells, following which they trigger an immune response by binding to their T-cell receptors (TCRs) with tumor-specific antigens on the surface of tumor cells. A single-cell sequencing study in patients with melanoma undergoing immune checkpoint therapy observed a strong correlation between the abundance of activated $CD4^+$ TEM cells and clonally diverse TCR repertoire in the peripheral blood and the therapeutic efficacy of patients [104]. Second, $CD4^+$ TEM cells can produce several cytokines, such as interferon-gamma (IFN-γ), and interleukin (IL)-2, IL-4, and IL-17. These cytokines can activate and regulate the activity of other immune cells, such as cytotoxic T lymphocytes (CTLs), NK cells, and macrophages, thereby enhancing their ability to attack tumor cells [80]. The transcriptome data obtained from NSCLC tissues demonstrated a significant proportion advantage of the TEM cell population compared to normal tissues [71, 105]. For example, Anna et al. reported that $CD103^+CD4^+$ TEM were the most effective producers of tumor necrosis factor (TNF)-α and IFN-γ, whereas other subsets of $CD4^+$ T cells lacked the production of these cytokines. In addition, IFN-γ induced the production of chemokines by the tissue and enhanced the expression of adhesion molecules in the vascular system, causing increased T cell infiltration [106], perhaps such cell populations could become targets for future targeted therapies. Moreover, a high level of $CD4^+CD161^+$ TEM is correlated with improved survival rates in squamous cell carcinoma of the oral pharynx. This particular population of cells enhances the TCR signal transduction by expressing *SOX4*; therapeutic vaccination can induce the generation of cytokine-producing $CD4^+CD161^+$ effector cells. However, inhibiting TGF-β could be a potential immune therapy strategy due to TGF-β downregulating *KLRB1/CD161* and *SOX4* [107]. The same study reported similar discoveries in other types of cancers, such as a high expression of *NKG7*, *PRF1*, granulysin (*GNLY*), and *human recombinant protein* (*GZMH*) in the $CD4^+$ TEM population in colorectal cancer [63, 80]. Moreover, similar findings were observed in renal clear cell carcinoma [89]. Multiple studies mentioned above conclude that the $CD4^+$ TEM population exhibits similar gene expression to the well-known $CD8^+$ T cell population in exerting anti-tumor effects. However, differences could be present in their internal regulatory mechanisms.

TCM is a specific type of immune cell, belonging to a subset within the $CD4^+$ helper T cell (Th) population. They possess memory functions, capable of long-term preservation of antigen recognition and response information. In addition, they can be rapidly activated for immune responses [108–110]. Compared with $CD4^+$ TCM, $CD4^+$ TRM largely exist in non-lymphoid tissues such as skin, intestines, and mucous membranes, and exhibit tissue specificity [111]. They possess highly specific antigen recognition on tumor cell surfaces and can generate immune responses within tumor tissues. This local surveillance allows identifying and eliminating tumor cells, and restricting tumor growth and spread [112]. These interactions between cell populations are complex and dynamic, involving the collective regulation

of tumor immune response intensity and effectiveness. Factors such as cytokine production and signaling, cell-cell contact, and expression of co-stimulatory molecules have been reported to be involved in this process [113]. The balance and regulation of these intercellular interactions are crucial for an effective anti-tumor immune response.

Helper T cells

Several Th subpopulations have been identified in the TME, including Th1, Th2, Th17, and Th9 [5] due to distinct transcriptional factors and functions. Recent studies have suggested T-bet as an important transcription factor for Th1, whereas STAT, IL-12, and IFN-γ promote the differentiation to Th1 [114, 115]. After activation, Th1 secretes TNF and IFN-γ to mediate macrophage activation and induce suppression and apoptosis of tumor cells [116]. Th1 cells exert potent anti-tumor immune effects against different types of cancers [5, 114]. The differentiation of Th2 depends on IL-4 and IL-25, whereas cytokines secreted by Th2 include IL-4, IL-5, IL-10, and IL-13 [117]. Th2 secretes different kinds of cytokines, so its effect on tumor cells is complex. Th17 cells are widely present in tumors. Although Th17 cells secret IL-17 to promote tumor progression, they can kill tumors by secreting IL-2, stimulating the production of pro-inflammatory cytokines and chemokines by other immune cells, and transdifferentiating into a Th1-like phenotype [63, 114]. Recently, Th9 cells have been found to possess anti-tumor properties. Th9 cells are generated from reprogrammed Th2 cells stimulated by TGF-β, and Th9 secretes IL-9, and IL-21, which triggers the activation of DCs, mast cells, NK cells, and anti-tumor $CD8^+$ T cells [118, 119]. The application of Th9 to tumor immunotherapy is highly promising due to its excellent anti-tumor effects.

