Review

Harnessing the Transcriptional Signatures of Car-T Cells and Leukemia/Lymphoma Using Single-Cell Sequencing Technologies

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Harnessing the Transcriptional Signatures of CAR-T Cells and Leukemia/Lymphoma Using Single-Cell Sequencing Technologies

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Abstract: Chimeric antigen receptor (CAR) T-cell therapy has greatly enhanced results for individuals experiencing relapsed or refractory hematological malignancies. However, challenges such as treatment resistance, relapse, and severe toxicity still hinder its widespread clinical application. Traditional transcriptome analysis has provided limited insights into the complex transcriptional landscape of both leukemia cells and engineered CAR-T cells, as well as their interactions within the tumor microenvironment. However, with the advent of single-cell sequencing techniques, a paradigm shift has occurred, providing robust tools to unravel the complexities of these factors. These techniques enable unbiased analysis of cellular heterogeneity and molecular patterns. These insights are invaluable for precisely designing the receptor, guiding T cell modifications based on genetic alterations, and fine-tuning manufacturing conditions. Consequently, this review utilizes modern single-cell sequencing techniques to clarify the transcriptional intricacies of leukemia cells and CAR-Ts. The aim of this manuscript is to discuss the potential mechanisms that contribute to the clinical failures of CAR-T immunotherapy. We will examine the biological characteristics of CAR-Ts, the mechanisms that govern clinical responses, and the intricacies of adverse events. By exploring these aspects, we hope to gain a deeper understanding of CAR-T therapy, which will ultimately lead to improved clinical outcomes and broader therapeutic applications.

Keywords: single-cell sequencing technologies; CAR-T therapy; tumor microenvironment; transcriptional complexity

1. Introduction

Chimeric antigen receptor (CAR)-T cell therapy has emerging as a crucial component of treatment for cancer, particularly in the immunotherapy for hematological malignancies [1–3]. CAR is a designed receptor comprising several principle components: the extracellular; hinge ; transmembrane, and intracellular signal transduction domains The extracellular domain comprises a single chain fragment variable (scFv) that can recognize and bind to the specific antigen on the malignant cells. The hinge, transmembrane, and signal transduction domains of CAR-T cells consist of a dimer of homologous or heterologous combinations of CD3, CD8 and CD28. These elements regulate receptor expression and signaling thresholds subsequently mediating optimal cellular activation [4,5]. The signal transduction domains of CAR-T cells can control the input efficiency of

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CAR signals facilitating the cytokine production function, and enhancing the tumor killing abilility of CAR-T cells [6]. The diversity within of CAR-T cell products is affected by the structure of the CAR [7–9], subtype of T cells [10] and the manufacturing process of the product [11], consequently impacting the fficacy and security of CAR T-cell therapy. Various configurations of molecular elements within the CAR, including the choice of the domain responsible for costimulation [9], and the potential for immune response of the single-chain variable fragment (scFv) [7,8] lead to varied impacts on the characteristics and activities of CAR-T cells.

CAR-T cells that display less-differentiated with characteristics of naive and early memory stages. have been observed to be associated with an increased rate of lasting clinical complete response[12,13]. Conversely, exhausted T cells,characterized by heightened immune checkpoint receptors with inhibitory functions are linked to worse clinical results [14]. Furthermore, variations in CAR-T cell lineage clones that exhibit unique patterns of clonal dynamics, contributing the variety the CAR-T cell population after administration. Notably, the manufacturing process introduces heterogeneity at each step, impacting the ultimate makeup of the CAR-T cell formulation [11].

In recent times, the U.S. Food and Drug Administration (FDA) has approved six commercial CAR-T cell products since 2017 [15–19]. However, obstacles such as treatment resistance to CAR-T, relapse after CAR-T therapy, and serious adverse effects hinder their clinical applications [20,21]. Moreover, CAR-T cell therapy exhibits restricted effectiveness in addressing solid tumors compared to its effectiveness in hematological malignancies [22–26]. This limitation may stem from insufficient tumor infiltration by CAR-T cells, the absence of stable expression of antigens unique to the tumor, and the tumor microenvironment with a high degree of immunosuppression [27–31].

After the completion of the Human Genome Project, the structure and functions of the genome have been extensively explored. The development of cancer is theoretically a buildup of genetic and epigenetic changes, including the unregulated growth and cell division, loss of responsiveness to regulatory factors,and the evasion of apoptosis in the cell. The genetic modifications and overall transformations within cancer cells could be dissected at genomic, transcriptomic, and proteomic levels. Each of these aspects has its unique advantages and limitations [32].

The genomic study level is the first aspect that has been extensively explored, with rapid progress made after the sequencing of the human genome [33]. Since then, specific mutations accountable for the conversion to a malignant state and inherited cancer predisposition syndromes have been pinpointed [34]. However, genomic studies have their limitations. First, only a relatively limited fraction of the human genome is transcribed and translated into proteins. Additionally, the expression of gene is a complex process regulated by various mechanisms, including DNA methylation [35], DNA-binding proteins [36], and small interfering RNA (siRNA) [37]. Therefore, the genomic level alone cannot fully represent the cancer phenotype, necessitating the exploration of other approaches to address this crucial issue.

The proteomic approach is more closely associated with unraveling the molecular processes governing the characteristics of a cell compared to the methodology related to genomics [38]. However, the proteomic approach, such as protein microarrays, also has its limitations, particularly regarding the diverse physical and chemical properties of monoclonal antibodies. Therefore, transcriptome profiling has risen as a potent instrument within the field of oncology over the past decades. Transcriptome encompasses the sum of all RNA transcripts in an individual or bulky tumor cells. It includes protein-coding RNAs, such as messenger RNAs (mRNAs), and non-coding RNAs (ncRNAs). Each of these RNAs plays a distinct role in the cell and responds variably to external cues from the environment [39,40].

The initial phases of transcriptome annotations commenced with the publication of cDNA libraries in the 1980s. Two common biological techniques employed for studying the transcriptome are DNA microarray, which is a method relying on hybridization, and RNA-seq, which is an approach centered on the analysis of genetic sequences [32]. Following this, the introduction of high-throughput technology resulted in accelerated and more effective methods for acquiring information regarding the transcriptome. Nowadays, Next generation sequencing has emerged as the favored approach and has been the prevailing method in the realm of transcriptomics since the 2010s.Data

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obtained from the transcriptome can be utilized for the research in cellular differentiation process, carcinogenesis, transcription regulation and biomarker discovery in the field of oncology. Traditional next-generation sequencing has the capability to capture transcriptomic information from cell populations, including as a multitude of cells, tissues from animals or plants, or even an entire organism. However, bulky next-generation sequencing has its limitation in dissecting cell heterogeneity within the bulky tumors.

While traditional techniques allow for the numerical evaluation of genomic, transcriptomic and proteomic patterns in bulk tumors, the advent of single-cell sequencing has catalyzed significant advancements in identifying of innovative biomarkers, novel cellular phenotypes, and new therapeutic targets that remain undetectable through sequencing methodologies for bulk samples [41–43]. Single-cell sequencing , with its heightened precision, serves as an invaluable tool for examining the characteristics of immune cells.This includes exploring various developmental lineages, antigen specificity, phenotypic plasticity, cellular transitions, interactions, cellular communications, and adaptability to various microenvironments [43–52].

Various modern single-cell sequencing methods have been utilized in the reserch of CAR-T cell, encompassing scRNA-seq (single-cell RNA sequencing), single-cell T-cell receptor sequencing (scTCR-seq), single-cell assay for transposase-accessible chromatin sequencing (scATAC-seq), cytometry by time-of-flight (CyTOF), cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), and single-cell multiplexed secretome proteomics. Of these tools, scRNA-seq presently stands out as most commonly employed single-cell sequencing method. The droplet-based system, specifically the 10x Genomics BD, stands out as the primary scRNA-seq system currently in utilization. Utilizing a microfluidic chamber, the droplet-based technique segregates single cells into microdroplets dispersed in an oil-based medium. Within each droplet, there are gel microbeads consist of mRNA-capturing primers, a distinctive molecular barcode, and an enzyme/reagent combination crucial for cell lysis and reverse transcription. After reverse transcription and cDNA amplification, a barcoded sequencing library is generated. Subsequently, the samples undergo processing for sequencing and subsequent analyses [53,54]. In scTCR-seq, the sequencing focuses on the 5′-end transcript, enabling the concurrent identification of the V(D)J sequence and transcript of an individual cell. Consequently, scTCR-seq proves particularly useful in characterizing the diversity of T cell population based on clonotypes, and dissecting functional phenotypes of T cells, such as memory, activation, and exhaustion T cells [55–57]. scATAC-seq is primarily designed for the identifying activated genes through genome-wide analysis of open chromatin regions, promoters, enhancers and subsequently postulate the transcription factors (TFs) that bind to the cis-elements [58] This is accomplished by incorporating sequencing adapters into regions of the genome that are readily available or open for molecular interactions through Tn5 transposase. Consequently, scATAC-seq is primarily employed to analyze and define the epigenetic features associated with T cell differentiation and exhaustion. [59,60]. CyTOF is a conjunction of flow cytometry with mass spectrometry, facilitating analysis with multiple parameters. Through conjugation with over 100 antibodies labeled with unique isotopically pure metals, CyTOF accurately detecting protein expression both intracellularly and extracellularly at the individual cell level [61,62]. When combined with single-cell RNA-Seq, CyTOF becomes a powerful tool for characterizing the heterogeneity of rare T cell subpopulations. CITE-seq exploits an an antibody labeled with a unique oligonucleotide barcode linked to the molecular marker on the cell surface to characterize cell populations guided by the markers on the cell surface and the transcriptome profile [63] This method allows for the simultaneous delineation of the immunophenotypic profile and transcriptome signature of T cell. SCBC (single-cell barcode chip) utilizes concentrated microfabricated compartments with spatially barcoded capture sites for analysis of single cells with a high degree of multiplexing up to 42 secreted cytokines associated with T cell function, including cytotoxic, regulatory ,stimulatory, and inflammatory molecules [64,65]. Given the marked diversity in the secretion of cytokines observed in CAR-T cells. The concept of polyfunctional CAR-T cells, along with the Polyfunctionality Strength Index (PSI), is used to characterize subsets of CAR-T cells which are able to simultaneously

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generation of various cytokines at the individual cell level. This methodology has been employed to deduce the patients' medical results. [66–69].

The aforementioned single-cell methods play a crucial role in facilitating improved design of receptors, directing modifying T cells based on genetic alterations and refine the conditions for manufacturing [60,70–73]. Furthermore, they contribute to clinical forecasting therapeutic effectiveness and potential toxicities, enabling the personalized treatment of CAR-T therapy [74,75]. Despite their significance, there have been relatively few reports reviewing the function of modern single cell sequencing methods in understanding the intricacies of CAR T-cell therapy [43,76–80].

In this review, our objectives are to address the potential mechanisms contributing to the clinical failures of CAR-T immunotherapy. We will delve into the biological characteristics of CAR-Ts, the mechanisms governing clinical responses, and the intricacies of adverse events. By exploring these aspects, we aim to acquire a more profound comprehension of CAR-T therapy, ultimately facilitating optimal selection of target antigens, CAR-transgene integration, and manufacturing processes (Table 1), which collectively help to improve clinical outcomes and broaden therapeutic applications.

Table 1. Summary of scRNA-seq based researches on CAR-T cells.

Technology	Disease	T Cell Source	CAR-T Antigen	Findings	Reference
CAR-T cell product heterogeneity					
scRNA-seq, 10 × Genomics Chromium Single Cell 3', Illumina HiSeq bulk RNA-seq	B-ALL	Activated and inactivated CAR-T cells from 5 healthy donors	LV αCD19–CD28–CD3ζ, LV αCD19– 4-1BB–CD3ζ	4-1BB based CAR T-cells have a central memory cell phenotype, enriched expression of fatty acid metabolism genes. Moreover, they exhibited heightened levels of MHC II genes, ENPP2, IL-21 axis genes, as well as a reduction in PD1 expression	[81]
Bulk RNA-seq, CITE-seq, scATAC-Seq, 10 × Genomics Illumina Nova-Seq 6000	B-ALL	Post infusion CAR-T cells from 71 patients.	LV αCD19–4-1BB–CD3ζ	The TCF7 regulon associated with the favorable naïve T-cell state and effector T cells maintenance in patients with long-term CAR T-cell endurance. IRF7 regulated chronic IFN signaling was associated with poor CAR T-cell endurance	[13]
scRNA-seq, scFTD-seq, Illumina HiSeq 4000 CITE-seq	R/R B-ALL	IPs, Activated and inactivated CAR-T cells of 1 healthy donor and 2 patients	LV αCD19–4-1BB–CD3ζ	CAR T-cells derived from healthy donors exhibit stronger functional activities and revealed the upregulation of MHC II genes	[71]
scRNA-seq, flow cytometry	B-ALL	Activated CAR-T cells of 3 donors	VSV-LV CART and CD8-LV CART	VSV-LV CAR T-cells revealed central memory phenotype, while CD8-LV CAR T-cells revealed cytotoxic activity	[70]
scATAC-seq, CHIP-seq, flow cytometry	B-ALL, MM	Activated and post- infusion	LV α CD19 -CD8-4-1BB-CD3ζ	BATF and IRF4 are CAR T- cell exhaustion regulators	[60]

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		CAR-T cells of 2 patients	BCMA		
scRNA-seq 10x Genomics Chromium Single Cell 3′; Illumina HiSeq 4000		Autologous CAR-T cells from human	(γ-Retroviral) APRIL–CD28–OX40–CD3ζ	Most the CAR-expressing cells exhibited transcriptional changes upon exposure to the CAR-specific antigen exposure. A tiny proportion of these cells displayed an exhaustion signature such as LAG-3 and TIM-3	[82]
Antigen-specific stimulation of CAR T-cells					
scRNA-seq, Illumina HiSeq 2500 scFTD-seq; single-cell cytokine assay, single-cell cytotoxicity assay	BCL	Activated and inactivated CAR-T cells from 3 healthy donors	LV αCD19-CD28-4-1BB- CD3ζ	Activated CAR T-cells exhibited a diverse composition, including TH1, TH2, Treg, and GM-CSF-expressing T cell subsets.	[83]
Dynamic behavior of CAR T-cells					
scRNA-seq, scTCR-seq 10 × Genomics Illumina NovaSeq	B-ALL	IPs, post-infusion CAR T-cells of 15 patients	LV α CD19-CD8-4-1BB-CD3ζ	$TIGIT^{+}CD27^{-}CD62L^{Low}$ is leads to a highly efficient post-infusion CAR T-cell phenotype.	[84]
scRNA-seq TCR-seq, 10xGenomics Chromium Single Cell 50 + V(D)J enrichment, Illumina HiSeq 2500	NHL	IPs, post-infusion CAR T-cells of 10 patients	LV αCD19-IgG4-CD28-4-1BB-CD3ζ	The heterogeneous of CAR T-cells was most pronounced in the infusion products (IPs). Clones higher expression of cytotoxicity and proliferation genes predominately expansion after administration	[85]
Cellular interactions with CAR T-cells					
scRNA-seq 10 × Genomics Illumina HiSeqx10 Image analysis, flowcytometry	BCL	BM cells of Mice	LV αCD19-CD28-CD3ζ	CAR T-cell activity relied on cytokine-mediated communication with the tumor microenvironment (TME). IFN-γ produced by CAR T-cells not only promotes endogenous T cells but also promotes CAR T-cell tumor killing effect	[86]
Primary resistance					
scRNA-seq 10xGenomics Chromium Single Cell 50 + V(D)J Enrichment; Illumina HiSeq 4000	LBCL	IPs from 24 patients	RV αCD19–CD28–CD3ζ (Yescarta)	Exhausted $CD8^{+}$and $CD4^{+}$ T cells were predominant in poor responder. Memory type CD8 T cells were predominant in excellent responder	[75]