A study revealed the interaction among different types of Th cells by analyzing single-cell sequencing data from patients with breast cancer [120]. Researchers reported that Th1-secreted IFN-γ induces the expression of *STAT1* and *STAT4* in $CD4^+$ Tn cells, promoting Th1 differentiation and inhibiting Th2 and Th17 differentiation. Th2-secreted IL-4 inhibits Th1 differentiation and promotes Th2 differentiation. A correlation analysis revealed that Th2 was positively correlated with Tregs, whereas Th1 was negatively correlated with Th2 and Tregs. Zhang et al. used Smart-seq2 single-cell sequencing to identify Th1 and Th17 in colorectal cancer and investigated their immune profiles [63]. They found that Th1-like $CD4^+$ T cells were enriched in patients with microsatellite-instable tumors and absent in patients with microsatellite-stable tumors. These $CXCL13^+BHLHE40^+$ Th1-like cells highly express *IFNG* and granzyme B (*GZMB*) and share TCRs with $CD4^+GZMK^+$ Th. The regulatory and effector functions of $CXCL13^+BHLHE40^+$ Th1-like cells result in a favorable response to immunotherapy. A study reported that Th17 cells were enriched in patients with MSS. Th17 share TCRs with Tregs and therefore display negative effects in immunotherapy in patients with MSS. The balance between Th1 and Th17 and the conversion with Th17 and Tregs are crucial for maintaining TME homeostasis and determining the efficacy of immunotherapy.

Differentiation and functional regulation of CD4 + T cell subsets

Activated CD4 + T cells initially undergo clonal expansion and differentiation, accompanied by epigenetic changes that lead to the formation of various Th cell subgroups. These subgroups are defined by the expression of specific transcription factors driving distinct effector functions, primarily determined by the production of different cytokines. For example, Th1 cells release IFN-γ and TNF; Th2 cells release IL-4, IL-5, and IL-13; Th9 cells produce IL-9; Tregs secrete IL-10 (a cytokine with immune suppressive function, which maintains the expression of FOXP3 required for the inhibitory function of Tregs on other cells) and TGF-β; Th17 cells produce IL-17 (a cytokine involved in anti-bacterial and anti-fungal host defenses) [5, 101].

We found that Tn differentiates into different subsets in different types of cancer. However, they share the overall and continuous differentiation of the entire $CD4^+$ T cell population, achieved through the expression of different chemokines and cytokines, as well as sharing TCR clones [103]. In HCC, the Tn population differentiates into an exhausted cell population, leading to the expression of *CXCL13*, *PDCD1*, *CTLA-4*, and *TIGHT*. In contrast, another population expresses *NKG7*, *GNLY*, and *GZMB*, indicating their cytotoxic status [72]. Similar findings have been discovered in NSCLC, and these differentiated cell populations affect the antitumor functions of $CD8^+$ T cells [80]. The TCM cell population is enriched in ascites in female ovarian cancer, whereas the Th1 cell population is relatively higher in tumor tissues(Fig. 1). The single-cell sequencing data demonstrated that they share TCR clones between them, and this sharing was not detected in other T cells. This suggests that the ascites-derived TCM population could be a direct source of the Th1 cell population [121]. In breast tumors, Tregs differentiate to suppress the function of Th1 and Th17, causing immune suppression [95]. These studies can be used to construct the differentiation spectrum of $CD4^+$ T cells in the TME through scRNA-seq data to explore the key transcription regulatory factors and marker genes. This will allow better understanding of the differentiation and functional shaping of $CD4^+$ T cells, further investigate the regulatory relationship between key transcription factors and target genes, and validate their functionality. However,

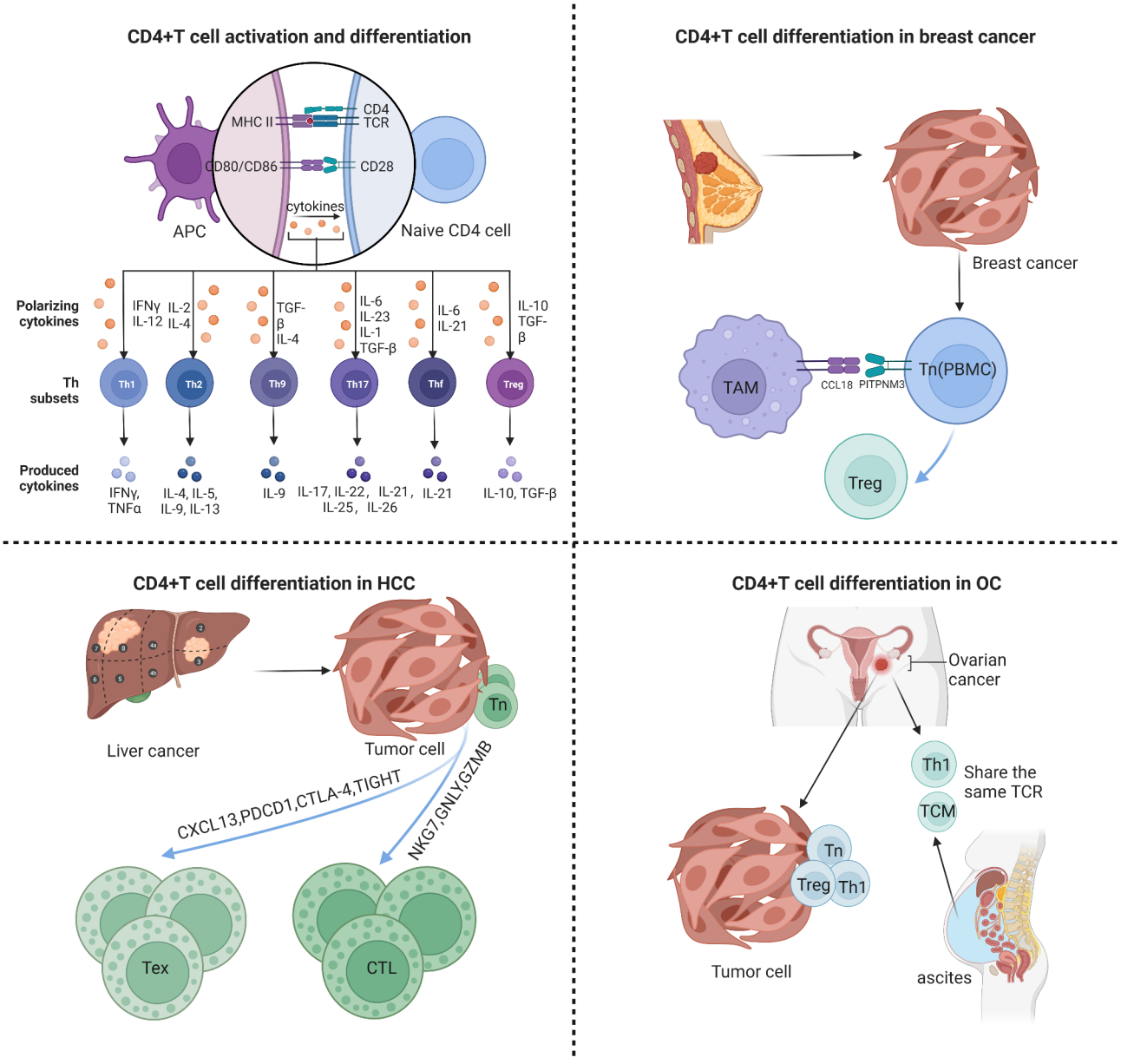

Fig. 1 CD4$^+$T cell differentiation identified by single-cell sequencing in TME. Naive CD4$^+$ T cells differentiate into different T cell subsets by interacting with APCs. In breast cancer, Tn cell populations in peripheral blood promote Treg differentiation by interacting with TAM. In hepatocellular carcinoma, Tn differentiates into CD4$^+$ CTLs, characterized by cytotoxicity, and exhausted T cell populations characterized by depletion. Tumor tissue shares TCRs with ascites tumor cells and promotes the differentiation of Tn cells in OC. APC, antigen-presenting cells; Tn, naïve T cell; Treg, regulatory T cell; Th, helper T cell; TAM, tumor-associated macrophages; TCM, central memory T cell; CD4$^+$ CTL, CD4$^+$ cytotoxic T lymphocyte; TCR, T-cell receptor; IL, interleukin; IFN, interferon; TGF, transforming growth factor; Tex, exhausted T cell; HCC, hepatocellular carcinoma; OC, ovarian cancer

the current study focuses on the supportive function of CD4$^+$ T cells for CD8$^+$ T cells. Therefore, more research is required to understand the role of the CD4$^+$ T cell population in tumor progression. We summarize the molecular markers and functions of currently known CD4$^+$ T cells in Table 2.