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scRNA-seq, 10 × Genomics, flow cytometry	NHL	IPs, post-infusion CAR T-cells from 17 patients	LV αCD19-CD8-4-1BB-CD3ζ	Exhausted CD8+ CAR T-cells expressing TIGIT were associated with poor responder	[87]
scRNA-seq, TCR-seq, 10 × Genomics Illumina NovaSeq S4, flow cytometry	BCL	Pre-infusion and post-infusion PBMCs, IPs from 32 patients	LV αCD19–4-1BB–CD3ζ (Kymriah)(tisa-cel) RV αCD19–CD28–CD3ζ (Yescarta)(axi-cel)	Proliferative memory-like CD8 clones were associated with of tisa-cel response. Axi-cel responders exhibited more heterogeneous features. The amount of CAR Treg cells linked to clinical progression	[88]
scRNA-seq, 10 × Genomics Chromium Single Cell V(D)J; Illumina HiSeq 2500 genome- wide CRISPR/Cas9 knockout screening	B-ALL	IP, post-infusion CAR T-cells from 2 patients	LV αCD19–4-1BB–CD3ζ	Death receptor signaling was asscoated with primary resistance to CAR T-cells. CAR T-cells from patients with primary resistance showed higher levels of exhaustion profiles	[89]
Antigen positive relapse					
scRNA-seq, CITE-seq, Drop Seq, Illumina HiSeq 4000, Flow cytometry, multiplexed secretomic assay	ALL	Activated and inactivated CAR T-cells, pre-infusion CAR T-cells from 61 patients	LV αCD19–4-1BB–CD3ζ	The absence of TH2 function in CAR T-cell products was linked to CD19-positive relapse. Early memory-like T-cell subsets, T_{SCM} (stem cell memory) and T_{CM} (central memory), were notably reduced in patients experiencing positive relapse	[90]
Antigen negative relapse					
Bulk RNA-seq, scRNA-seq, flow cytometry	B-ALL	2 second-trimester human fetus HSPCs	CD19 (detail not available)	Phenotypic escape after CD19-directed immunotherapies is underlined by CD34+CD19−CD22+ progenitors	[91]
CRS Toxicity					
scRNA-seq	B-ALL	Post-infusion CD45+immune Cells from 8 mice	?V αCD44v6 - CD28–CD3ζ ?V αCD19–CD28–CD3ζ	Human circulating monocytes responsible for the CRS related IL-6 production	[92]

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Single-cell cytokine pro-filing, flow cytometry	NHL	IPs from 20 patients	RV αCD19–CD28–CD3ζ (Yescarta)	Higher PSI was link to severe CRS. IL-17A-producing polyfunctional CAR T (Th17)-cells were linked to ICANS	[69]
ICANS Toxicity					
scRNA-seq, 10 × Genomics Illumina HiSeq 2500	NA	Human brain, lung pericytes, PBMCs, mice brain cells Mice and the BRAIN Initiative Cell Census Network Public data from GEO	LV αCD19–4-1BB–CD3ζ RV αCD19–CD28–CD3ζ	CD19 postive human brain mural cells might partly responsible for the neurotoxicity	[93]
scRNA-seq	BCL	IPs, post-infusion CAR T-cells from 72 patients	LV αCD19–4-1BB–CD3ζ RV αCD19–CD28–CD3ζ	Reactivated HHV-6 infection shows symptoms are similar to ICANS	[94]
scRNA-seq, scTCR-seq, CITE-seq, CyTOF, 10 × Genomics Illumina NovaSeq 6000 or HiSeq 4000	LBCL	Post-infusion CAR T-cells from 32 patients	RV αCD19–CD28–CD3ζ	$CD4^+Helios^+$ CAR T-cells one week after administration were Treg cells like cells and were associated with less severe neurotoxicity and progressive disease	[95]
scRNA-seq	B cell lympho ma	Infusion products of Public data from GSE150992		Neurotoxicity is linked to decreasing cycling activity, the quantity of CAR+ cells, and the expression of cell cycle genes and exhaustion-related genes	[96]
Hematological toxicity					
scRNA-seq, scTCR-seq, 10 × Genomics Illumina, NovaSeq	Richter-transfor med DLBCL	Pre-treatment and post-treatment PB samples	LV αCD19–4-1BB–CD3ζ	Oligoclonal CAR-T cell expansion as a possible contribution to hematological toxicity	[97]
On-target off-tumor effects					
scRNA-seq, flow cytometry	B-lineage derived	Cells in normal		Acquired of around one hundred of taget molecules in	[98]

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	malignant cells, solid tumors , AML	Tissues and organs from healthy donors of the public scRNA-seq datasets		normal specimens at the single cell level	
scRNA-seq		Cells from the human cell landscape and the adult human cell atlas from 40 donors cell		Find out the targets for possible on-target off-tumor toxicity	[99]
Others					
scRNA-seq, 10 × Genomics Illumina, CRISPR/Cas9 genome editing system	B-NHL	IPs, engineered CAR T-cells , PBMCs from 3 patients	Non viral AAVS1-αCD19-CD8-4-1BB-CD3ζ	PD1-targeted CAR T-cells by CRISPR/Cas9 technology within virus-free method	[100]
CyTOF	B-ALL, NHL, DLBCL	IPs, PMBCs, BM, post-infusion CAR T-cells from 3 patient	LV αCD19–IgG4-CD28–huEGFRt-CD3ζ	CAR T cells from patients displayed spatiotemporal alterations in trafficking, activation, maturation, and exhaustion expression. A distinct signature was observed in the CSF niche	[101]
scATAC-seq, 10 × Genomics Illumina NextSeq 550	BNHL	CAR-T cells		EpiVIA for the profiling of the chromatin accessibility and lentiviral integration site analysis simultaneously	[102]
scRNA-seq, scCAR-seq, 10 × Genomics Illumina NovaSeq		CAR-T cells		Create an alta of 180 distinct CAR targets integrated into primary human T cells using CRISPR-Cas9. Discovered multiple targets demonstrating tumor-killing abilities and T cell subgroups significantly divergent from standard CARs	[103]
Single-cell, 16-plex cytokine profiling, Single-cell barcode chip		CAR-T cells		Various immune effector response of CD19 CAR-T cells when treated with target stimulation. Significant subgroup of activated CAR-T cells displays strong polyfunctionality, characterized by a dominant antitumor effector cytokine profile	[67]

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Abbreviations: axi-cel, axicabtagene ciloleucel (Yescarta); tisa-cel, tisagenlecleucel (Kymriah); ALL, acute lymphoblastic leukemia; AML acute myeloid leukemia; APRIL, 'a proliferation-inducing ligand', a high-affinity ligand for the receptors BCMA and TACI; B-ALL B-cell acute lymphoblastic leukemia; BM, blood marrow; CAR, chimeric antigen receptor; ChIP-seq, chromatin immunoprecipitation sequencing; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CRS, cytokine release syndrome; CR, complete remission; CRISPR, clustered regularly interspaced short palindromic repeats CSF, cerebrospinal fluid; CyTOF mass cytometry by time-of-flight; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; GM-CSF, granulocyte-macrophage colony-stimulating factor; HHV, human herpesvirus; ICANS, immune effector cell-associated neurotoxicity syndrome; IFN, interferon; IPs, infusion products; IRF7, interferon regulatory factor 7; IRF4, interferon regulatory factor 4; LAG-3, lymphocyte activating 3; LBCL, large B cell lymphoma; LV, lentiviral vector; MCL, Mantle cell lymphoma; MHC, major histocompatibility complex; MM, multiple myeloma; NA, not available; NHL, non-Hodgkin lymphoma; NK, natural killer; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PD, progressive disease; PFS, progression-free survival; PR, partial response; PSI, polyfunctional strength index; R/R, relapse and refractory; RV, retroviral vector; scATAC-seq, single-cell assay for transposase-accessible chromatin using sequencing; scFTD-seq, single-cell freeze–thaw lysis directly toward 3′ mRNA sequencing; scRNA-seq, single-cell RNA sequencing; scTCR-seq, single-cell T-cell receptor sequencing; TACI, cyclophilin ligand interactor; TCF7, transcription factor 7; TCM, central memory T cell; TH, T helper cell; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TILs, tumor-infiltrating lymphocytes; HAVCR2, hepatitis A virus cellular receptor 2; TLE4, transcription factor transducin like enhancer of split 4; TME, tumor microenvironment; TRAC, T cell receptor alpha constant; Treg, regulatory T cell; TSCM, stem cell-like memory T cell; VSV, vesicular stomatitis virus.

2. Primary Resistance

Primary resistance to CAR-T treatment is defined as CD19+ progressive disease during CD19 CAR-T treatment, with the underlying mechanisms remaining elusive [104]. Several intrinsic mechanisms in leukemia have been identified (Table 2), including the decrease in the expression of pathways related to death receptor signaling such as TRAIL and Fas pathway functions [89,105]. Notably, leukemia cells with exhibiting decreased the activity of death receptor genes (TNFRSF10B, CASP8, BID, and FADD) have been linked to patients who show no response [89]. Additionally, a stem cell epigenome in leukemia cells exhibiting myeloid and stem cell like features is also linked to primary resistance to CD19 CAR-T treatment [106]. Brian J. et al reported changes in various categories of genes in DLBCL cells including those related to B cell identity (PAX5 and IRF8), immune checkpoints (CD274), and the microenvironment (TMEM30A), are associated with resistance [107].