Single-cell sequencing promotes the research and application of CD4+T cells in tumor immunotherapy

Tumor immunotherapy strategies include immune checkpoint blockade (ICB), adoptive cell transfer therapy (ACT), and tumor vaccines, which target cancer cells by activating or enhancing the immune system's response. Although tumor immunotherapy has demonstrated considerable efficacy in several cancer types, certain patients

Table 2 Summary of molecular markers and functions of $CD4^+$T cells

Cell type	Molecular markers	Function	
Treg	CD3, CD4, IL-2RA, CD39, FOXP3, GARP, GITR, CTLA-4, LAP	Immune suppression and immune tolerance effect.	[1, 45]
Tn	CCR7, LEF1, TCF7, CD4, CD3, CD28, CD62L, SEEL	Tn differentiates into different cells after exposure to antigen.	[72–74]
Tem	CD45RO, CD95	Immune-protective role, produce cytokines activate other immune cells.	[55, 61, 88]
Tcm	CD45RO, CD62L, CD95	Long-term preservation of antigen recognition and response.	[1, 114]
Trm	CXCR6, CD49A	Prevention of infections and control of tumor progression.	[1, 114]
Tfh	CXCR5, CD40L, ICOS, CD3, CD4, CD126, SLAM, CXCL13	Tfh activates B cell proliferation and differentiation.	[93–95, 115]
Tph	PD-1, ICOS, CXCL13, CCR2, CCR5	Tph collaborates with Tfh to activate B cells.	[101, 104]
Th1	CD3, CD4, CD119, CCR5, STAT, IFN-γ, TNF-α, PDCD1, IFNG, CXCL13	Th1 mediates macrophage activation and has anti-tumor effects.	[105–107]
Th2	CD3, CD4, CD119, CCR3, CCR4, CD365	Th2 promotes the production of antibodies by B cells.	[108]
Th17	CD3, CD4, CD161, CCR4, CCR6, IL-23R	Th17 participates in inflammatory response.	[55, 105]
Th9	CD3, CD4, IL-9, IL-10, TCR-α/β	Activation of other immune cells and anti-tumor effects.	[109, 110]
$CD4^+$CTL	EOMES, CRTAM, IFN-γ, CD3, IL-2R, GZMA, GZMB, GZMK, NKG7	Cytotoxic effects, direct and indirect killing of tumor cells.	[36, 37, 40, 41]

Abbreviations: Treg, regulatory T cell; Tn, naïve T cell; Tem, effector memory T cell; Tcm, central memory T cell; Trm, resident memory T cell; Tfh, T **f**ollicular helper cell; Tph, peripheral helper T cell; Th, helper T cell

could become resistant to it, resulting in reduced or ineffective therapeutic efficacy. Thus, not all patients can obtain a sustained therapeutic effect from immunotherapy. $CD4^+$ T cells in the TME affect tumor progression directly and indirectly in different ways and therefore are crucial to developing immunotherapies [53, 122, 123]. Single-cell sequencing has been applied to immunotherapy-related studies in lung cancer [124, 125], bladder cancer [126], colon cancer [127], melanoma [128] and renal cell carcinoma [129], contributing to the research and application of $CD4^+$ T cells in tumor immunotherapy (Fig. 2).

Single-cell studies of CD4+ T cells in ICB

ICB has emerged as a revolutionary advance in the field of oncology treatment, however, its effectiveness is limited to a subset of patients. Certain biomarkers can be used for prognostic prediction to substantially improve the effectiveness and accuracy of ICB. Currently, the major ICB-related studies focused on the blockade of PD-1/PD-L1 and CTLA-4. Several studies using single-cell sequencing to discover novel $CD4^+$ T cell subsets associated with ICB have recently emerged. A recent single-cell sequencing research identified novel $CD4^+$ T cell clusters consisting of $CXCR3^+CCR4^-CCR6^+CD4^+$ T cells and $CXCR3^-CCR4^-CCR6^+CD4^+$ T cells from patients treated with anti-PD-1 therapy. These cell clusters are characterized by high expression of the *IL-7R* and *TCF7* [124]. The frequency of these cells in peripheral blood was correlated with survival. Thus, it is feasible to use patient peripheral blood samples to predict immunotherapeutic response by this novel $CD4^+$ T cell cluster. For instance, Zheng et al. analyzed two scRNA-seq datasets from ICB-treated renal cell carcinoma patients and identified two $CD4^+$ T cell subtypes related to prognosis [129]. The two subpopulations were a proliferative $CD4^+$ T cell subtype ($MKI67^+CD4^+$ T) and a regulatory T cell subtype ($MKI67^+$ Treg), contributing to ICB resistance. The marker genes of these clusters can be integrated to construct a predictive model and guide clinical ICB therapy.

In addition to discovering novel $CD4^+$ T cell subpopulations, single-cell sequencing has been used to identify certain novel ICB targets or regulators. Avery et al. used single-cell sequencing and reported that ICB treatments upregulated the transcription factor *BHLHE40* in anti-tumor $CD4^+$ T cells, whereas intratumoral $CD4^+$ T cells from *BHLHE40*-knockout mice exhibited higher expression of the inhibitory receptor gene *Tigit* [130]. This suggests that *BHLHE40* exerts a critical and positive function in ICB and is a potential prognostic marker for ICB. Using single-cell sequencing, another team reported that *CD226* was differentially expressed in different tumor types and that its elevated expression correlated with better clinical outcomes. The expression of *CD226* on effector $CD4^+$ T cells was higher than that on Tn, and GSEA analysis revealed that CD226 was intricately associated with T cell activation and T cell receptor signaling pathways [128]. A recent single-cell study demonstrated that the MHC-II signature of $CD4^+$ T cells significantly correlated with the levels of immune-related gene expression and immune cell infiltration. Further transcriptome analyses revealed immune activation in high MHC-II signature subgroups, whereas fatty acid metabolism and glucuronidation were present in the low MHC-II signature subgroups. This difference could be attributed to the tumor protein p53 and fibroblast growth factor receptor 3. This study suggested MHC-II signature