Table 2. Overview of scRNA-seq studies on acute lymphoblastic leukemia / lymphoma cells.

Technology	Disease	Cell Source	Target Antigen	Findings	Reference
scRNA-Seq,10x Chromium Single Cell 5′	B-ALL	Bone marrow leukemia cells	CD19	Subgroup of leukemic cells with negative CD19 expression were existed prior the initiation of CAR T-cell therapy. This subclone might related to relapse	[108]
scRNA-Seq	B-ALL	CAR-T cells, leukemic cells	CD19	Leukemic cells that survived were co-cultured under various conditions (CAR T-cells and T cells) for one day was analyzed. Low CD19 expression leukemic cells maintained the reduced CD19 levels through transcriptional programs associated with normal activation of B-cell and germinal center reactions. This process facilitating leukemia cell immune escape	[109]

Abbreviations: ALL, acute lymphoblastic leukemia.

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More significantly, novel single cell technologies have played a crucial role in uncovering novel mechanisms involving T cell dysfunction, such as elevated markers associated with T cell exhaustion and reduced expansion of CAR-T cells, which are factors associated with primary resistance disease [75]. Deng Q et al utilized whole- transcriptome scRNA-seq analysis and discovered an enrichment of exhausted CD4+ along with CD8+ T cells in the product of patients exhibiting only a partial improvement or even worsening disease. In their analysis, genes such as lymphocyte activation gene 3 (LAG-3) and TIGIT, along with the basic leucine zipper ATF-like transcription factor (BATF) and inhibitor of DNA binding 2 (ID2), were observed to have elevated expression levels in the exhausted T cells [75]. Jackson et al utilizing scRNA-Seq also identified the increased expression of the exhaustion marker TIGIT in CAR-T cells was linked to a diminished reaction in patients with recurrent or resistant B-cell lymphoma [87]. CAR Treg cells have also been implicated in primary resistance [74,88]. Haradhvala et al, using scRNA-seq analysis, discovered that an increasing count of CAR Treg cells correlated with the nonresponse rate [88]. Good et al utilizing CyTOF combined with single-cell RNA-Seq and found that increased counts of CD4+ HELIOS+ CAR-T cells showing characteristics of Treg cells were linked to the advancement of the disease [74].

3. Relapse

3.1. CD19 negative relapse

A common reason for recurrence after CAR-T cell therapy is the absence of the target molecule or loss of antigen expression [110]. Possible mechanisms include the pre-exiting of antigen negative clones, such as CD34+CD19-CD22+ B-cell progenitors [91,108], gene mutation [111], splicing events [112], transcriptional plasticity of leukemia cell [109], reversible antigen loss through trogocytosis [113], instant introduction of the CAR-T gene to the leukemia cell [114], and lineage switch induced antigen loss [115].

On the other hand, factors associated with CAR-T cells may also contribute to therapeutic failure [51,64–75,116–119]. It has been proposed that CD19 negative relapse is related to immune escape. Fraietta et al reported that CAR-T cells obtained from individuals who did not exhibit a positive response exhibited upregulation of signatures related to effector differentiation, apoptosis, exhaustion, and glycolysis in individuals diagnosed with chronic lymphocytic leukemia (CLL)[12]. Moreover, the exhaustion of T cells mediated by Programmed Death-1 (PD-1) is also associated with lack of responsiveness to CAR-T cell therapy [120–122].

3.2. CD19 positive relapse

The most common reason for recurrence following CAR-T cell therapy was noted in many clinical trials is CD19 positive relapse [1]. The Leukemia intrinsic factors contributing to CD19 positive relapse are still poorly understood. Additionally, CAR-T associated factors, such as impaired CAR T-cells activation, expansion, in vivo resistance, and poor antitumor potency [123,124] , have also been found to be associated with CD19 positive relapse [124]. Bai et al, using scRNA-seq analysis, discovered that a scarcity of TH2 function, as well as lack of TH2 cytokine molecules such as IL-4, IL-5, and IL-13, is link to CD19-positive relapse in patients [90]. They also applied CITE-seq analysis to identify novel subgroups of T cells, revealing that early memory-like T cell subgroups such as T_{SCM} and T_{CM} were significantly reduced among patients with positive CD19- positive relapse [109].

3.3. Crosstalk between CAR-T cells and TME

The tumor microenvironment (TME) takes a vital part in cancer development, relapse, and is closely linked to the ineffectiveness of cancer immunotherapy. The Immunosuppressive microenvironment includes cells and cytokines that hinder or suppress the immune response. Cell types like regulatory T cells, tumor associated macrophage, myeloid derived suppressor cells, have the ability to impede the proliferation and cytotoxic abilities of CAR-T cells [125–128]. Deficiencies in chemokines also contribute to reduced chemotactic capabilities of CAR-T cells [129]. Additionally,

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the presence of abnormal blood vessel structure along with cancer-associated fibroblasts poses challenges for impairing the effective migration of CAR-T cells to the neoplasm site [130,131].

4. Adverse Events

The frequently occurring adverse events (AEs) of CAR-T treatment include cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), cytopenia, infection, as well as on-target off-tumor effect [132,133]. Single-cell analysis has played a significant role in deeper research into the underlying mechanisms of these AEs.

4.1. CRS

The most prevalent advese effect associated with CAR-T cell therapy is Cytokine Release Syndrome (CRS) [134]. While reversible in the most patients, severe CRS can be fatal. Endothelial cell activation holds a pivotal role during the initiation of CRS [135]. CRS is a systemic inflammatory reaction triggered by dysfunction of endothelial cells, abnormal activation of macrophages, followed by the release of different proinflammatory signaling molecules, such as IL-1, IL-6, and IL-8 [92,136]. Furthermore, single-cell cytokine profiling has showed that greater T-cell polyfunctionality strength index (PSI) is associated with grade ≥ 3 CRS [69]. Additionally, the combination of PSI with CAR T-cell expansion or plasma levels of IL-15 before treatment provides a reliable indicator for severe CRS [69].

While it is renowned that IL-6 is the main cytokine for CRS, the primary cellular source of IL-6 remains unknown. Norelli et al, utilizing scRNA-seq, sought to answer this important question. Their analysis of leukocytes marked by CD45+ isolated from humanized mice that experienced CRS following CAR-T cell adminstration confirmed that circulating monocytes were the sole cell population expressing high levels of IL-6 [92]. Furthermore, with the assistance of single-cell analyses, Deng et al reported a positive association between exhausted CD4+ T cells and a severe grade of CRS, while exhausted CD8+ T cells had a negative association with higher grade of CRS [75]. When comparing patients with grade 0-1 CRS to those with higher grade, it was found that the latter group exhibited a higher PSI of CD4+ CAR T-cells, particularly in terms of PSI for IL-8 and MCP-1. [68]. These cytokines play a role in recruiting neutrophils, monocytes, and macrophages [137,138].

4.2. ICANS

Immune effector cell-associated neurotoxicity syndrome (ICANS) is another common adverse event linked to CAR-T cell therapy. The pivotal factor in the development of ICANS is the activation of endothelial cells in the brain, which leads to inflammation and the disruption of the integrity of the blood-brain barrier (BBB) [134,139]. With the assistance of single-cell analyses, Deng and colleagues recognized a unique cell population exhibiting transcriptional characteristics similar to monocytes, and this was linked to severe ICANS [75]. On the other hand, Parker et al discovered that CD19 is expressed during brain development, especially in the development of mural cell lineages. Mural cells envelop the endothelium and are linked to the integrity of the blood-brain barrier. The expression of CD19 remains present throughout adulthood in all regions of the brain as is revealed by single-cell analysis. Consequently, mural cells in the brain expressing CD19 can become unintended off-tumor targets for CAR-T therapy [93]. Furthermore, Loeffler et al, utilizing scRNA-seq of 24 patients, found that number of CAR+ cells, decreasing cell cycling activity, and the expression of cell cycle and exhaustion-related genes such as LAG3 and TIM3 are associated with higher neurotoxicity [96]. In conclusion, single-cell sequencing technologies possess the capability to identify biomarkers from CAR-T cell products that deduce an increased risk of severe ICANS. These biomarkers include cells associated with ICANS (IACs) exhibiting a transcriptional profile resembling that of monocytes [75], an elevated count of polyfunctional CAR T-cells capable of producing IL-17A[69], and diminished levels of CD4+Helios+ CAR-T cells [74].

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4.3. Cytopenia

Emerging data indicate the incidence of cytopenia resulting from CAR-T therapy [140]. Grade 3 or higher cytopenia, persisting for more than one month following CAR-T cell infusion, is observed in approximately 20–40% of patients [141]. The mechanisms behind delayed and persistent cytopenia following CAR-T cell therapy are not well comprehended. Various hypotheses have been suggested, including the impact of prior lines of therapies, advanced age, diminished bone marrow reserve, the severity of cytokine release syndrome (CRS), and the involvement of various inflammatory cytokines [141]. Singe cell methods also help to further delineate the novel mechanisms. Rejeski et al reported a patient exhibiting prevalent oligoclonal T cell expansion in both populations, whether they bear CAR or not. These oligoclonal T cells exhibited heightened expression of cell cycle-related genes and reduced expression of genes associated with cell apoptosis, immune response, and inflammation [97]. Besides, Paolo et al discovered that long-lasting cytopenia after CD19 CAR-T cell therapy is linked to the infiltration of clonally expanded CD8 T cells expressing IFN-γ into the bone marrow, as identified through single-cell RNA-Seq and paired B-cell or T-cell receptor (BCR/TCR) sequencing [142]. In conclusion, single cell technology provides previously undiscovered mechanisms of CAR-T related hematologic toxicity.

4.4. Infection

Infection-related complications are also common among the CAR-T treatment patients with reports indicating that around 30% of patients will experience a serious bacterial infection in the initial 30 days after CAR-T treatment. Viral respiratory tract infection tend to occur during the late phase, while fungal infections and cytomegalovirus (CMV) reactivation are relatively uncommon [143]. Single-cell methods have also contributed to research in this field. Chen et al, using single-cell transcriptomics analysis, discovered that monocyte loss is one of the possible factors contributing to infections after CAR-T infusion [144].

5. CAR-T Cell Behaviors Correlate with Clinical Therapeutic Response

5.1. The dynamic and kinetic performance of CAR-T cells

CAR-T cell characteristics undergo variations from the pre-administration sample throughout the process of therapy. The heterogeneity in T cell composition of CAR-T cell products influences the maturation process of CAR-T cells, resulting in distinct CAR-T cell behaviors, kinetics, and cell destinies [145–147]. Haradhvala and colleagues applied scRNA-seq and scTCR-seq to identify that among tisa-cel responders, there was an expansion and proliferation of memory-like CD8+ CAR-T cell clones that transformed into IL7R+ effector memory CAR-T cells. In contrast, responders to axi-cel demonstrated more diverse populations [88]. Those CD8+ CAR-T cells exhibited a more pronounced upregulation of the stimulation indicator PDCD1 and the immune checkpoint controller SLAMF6 [88].

Although activated by the identical antigen, CAR-T cell sub-populations of individual patients demonstrated distinct patterns of expansion and diverse differentiation trajectories[45,88]. In general, clusters characterized by strong activities of cytotoxicity and proliferation genes typically dominated [84,85]. Furthermore, Wilson et al applied scRNA-seq combined with scTCR-seq methods and discovered an effector precursor CD8+ CAR-T cell exhibiting a distinctive transcriptional signatures, $TIGIT^{+}CD27^{-}CD62^{Low}$ among the typical pathways taken by highly effective CD19-specific CAR-T cells [84]. These CAR-T cells matured into persisted memory cells and maintained a "resting primed" state with minimal metabolic expenditure to prevent recurrence [85,148]. For example, Melenhorst and colleagues reported decade-long leukemia free with the endurance of $Ki67^{hi}CD4^{+}$ CD4+ CAR-T cells in two patients with CLL [149]. These CD4+ CAR-T cells harboring an uncommon memory phenotype characterized by ongoing stimulation and replication, expressing cytotoxic genes including GZMA, GZMK and PRF1, and other genes associated with oxidative phosphorylation pathways. Those prolonged persistence CD4+ CAR-T cells showed tumor killing features,

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proliferation, metabolic activity, cytokine expression and strong response to CAR stimulation in cell level, indicating functionally active rather than exhausted [149]. More recently, Zhu and colleagues utilizing scRNA-seq in combination with scATAC-seq simultaneously in the same CD8+CAR-T cell. They identified that FOXP1 play a crucial role in the stem-like network to facilitate extension, maintain stemness of CAR T cells and prohibit redundant effector differentiation. Meanwhile, KLF2 promote effector CD8+ T cell differentiation and hindered terminal exhaustion [150].

A dynamic model of CAR-T cells changes after administration has been suggested [148]. Initially, the infusion products exhibit high metabolic activity, characterized by elevated glycolysis and biosynthetic gene expression. They then gradually transition to a highly cytotoxic state. Subsequently, the CAR-T cells become non-proliferative while maintaining their cytotoxicity. Finally, the signatures of replication and cell-killing activity in the CAR-T cells decline during the tumor free phase [148]. Goldberg et al adapted single cell analysis combined with mass cytometry simultaneously and discovered that patient' infusion products showed a heightened activity of numerous migration and activation markers when using T cells obtained through leukapheresis as a baseline. Additionally, they found that CD4 and CD8 trafficking and memory phenotype markers are significantly enriched in cerebrospinal fluid compared to peripheral blood samples [101]. However, it remains to be clarified, whether this is the memory phenotype responsible for CAR-T cell CNS movement, or if the migrated CAR-T cell is undergoing reprogramming to a memory phenotype within the CNS niche.

5.2. Response-associated CAR-T cell behaviors

As mentioned earlier, Bai and colleagues utilized single cell transcriptomes combined with surface protein method to analyze the landscapes of 12 ALL patients and observed that an association was observed between impaired Th2 cell function and a dominant shift towards cytotoxic cell subtypes, contributing to CD19-positive recurrence [90]. The proteomic data in their study likewise demonstrated that a reduced amount of early memory T cells may serve as a predictive factor for relapse [90]. Consistently, Rabilloud et al used scRNA-seq analysis and reported the existence of pre-existing CD19-negative B-ALL subpopulations in leukemic cells from one B-ALL patient prior to CAR-T treatment. There was an observed elevation in regulatory (CAR-Treg) cell subroups among axi-cel resistant patients, suggesting their potential to inhibit the expansion of conventional CAR-T cells and contribute to delayed relapses [74,88]. Furthermore, Good et al also identified that an elevated level of CD4+Helios+ CAR-T cells at one week post-administration was linked to clinical advancement and less serious neurotoxicity, as determined through single-cell proteomic profiling [74].

5.3. CAR-T persistence

With the help of scRNA-seq, Deng et al demonstrated that among axi-cel treated LBCL patients those who with a complete clinical remission was linked to expanded amount of CD8+ T cells demonstrating memory-related profiles. In contrast, poor clinical reaction was linked to a malfunctioning CD8+ T cell profiles dominated for exhaustion and activation markers, as well as genes encoding MHC class II proteins [75]. Transcription factors related to CAR-T treatment failure-were also highly expressed among poor responders. Conversely, patients with complete remission had increased amount of memory CD8+ T cells, as indicated by scatter plots of CCR7+CD27+ CD8+ T cells assessed by CapID in their study [75].

5.4. CAR-T cell exhaustion

T cell exhaustion, resulting from prolonged exposure to tumor antigens, is considered as one of the principal reasons for treatment failures in immunotherapy. Exhaustion-related transcription factors, such as TOX, NR4A1, and IRF4, play a central role in influencing the activity of immune checkpoint genes and driving T cell exhaustion [59,151–154]. Nevertheless, the processes of CAR-T cells exhaustion differ from the non-transformed T cells. For example, Deng et al demonstrated that

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those DLBCL patients treated with axi-cel, those with a poor clinical response exhibit a CD8+ T cell dysfunction profile increased for exhaustion and activation indicators, as well as genes encoding MHC class II proteins, as identified through single-cell RNA sequencing [75]. Consistently, Singh and colleagues also reported that the development of treatment resistance is linked to the increased expression of exhaustion markers. [89].