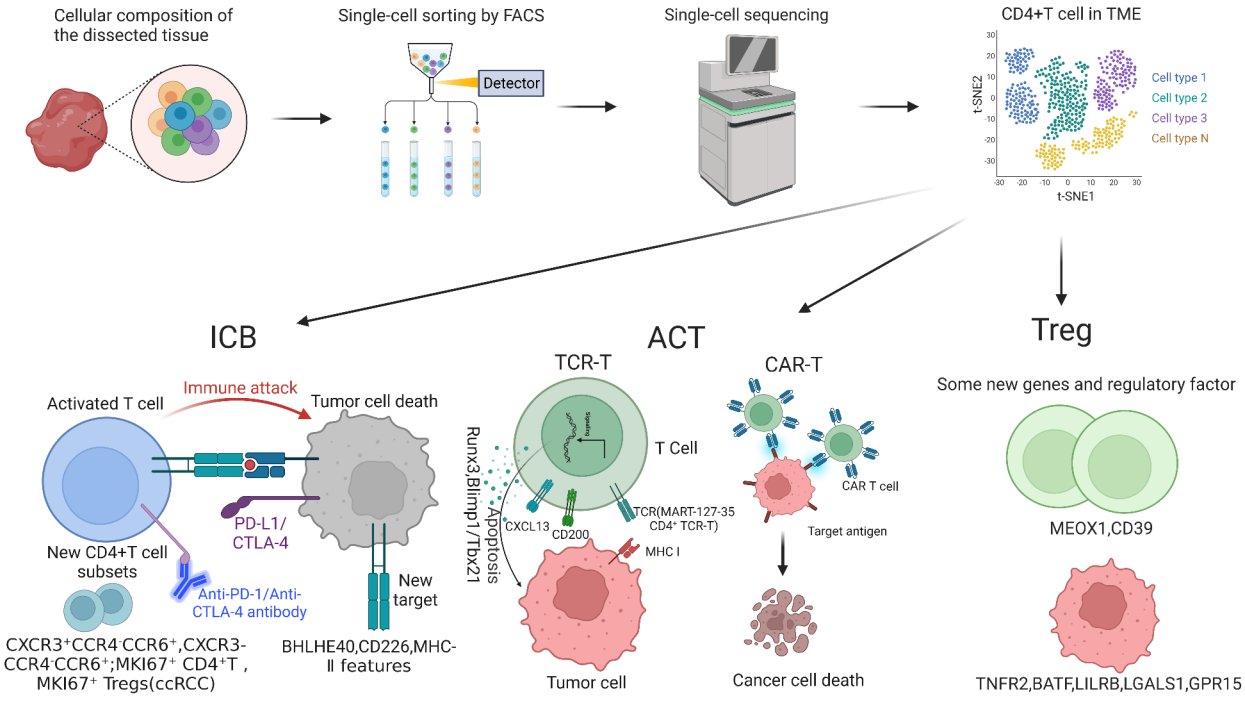

Fig. 2 Application of CD4+T cells in tumor immunotherapy by single-cell sequencing. Upper Panel: Single-Cell Sequencing Workflow. This panel outlines the process of isolating cells from tumor tissue, followed by single-cell sorting via FACS to enrich for CD4 + T cells. Subsequent single-cell RNA sequencing reveals the transcriptional landscape, highlighting the diversity of CD4 + T cell subsets within the TME. Lower Panel: Immunotherapy Approaches. ICB: The figure illustrates the blockade of PD-1 and CTLA-4 pathways to enhance T cell activation and tumor cell elimination. It also identifies CD4 + T cell subsets that may predict responses to ICB therapy. ACT: Two ACT strategies are depicted: TCR-T cells, which recognize tumor antigens like MART-127-35, and CAR-T cells, which target cancer cells via engineered chimeric antigen receptors. Treg: The figure highlights Tregs, marked by genes such as MEOX1 and CD39, which modulate immune responses and represent potential therapeutic targets within the TME. ICB, immune checkpoint blockers; TCR-T, T cell receptor-gene engineered T cells; CAR-T, chimeric antigen receptor T cells; Treg, regulatory T cell; MHC, major histocompatibility complex

as an independent and favorable predictor of bladder cancer treated with ICB [126]. Several previously identified biomarkers are effective in predicting survival in patients treated with ICB; however, it is unclear whether these markers can contribute to predicting sequential ICB treatment (anti-CTLA4 ICB therapy followed by anti-PD1 ICB therapy). To explore the range of validity of these biomarkers, several investigators analyzed the scRNA-seq data from patients with melanoma [131]. They observed that IFN-γ and cytotoxic activity features associated with mature $CD4^+$ T cells could not be used for prognostic prediction of sequential ICB. However, features associated with early memory $CD4^+$ T cells (integrating TCF1-driven stem cell-like transcriptional programs) can be accurately used for the prognostic prediction of sequential ICB therapy. Thus, the predictive value of tumor biomarkers is greatly influenced by the treatment regimen, tumor type, and stage, and the cell type selected for the assay. We believe that it is necessary to select suitable biomarkers for each specific situation of immunotherapy. In addition, single-cell sequencing must be applied to identify more novel tumor biomarkers.

Single-cell studies of CD4+ T cells in ACT

Another mainstream cancer immunotherapy is ACT including tumor-infiltrating lymphocyte, TCR-gene engineered T cells (TCR-T), and chimeric antigen receptor T cells (CAR-T). An important component of ACT is the analysis of T cell clonotypes; single-cell sequencing can be used to identify TCRs, thus detecting the heterogeneity of T cells [132]. The information obtained from single-cell sequencing not only helps explain the causes of resistance to ACT therapies but also identifies high-affinity anti-tumor TCRs to develop new ACT [133]. Although most current studies have focused on cytotoxic $CD8^+$ T cells, the application of $CD4^+$ T cells in ACT has recently attracted extensive interest from oncology researchers [134].

The cell surface markers and cytosecretory factors of ACT in preclinical models can be used to develop ACT engineer cells and prove their tumor-killing effect. However, these indicators cannot completely reflect the anti-tumor immune effects of T cells. ScRNA-seq can be used to identify subpopulations of cells contributing to the overall response by providing an unbiased transcriptome

profile for each cell. Thus single-cell sequencing can be applied to completely characterize the interactions of T cell subtypes in ACT [135]. Certain researchers obtained MHC class I-restricted MART-127-35-specific TCR sequences based on single-cell sequencing and constructed MART-127-35-specific $CD4^+$ TCR-T cells. They reported that $CD4^+$ TCR-T cells adopted the classical granule-dependent and cytolytic-independent pathway to kill the tumor cells. Signaling pathway analyses revealed that transcription factors *Runx3* and *Blimp1/Tbx21* contribute to the development and killing function of cytotoxic $CD4^+$ T cells. These results suggest that MHC class I-restricted $CD4^+$ TCR-T cells could be used for potential ACT therapy [136]. The important correlation of *Blimp-1* with $CD4^+$ T cytotoxic activity has been demonstrated in another single-cell sequencing study of TCR-T, and Blimp-1 could be a valuable potential gene [55].

Following their infusion into patients, CAR-T cells get triggered by cancer cells in TME to kill tumors. However, it is unknown how changing the TME affects the status of CAR-T cells over time. Single-cell sequencing can be used to analyze the cellular heterogeneity, trajectory analysis, and cell lineage tracking of CAR-T cells during treatment or prognostic follow-up to gain insights into the correlation between CAR-T cell status and clinical outcomes. A study conducted a long-term investigation of two children with leukemia who were treated with $CD4^+$ CAR-T. The $CD4^+$ CAR-T cells were still detectable 10 years after infusion. Single-cell multiomics analyses demonstrated that these long-standing $CD4^+$ CAR-T cells exhibited cytotoxic signatures and sustained functional activation and proliferation, leading to sustained remission in these two patients [137]. Furthermore, single-cell sequencing can contribute to developing personalized CAR-T regimens by linking the characteristics of pre-infusion CAR-T cells to patients. For example, a new study proposed personalized TCR-T cell therapies involving the modification of T cells using TCR derived from tumor antigen-specific T cells (Tas), which naturally exist in the patient's body [138]. This study used scRNA-seq and demonstrated the screening of $CD4^+$ Tas cells using CXCL13 and CD200 as markers. CXCL13 has been validated as a marker to identify T cells in another recent single-cell immunotherapy study as well [73]. Ultimately TCR-T modified from $CD4^+$ Tas displayed significant therapeutic effects in tumors [138]. A recent study demonstrated the feasibility and tumor-killing mechanisms of the application of $CD4^+$ T cells in ACT using single-cell sequencing [53]. Although certain tumor cells achieve immune evasion by not expressing MHC, the authors demonstrated that adoptively transferred TPR-1 $CD4^+$ T cells can indirectly kill the tumor cells lacking MHC. These specialized $CD4^+$ T cells can localize to the tumor periphery, secrete IFN-γ, and interact with antigen-presenting DCs. This results in the reprogramming of monocytes in the TME, promoting their recruitment to the TME, and ultimately indirectly eliminating the tumor. The results provide a significant theoretical basis for the development of $CD4^+$ T cell-based ACT therapeutic strategies. Another study used single-cell sequencing and investigated signal transducer and activator of transcription 5 (STAT5) of $CD4^+$ T cells used in ACT. They found that STAT5 effectively promoted tumor infiltration of $CD4^+$ T cells within the tumor and drove the development of Tn cells to multifunctional effective T cells. Therefore, STAT5 can be utilized to construct $CD4^+$ CAR-T cells [139]. In addition to helping develop novel CAR-T strategies, single-cell sequencing can be used to evaluate and improve the existing CAR-T therapies. For instance, Boroughs et al. used scRNA-seq to compare the transcriptional status of human T cells with CD3z4-1BB-CD3z, or CD28-CD3z costimulatory domains [140]. They found high Th1-polarized gene expression in the T cells with CD3z4-1BB-CD3z and high Th2-polarized gene expression in the T cells with CD28-CD3z, which could be attributed to the high expression of Th2 differentiation suppressor *TRAF1* in T cells with CD3z4-1BB-CD3z. Due to Th1 cells contribute to the antitumor activity of $CD8^+$ T, a shift toward the Th1 phenotype could lead to better CAR-T efficacy.

In summary, single-cell sequencing promotes the development of ACT in two main aspects. On the one hand, ACT has both individual and cellular heterogeneity, which is intricately linked to the therapeutic effect, whereas single-cell sequencing can efficiently detect these heterogeneities. On the other hand, the changes in cell population and transcriptional dynamics of ACT cells infused in vitro can reflect the therapeutic effect, and scRNA-seq can be used to obtain these dynamic features. Therefore, scRNA-seq can be applied to develop biomarkers for efficacy response and relapse monitoring and improve therapeutic regimens to enhance the effectiveness and safety of ACT.

Application and study of Tregs in tumor immunotherapy

In most cases, Tregs in the TME exert complex effects are related to tumor immunosuppression [68]. Previous studies have observed Treg expansion and driving tumor immune rejection in patients after immunotherapy [134, 141]. Thus, a combination of suppression or elimination of Tregs with immunotherapeutic strategies such as ICB or ACT was once considered a potential option to enhance the efficacy of immunotherapy [142, 143]. Recently, relevant single-cell studies have identified gene loci or transcription factors associated with Treg activation and proliferation as well as poor prognosis of immunotherapy. For example, a few researchers analyzed the