Moreover, scRNA-seq of CAR-T cells from a trial in metastatic prostate tumor showed that the CAR-T cells of poor responders undergo a transition towards a highly differentiated, non-proliferative, and exhausted state. This group showed that an enriched exhausted signature was identified in CAR-T cells from poor responsers, defined by TIGIT expression [87]. Two transcription factors, BLIMP1 (B lymphocyte-induced maturation protein 1) and NR4A3 (nuclear receptor subfamily 4 group A member 3), also responsible for the regulation of dysfunction of CAR-T cell. Double deletion of these two transcription factors induced a change in CAR-T cell phenotypes, shifting them away from TIM-3+CD8+ towards TCF1+CD8+, countering tumor-infiltrating CAR-T cells exhaustion and enhancing their cancer killing activity in animal experiments [155]. In line with this, Jiang and collogues used scATAC-seq to demonstrate the pattern of chromatin accessibility in CAR-T cells while being stimulated by tumor cells revealed significant enrichment of BATF (Basic Leucine Zipper ATF-like Transcription Factor) and IRF4 (Interferon Regulatory Factor 4) in terminally exhausted CAR-T cells [60].

6. Preclinical Stage

6.1. On-target, off-tumor toxicity

The choice of target molecules is crucial for CAR-T therapy. However, many target molecules with widely applicable significance are frequently expressed in non-cancerous cell, giving rise to a significant issue referred to as “on-target, off-tumor” effect. To tackle the challenge, Zhang and colleague utilized single-cell analysis datasets of CAR-T target molecules [98] and developed all-encompassing single-cell atlas for target molecules used in CAR therapy across non-cancerous tissues. This study aids in the identification of rare antigen-expressing cell types that might be overlooked in bulk tissue assessments. Similarly, Jing et al [99] employed a single-cell approach (CARTSC) to analyze CAR-target gene on target toxicity at the individual cell level. This method enables the identification of specific targets and facilitates the recognition of the patterns of expression of chosen target genes in immune cells and tissues.

6.2. Target antigens and antigen-specific stimulation of CAR-T cells

Target antigens and the antigen-specific stimulation of CAR-T cells play distinctive roles during CAR-T treatment, and the stimulation pathways of CAR-T cells differ significantly from those of innate T cells. Several studies have utilized single-cell RNA sequencing (scRNA-seq) combine with other single-cell sequencing methods to investigate the variability in CAR-T cell products under different situations, including unstimulated, CAR-induced stimulated, TCR-induced stimulated, and CAR-induced stimulated CAR-T cells [71,81–83,90]. In detail, under the existence of ligand-independent tonic signaling, unstimulated CAR-T cells exhibit a combination of exhaustion signatures, early activation, and tumor killing activities [71]. Upon CAR-induced activation, CAR-T cells exhibit a strongly mixed TH1/TH2 cell signaling profile, and high cytokines levels, such as TNF-α, IFN-γ, GM-CSF, IL-5, and IL-13, reveal substantial diversity across distinct cell subpopulations [83]. Notably, GM-CSF+ CAR-T cells are considered functionally active due to the high expression of GM-CSF [71,83]. Strikingly, both CD4+ and CD8+ CAR-T cells exhibit elevated levels of tumor killing related cytokine expression, suggesting their killing functions [83]. Furthermore, to preserve immune balance after activation, specific CAR-T cell subgroups upregulate the presence of immune checkpoint related genes such as CTLA4 and PD-1, as well as immune-suppressing cytokine related genes such as IL-10 and TGFB1.Simultaneously, they downregulate co-stimulator genes such as inducible costimulatory (ICOS) and OX40 [83]. Interestingly, early signs of exhaustion have been observed in a subset of CAR-T cells in the initial stages after activation [82,84]. Additionally, gene

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expression differs between CAR-T cells stimulated by TCR and those stimulated by CAR, with the former showing specific enrichment in genes associated with T cell activation such as IFN-γ, IL-3, and CCL4 [81].

6.3. Integration of CAR-encoding vectors

The integration site of the lentiviral vector within the TET2 gene has been recognized as a factor contributing to clonal expansion [156]. Hence, the accurate positioning of the CAR vector in the T cell genome is a crucial factor for the success of CAR-T therapy [157,158]. Wang et al. introduced an approach named EpiVIA for the analysis of chromatin accessibility and identification of lentiviral integration sites at both the cell levels and single-cell levels [102]. Additionally, Charitidis et al devised a scRNA-seq-based approach to observe both transduced and untransduced cells during the manipulation of CAR T cells using lentiviral vectors [70].

6.4. CAR-T cell manufacturing

Bai et al utilizing scRNA-seq in conjunction with CITE-seq to unveil variabilities in the phenotypic, metabolic, transcriptional, and functional characteristics of CAR-T cells both before and following antigen-specific stimulation [71]. Following stimulation with CD19, donor originated CAR-T cells demonstrated heightened activation, which was associated with extended activity of major histocompatibility complex class II genes, as compared to autologous CAR-T cells from patient [71]. Moreover, the state of differentiation and costimulatory domain utilized during the production process could also impact the role of CAR-T cells. Xhangolli and colleagues [83] applied high-throughput single-cell transcriptomic sequencing, combined single-cell cytokine secretion assays, and real time cell imaging of cancer killing activity for studying CAR-T cells following antigen-specific activation. They discovered that despite the differentiation state, both CD4+ and CD8+ CAR-T cells exhibited the capacity for cell-mediated cytotoxicity. The stimulation of CAR-engineered T cells was recognized as a central process, resulting in a diverse reaction characterized by the production of both type 1 and type 2 cytokines, as well as granulocyte-macrophage colony-stimulating factor [83]. CAR tonic signaling, emanating from both CD28z and 4-1BBz CARs, has the capacity to influence the prevalence of specific T cell subgroups within quiescent CAR-T cells. Notably, the CD8+ central memory cell population was enriched in 4-1BBz CARs, while the CD28z CAR+ T cell population exhibited an elevated percentage of CD8+ effector cells and CD4+ central memory cells [81]. Castellanos-Rueda et al created a library comprising 180 unique CAR configurations incorporated into the genomes of primary human T cells through the use of CRISPR-Cas9 technology [103]. This library of CARs provides a comprehensive approach to the evolution of CAR-T cells by utilizing both shuffling of signaling domains and functional screening through pooling and pooled functional screening. ScRNA-seq and single-cell CAR sequencing (scCAR-seq) offer high-throughput assessment capabilities, enabling the discovery of various variants exhibiting cytotoxic abilities and distinct T cell subtypes compared to conventional CARs. These advanced methodologies play a vital role in increasing the range of possible combinations of CAR signaling domains, thereby facilitating the potential therapeutic development of novel CAR-Ts [103].

6.5. Behaviors and phenotypes of individual CAR-T cells

The diversity in cellular composition within administrated CAR-T products is a pivotal factor contribute to the fluctuating effect of CAR-T cell treatment. Grasping the individual cytotoxic activity and observable property of CAR-T cells has posed a significant difficulty. LaBelle and colleagues [159] pioneered a system for quantifying time-dependent CAR-T cell associated cytotoxic activity and isolating individual cells for subsequent studies. This methodology proves incredibly significant for the thorough depiction of CAR-T cells. Considering that single-cell methods precisely captures the level of resemblance among specimens, it becomes crucial to investigate cellular heterogeneity and thoroughly define the interactions among T cell subgroups in CAR-T cell treatment. Xue and colleagues [67] employed a single-cell barcode chip microdevice to illustrate the varied profile of the

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immune response in CAR-T cells, introducing a novel system for capturing data on CAR-T products for correlational examination. A thorough assessment of pre-administration products lays the foundation for comprehending the connection between in vitro functional characteristics and treatment results.

In recent times, the research team led by Wang conducted single-cell RNA sequencing to investigate T cell phenotypes linked to distinct phases of the dual BCMA (B cell maturation antigen) and TACI (transmembrane activator and CAML interactor)-targeting CAR-T cell production [146]. They showed that tonic signaling manifests in a minority of unactuated CAR-T cells. Furthermore, the researchers discovered enduring and distinct molecular signatures induced by CAR activation in T cells, marked by heightened expression of genes induced by the MYC transcription factor, restricted exhaustion, as well as a mixture with CD4+ CD8+ effector response.