transcription factor *MEOX1* on Tregs in patients with intrahepatic cholangiocarcinoma using single-cell techniques. The results demonstrated that the overexpression of *MEOX1* reprogrammed the circulating Tregs to acquire the transcriptional and epigenetic landscapes of tumor-infiltrating Tregs. Moreover, a high expression of *MEOX1* is intricately associated with poor prognosis of patients with intrahepatic cholangiocarcinoma, indicating that *MEOX1* could be a potential target [144]. Liao et al. used scRNA seq and found that *TNFR2* expressed by Tregs was associated with poor prognosis in ICB and could be used as a biomarker for predicting ICB efficacy [145]. Kota et al. used single-cell sequencing to reveal a key regulator of Treg, *BATF*, which regulates Treg cell differentiation by epigenetically controlling the activation of gene expression [146]. Tumor growth is significantly inhibited when *BATF* is deficient in Tregs, and high *BATF* expression is associated with poor prognosis. The expression of *BATF* in $CD4^+$ T cells has been detected in another single-cell study on CAR-T [135]. These findings suggest the potential of *BATF* in Treg-targeted therapies. Several investigators reported that blocking the *LILRB4* gene can significantly reduce tumor immunosuppression in TME [147]. Blocking *LILRB4* increases the ratio of effector T cells to Tregs in TME, promoting the polarization of $CD4^+$ T cells to Th1 cells and suppressing T cell exhaustion. Thus *LILRB* could serve as a potential target for tumor immunotherapy. Similarly, *LGALS1* was found to be highly expressed in Tregs in a single-cell sequencing study of nasopharyngeal carcinoma [148]. The proportion of immunosuppressive Tregs was significantly higher in patients with high *LGALS1* expression, suggesting its key role in Treg activation. The gene regulatory networks demonstrated that researchers believe that the regulatory function of *LGALS1* could be dependent on calcium channels. Another single-cell study in colorectal cancer demonstrated that the infiltration of Tregs was associated with *GPR15* expression on Tregs, and *GPR15* is considered a promising novel target for reducing the number of Tregs [149]. In addition to these Treg activation regulatory, Treg inhibitory regulators have been identified. For instance, researchers found that CD39 decreased the infiltration of Tregs, and CD39 inhibitors improved the survival rate of hormonal mice in a single-cell study of bladder cancer [150]. In addition, they observed that CD39 inhibitors exerted no synergistic effect with anti-PD-1 therapy. These results suggest that CD39 could be a potential new target for bladder cancer immunotherapy independent of PD-1. The complexity of Treg interactions with tumor cells in TME could result in the emergence of new studies in the future on introducing Tregs into tumor immunotherapy.

Prospects

CD4 + T cells play a role in the TME, and our understanding of their various types and functions is continually evolving. Single-cell sequencing has emerged as a powerful tool to comprehensively understand TME, and identify distinct $CD4^+$ T cell populations and their functions. It can be used to identify pathways driving the behavior of different $CD4^+$ T cell subsets and analyze their intracellular and intercellular interactions within the TME. These findings will have significant implications in the current clinical research in tumor immunology, offering novel targets and ideas for cancer treatment. Future studies should focus on unraveling the complexity, plasticity, and spatial organization of $CD4^+$ T cell subsets using a combination of multiomics, and translating these findings into improved clinical applications for disease diagnosis, treatment, and prognosis. However, research on the TME is still inadequate. For example, the heterogeneity of $CD4^+$ T cells, their spatial distribution in tumors, and the classification of their subtypes in different cancers are still unclear and warrant further research. We believe that single-cell sequencing can be refined in the future to better investigate the functions of $CD4^+$ T cells and the underlying mechanisms.

Abbreviations

ACT	Adoptive cell transfer
APCs	Antigen-presenting cells
CAR-T	Chimeric antigen receptor T cells
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DCs	Dendritic cells
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box protein 3
GZMB	Granzyme B
HCC	Hepatocellular carcinoma
ICB	Immune checkpoint blockade
IFN-γ	Interferon-gamma
IL	Interleukin
MHC	Major histocompatibility complex
NK cells	Natural killer cells
PD-1	Programmed cell death protein 1
scRNA-seq	Single-cell RNA sequencing
TAM	Tumor-associated macrophages
TGF-β	Transforming growth factor-beta
TME	Tumor microenvironment
Tregs	Regulatory T cells
TCR	T-cell receptor
Tn	Naïve T cells
TCM	Central memory T cells
TEM	Effector memory T cells
TRM	Tissue-resident memory T cells
Tfh	T follicular helper cells
Tph	Peripheral helper T cells
Th	Helper T cells

Supplementary Information

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Supplementary Material 1

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Author contributions
C.L, Q.Y.H conceived the review. Q.A and L.D were responsible for writing the manuscript. Y.Y.W and F.X.W helped to retrieval literature. Q.Y.H, C.L, and X.L instructed the study, as well as revised and finalized the manuscript. All authors read and approved the final manuscript.

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Data availability
The materials that support the conclusion of this review have been included within the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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