Qin and colleagues [160] engineered a variant of CAR-T cells that simultaneously express chimeric switch receptors targeting PD-L1. The research demonstrated that these CAR-T cells could facilitate differentiation into central memory-like T cells, elevate the expression of genes associated with T helper 1 (Th1) cells, and reduce the levels of Th2-associated cytokines via the CD70-CD27 axis [160]. Crucially, the combined analysis of bulk and single-cell profiles of pre-manufactured T cell subgroups uncovered specific factors influencing the prolonged endurance of CAR-T cells. They also conducted RNA-sequencing analysis performed on sorted T cell subgroups from all 71 patients, and subsequently performed paired CITE-seq and scATAC-seq on T cells from six of these patients. This investigation unveiled that chronic interferon signaling, governed by interferon regulator factor 7 (IRF7), was associated with diminished CAR-T cell perseverance across various T cell subgroups. Conversely, the transcription factor 7 (TCF7) was not only linked to the advantageous naïve T cell state but also correlated with a substantial count of effector T cells sustained in patients exhibiting prolonged CAR-T cell endurance [160]. Together, analyzing the actions and traits of pre-administration CAR-T cells at the single-cell level offers crucial observations into the underlying molecular determinants and may serve as predictive indicators of treatment outcomes.

6.6. CAR-T cell product heterogeneity

Considering chimeric antigen receptor (CAR) structures, the careful choice of the costimulatory domain emerges as a pivotal determinant. Despite the comparable clinical effectiveness exhibited by CAR-T cells incorporating CD28 or 4-1BB, distinctions exist in terms of kinetics and phenotype [9]. ScRNA-seq has revealed that they demonstrate unique transcriptional expression patterns, both in their baseline and activated states. This divergence may reflect the various transcriptional regulatory processes inherent in these CAR constructs [71,81,161].

It is widely recognized that CAR-T cells incorporating the 4-1BB co-stimulatory domain encode a memory phenotype and demonstrate prolonged persistence in opposition to CD28 CAR-T cells [81,161]. Research findings have suggested that 4-1BB CAR-T cells demonstrate a higher number of MHC II genes [71,81,161]. This characteristic is assumed to be advantageous for concurrent use with tumor vaccines, as it may augment antigen presentation and facilitate epitope spread. However, it could potentially increase the likelihood of host-graft rejection in allogeneic 'off-the-shelf' CAR-T cells. [81].

The distinction in manufacturing procedures is also pivotal and linked to the heterogeneity observed in CAR-T cell products. Earlier investigations have noted variations in the effectiveness of CAR T-cell products, depending on whether fresh or cryopreserved peripheral blood mononuclear cells (PBMCs) were chosen as the primary material [162,163]. A recent investigation of single-cell conducted by Haradhvala et al suggested that this inconsistency may be linked to a decrease in the quantity of regulatory T cells (Treg cells), as they are known to be sensitive to the freezing process [88]. Additionally, the effectiveness of viral transduction of CAR antigens also impacts the fitness and tumor killing capability of CAR-T cells. Diverse profiles of CAR-T cells, expressed through distinct CAR molecules (CAR^{High}, CAR^{Low}), have revealed that CAR^{High} T cells display heightened tonic signaling, exhaustion, and activation capabilities, both at the bulk and single-cell levels [164]. Furthermore, the analysis of gene regulatory networks disclosed that CAR^{High} T cells were under the

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regulation of exhaustion-related transcription factors, including RFX5, NR4A1, and MAF [164]. On the contrary, cells exhibiting limited or even absent expression expression of CAR demonstrate the presence of interferon-induced transmembrane (IFITM) 2 and IFITM3. These proteins serve to impede viral vector entry and could potentially serve as therapeutic opinions to address the inefficient CAR transduction [70]. In terms of vector bias affecting CAR T-cell activity, single-cell RNA sequencing indicated that the utilization of vesicular stomatitis virus (VSV)-lentiviral vectors (LV) for transduction facilitated the shift of CAR T-cells in the direction of a central memory phenotype. In contrast, the transduction with CD8-LV prompted the transition of CAR T-cells in the direction of a cytotoxic phenotype [70]. Moreover, CAR-T cells display notable diversity in cytokine production. Polyfunctional CAR-T cells, along with the Polyfunctionality Strength Index (PSI), are utilized to characterize subsets of CAR-T cells able to simultaneously secrete numerous cytokines at the single-cell level. These metrics have been employed to forecast the therapeutic results in patients [66–69].

7. Dynamic Connections between Tumor Cells, CAR-T Cells, and TME

The tumor microenvironment (TME) plays a pivotal role in the progression of cancer, tumor relapse, and is associated with the failure of cancer immunotherapy [165,166]. The functionality of CAR-T cells is intricately connected to immune cells, tumor cells, and stromal cells within the TME [167]. Moreover, single-cell analysis of both the TME and the cancer tissue helps gain new insights into antigen identification for CAR-T therapy [94,168,169]. Gottschlich et al. employed a compendium consisting of RNA sequencing database that is publicly available from more than 500,000 single cells. They recognized CD86 (cluster of differentiation 86) and CSF1R (colony-stimulating factor 1 receptor) as plausible candidates for CAR-T cell therapy for acute myeloid leukemia (AML) patients [168]. Boulch et al reported that the communication between different CAR-T cell subgroups and the surrounding environment of the tumor plays a crucial role in sustaining cytotoxic activity. Using single-cell RNA sequencing, the authors noted substantial alterations in the surrounding environment of the tumor throughout CAR-T cell therapy. [86]. CAR-T cells do not operate independently within the tumor TME but depend on communication with the tumor microenvironment mediated by cytokines for optimal activity [86]. In line with this, Romain et al [170], using high-throughput single-cell technologies, demonstrated that the existence of CD2 expression on T cells is linked to the directional migration of T cells. The engagement between CD2 on T cells and CD58 on lymphoma cells expedited the sequential elimination of tumor by CAR-T cells [170]. Additionally, CD4+ CAR-T cells exhibit greater effectiveness in activating the immune response of the host, whereas CD8+ CAR-T cells exhibit more potent capabilities in directly killing tumor cells. Both processes necessitate the inherent production of IFN-γ by CAR-T cells. Additionally, the host's ability to sense IFN-γ and produce IL-12 is crucial for CAR-T cell function. Therefore, effective CAR-T cell performance against tumors relies significantly on the communication between CAR-T cells and the tumor microenvironment (TME) [86]. Li X et al, using scRNA-seq analysis performed on CAR-T cell products and peripheral blood mononuclear cells (PBMCs) derived from plasma cell leukemia (PCL), illustrated comprehensive interactions among cytotoxic CAR-T cells, proliferating CAR-T cells, and also between CAR-T cells and endogenous T cells [148]. The authors demonstrated discrete subsets of CAR-T cells and endogenous T cells, revealing expression specific to different stages in cytotoxicity, proliferation, and intercellular signaling pathways. Additionally, they discovered that CAR-T cells undergo a gradual transitioning from a state of high proliferation to a state characterized by high cytotoxic activity along a developmental trajectory. This suggests that CAR-T cells possess the capability to establish a novel immunological milieu by recruiting endogenous T cells [148].

Indeed, CAR-T cells function not only as cytotoxic effectors but also as controllers that modify the environment surrounding the tumor and stimulate both innate and adaptive immunity. This results in synergistic antitumor immunity through the release of IFN-γ [171,172]. Alizadeh et al reported that the enhancement of an activated and less suppressive tumor microenvironment (TME) is facilitated by Interferon-gamma (IFN-γ) generated by CAR-T cells. This augmentation results in

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the simultaneous activation of host T lymphocytes and natural killer (NK) cells. In consequence, this process elevates the expression of myeloid cells that exhibit increased levels of genes associated with antigen processing and presentation, subsequently affecting the cytotoxic ability of CAR-T cells in vivo [173]. However, prolonged communication between CAR-T cells and tumor cells results in exhaustion of CAR-T cells. Good et al. reported that CD8+ CAR-T cells, upon reaching an exhausted state, undergo a transition to a phenotype resembling that of natural killer (NK) cells [95]. Surprisingly, transcriptional regulators ID3 and SOX4 exhibited specific expression within clusters of exhausted NK-like CAR-T cells. [168]. These groupings of CAR-T cells with NK-like characteristics, consisting of CD8+ CAR-T cells develop natural killer (NK) receptors through adaptability during sustained interaction to antigens [173,174]. Jiang et al using scATAC-seq discerned two types of exhausted CAR-T cells. One subset is comprised of terminal exhausted CAR-T cells characterized by heightened motifs of BATF, IRF4, and PRDM1. The second subset comprises subset consists of intermediate exhausted CAR-T cells exhibiting heightened motifs of transcription factors, including JUN, FOS, NFKB1, and BACH2. In conclusion, the significance of IFN-γ is crucial for the dynamic communications between cancer cells and CAR-T cells within the TME [60].

8. Further Strategies for Future CAR-T Cell Therapy

8.1. Combination therapy

Integrating CAR-T cell therapy with additional treatments, such as cytotoxic agents [175], radiotherapy [176], hematopoietic stem cell transplantation [177], oncolytic virus therapy [178], small molecules [179], and alternative immunotherapeutic approaches have emerged as a potentially effective method for further improve the efficacy of CAR-T cell therapy [180]. Srivastava et al [175] utilized single-cell RNA sequencing to recognize the immune cells within mouse lung tumors subjected to Oxaliplatin (Ox)/ Cyclophosphamide (Cy) treatment along with CAR-T cell therapy. Their analysis revealed the activation of T-cell- attracting chemokine genes, including CXCL16 and CCL5, in diverse cell types including dendritic cells and macrophages within the tumor microenvironment (TME) [170]. Furthermore, the IFN-γ produced by CAR-T cells that infiltrating within the tumor led to the recruitment and activation of tumor macrophages expressing iNOS. This process further upregulated activity of the ligands of CXCR3 including CXCL10 and CXCL9. The communication between CAR-T cells and tumor macrophages within the tumor microenvironment abundant in chemokines facilitated a positive feedback loop [175]. In addition, Sen and colleagues employed scRNA-seq technology for investigate the effect of connecting CAR-T cells with stimulator of interferon genes (STING) DMXAA/cGAMP in breast cancer mouse model. The findings indicated a decrease in myeloid-derived suppressive cells, an augmentation of pro-inflammatory myeloid cells, and an increase in the production of chemokines that promoted the mobilization and sustained presence of CAR-T cells within the tumor microenvironment [181]. Furthermore, Lelliott and colleagues applied scRNA-seq combined with CITE-seq in an ovarian cancer mouse model demonstrating that the combine with CDK4/6 inhibitors might enhance the tumor killing effects CAR-T cells, thereby improving the efficacy and lasting impact on tumor killing effects [182].

8.2. Engineered CAR-T cells

Single-cell sequencing methods play a crucial role in the design of novel engineered CAR-T cells [183–185]. Wang et al., through the application of genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) screening, discovered that transducin-like enhancer of split 4 (TLE4) and Ikaros family zinc finger protein 2 (IKZF2) are potentially associated with exhaustion and the functional inhibition of T cells in glioblastoma multiforme (GBM). ScRNA-seq of CAR-T cells with double knockdown of IKZF2 and TLE4 revealed augmented cytotoxicity and an activated immune response transcriptional patterns concurrently inhibiting exhaustion associated signatures [186]. Zhang et al. and Mueller et al., utilizing single-cell RNA sequencing (scRNA-seq), discovered that CAR T-cells incorporating the CAR gene into the T-cell receptor α constant (TRAC) genetic location and the PD1 genetic location exhibited a phenotype resembling memory cells and fewer

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transcriptional patterns associated with T cell exhaustion [100,187]. The precise incorporation of CAR genes using CRISPR might offer advantages over the conventional haphazard insertion that achieved through transduction by viral vector. Additionally, Johnson et al. introduced an innovative CAR-T cell (RN7SL1 CAR-T cell) capable of releasing non-coding RNA RN7SL1 in a format of extracellular vesicles (EVs), hat are absorbed by immune cells. The authors performed single-cell RNA sequencing (scRNA-seq) on tumorous tissues obtained from rodent models that received RN7SL1 CAR-T cells and discovered a decrease in suppressive myeloid cell subsets, an augmentation of inflammatory dendritic cells bearing the expression of genes linked to costimulatory signals, and the activated enhancement of endogenous CD8+ T cells with effector-memory properties [188]. Brog et al developed a novel CAR-T cell (Super2+IL-33+ CAR-T cells) exhibiting increased expression of superkine IL-2 (Super2) and IL-33. They utilized single-cell RNA sequencing and observed a transformation of macrophages displaying M2-like polarization into macrophages displaying M1-like polarization with high expression of antigen presentation genes in the tumor microenvironment (TME). Additionally, there was an expansion in the proportion between CD8+ cytotoxic T cells and regulatory T cells with immunosuppressive properties in this animal models [189].

8.3. Locoregional delivery of CAR-T cells

For solid tumors, the application of locoregional and intratumoral CAR-T cell delivery is also considered [28,190]. Both preclinical investigations and preliminary medical trials have shown positive outcomes with the intraventricular and administering via intrathecal route of CAR-T cells [22,190–195]. Wang et al., utilizing single-cell RNA sequencing (scRNA-seq) and cytometry by time-of-flight (CyTOF), respectively, illustrated that CAR-T cells, when exhibited to cerebrospinal fluid (CSF), have the potential to prompt the development of a memory-like cellular characteristics through alterations in metabolic processes. This can result in heightened tumor killing ability [193]. This type of metabolic reprogramming is driven by an expansion in the expression levels of markers associated with cellular activation and signatures associated with CAR-T cell trafficking and CAR-Tell homing, potentially enhancing the migration of CAR-T cells toward the central nervous system.[101].

8.4. Perspectives for CAR target selection

Bosse et al. conducted a comparative analysis of bulk RNA-seq findings in neuroblastomas and healthy cells, pinpointing Glypican 2 (GPC2) as a promising candidate antigen for CAR-T cell therapy [196].

8.5. Challenges

Single-cell sequencing has its limitations and challenges. First, incurs high sequencing costs, making it only suitable for innovative discoveries but impractical for extensive validation on a large scale. Second, technologically, single-cell sequencing faces several limitations, primarily being applicable mainly to fresh tissue samples [197]. Additionally, sample preparation for certain large tissues, such as bone, poses difficulties. Third, the spatial information of single cells within the sample is frequently forfeited during processing. Consequently, researchers should consider incorporating spatial transcriptomics into their studies [198,199]. Lastly, while the most widely employed scRNA-seq technology can reveal novel cellular categories and their respective cellular conditions within diverse tissue compositions, distinguishing immune cells exhibiting comparable transcriptomes but fulfilling distinct activities presents a challenge. In conclusion, incorporating diverse forms of information is indispensable and there is a growing requirement for single-cell multi-omics technology.

9. Conclusions

CAR-T cell immunotherapy has significantly improved the treatment of hematological malignancies over the past decade. Despite these advancements, challenges such as intrinsic

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resistance to CAR-T cell therapy, tumor relapse, and serious adverse events persist. Moreover, the effectiveness of CAR-T cell therapy in treating solid tumors remains unsatisfactory. The future advancements in CAR-T therapy will rely on our increasing comprehension of the behavior of engineered T cells, both in preclinical and clinical contexts.

For precise identification of individual CAR-T cell phenotypes and their dynamics during treatment, single-cell sequencing technologies have emerged as essential tools for research. The analysis of molecular characteristics of individual CAR-T cells aids in screening for the exploration of molecular features in individual CAR-T cells contributes to the identification of optimal antigen receptor design. This aids in mitigating potentially harmful off-target effects, providing insights for gene-based T cell modifications, refining conditions for CAR-T manufacturing, understanding the attributes of CAR-T cell products, and unraveling the connections between key drivers of cancer phenotypes and the treatment outcomes associated with CAR-T therapy [200]. Single-cell multi-omics technologies play a crucial role in the execution of research, covering a range of disciplines such as single-cell genomics, transcriptomics, proteomics, epigenomics, and spatial transcriptomics. These advanced methodologies facilitate the discovery of targets, validate experimental results collaboratively, and explore molecules and pathways both upstream and downstream.

In summary, single-cell sequencing technologies have illuminated the path for advancing CAR-T cell therapy in various aspect, spanning stages preceding CAR-T therapy, modification of CAR-T cells, monitoring the progress of CAR-T therapy, and holding the promise to facilitate the progress in cancer treatment. By exploring these aspects, we can gain a deeper understanding of CAR-T therapy, ultimately contributing to improved clinical outcomes and broader therapeutic applications.

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