Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) *2022 23(10):793-811*
www.jzus.zju.edu.cn; www.springer.com/journal/11585
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Review

https://doi.org/10.1631/jzus.B2200256

Relapse after CAR-T cell therapy in B-cell malignancies: challenges and future approaches

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Abstract: Chimeric antigen receptor-T (CAR-T) cell therapy, as a novel cellular immunotherapy, has dramatically reshaped the landscape of cancer treatment, especially in hematological malignancies. However, relapse is still one of the most troublesome obstacles to achieving broad clinical application. The intrinsic factors and superior adaptability of tumor cells mark a fundamental aspect of relapse. The unique biological function of CAR-T cells governed by their special CAR construction also affects treatment efficacy. Moreover, complex cross-interactions among CAR-T cells, tumor cells, and the tumor microenvironment (TME) profoundly influence clinical outcomes concerning CAR-T cell function and persistence. Therefore, in this review, based on the most recent discoveries, we focus on the challenges of relapse after CAR-T cell therapy in B-cell malignancies from the perspective of tumor cells, CAR-T cells, and the TME. We also discuss the corresponding basic and clinical approaches that may overcome the problem in the future. We aim to provide a comprehensive understanding for scientists and physicians that will help improve research and clinical practice.

Key words: Chimeric antigen receptor-T (CAR-T); B-cell malignancies; Mechanisms of relapse; Strategy

1 Introduction

In recent decades, cancer treatment strategies have progressed through surgical resection, radiotherapy, chemotherapy, and targeted therapy, each of which has marked a milestone in treatment history. Chimeric antigen receptor-T (CAR-T) cell therapy, the adoptive transfer of T cells redirected to target tumor cells, has achieved remarkable outcomes in the treatment of B-cell malignancies.

For the first time, complete remission (CR) has been achieved and maintained in a girl with B-cell acute lymphoblastic leukemia (B-ALL) who received cluster of differentiation 19 (CD19) CAR-T cell therapy a decade ago (Grupp et al., 2013). Based on their outstanding clinical efficacy, a total of six CAR-T cell-based products have been approved for the treatment of B-cell malignancies, including tisagenlecleucel (tisa-cel) and axicabtagene ciloleucel (axi-cel), with an overall CR rate of 80%–90% in relapsed/refractory (R/R) B-ALL patients and 50% in R/R B-cell lymphoma patients (Larson and Maus, 2021). Ever-increasing clinical trials are being conducted and optimized treatment strategies measured.

Nevertheless, relapse remains the major obstacle to be addressed (Miao et al., 2021). Clinical evidence indicates that a large proportion of patients with B-cell malignancies suffer from relapse after CAR-T cell therapy, which brings even greater efforts to achieve remission again. Recent discoveries have uncovered mysterious mechanisms underlying relapse after CAR-T cell therapy. Tumor cells, CAR-T cells, and the tumor microenvironment (TME) integrally contribute to the course of relapse, with dismal outcomes. Relevant optimization and breakthroughs to promote antigen recognition, enhance CAR-T cell function, and develop

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Received Apr. 30, 2022; Revision accepted Aug. 10, 2022;
Crosschecked Sept. 19, 2022

combinational treatment strategies provide insights for better clinical efficacy.

In this review, based on the most recent discoveries, we focus on the challenges of relapse after CAR-T cell therapy in B-cell malignancies by discussing the underlying mechanisms from the perspective of tumor cells, CAR-T cells, and the TME, and propose promising basic and clinical approaches that may overcome the obstacle in the future.

2 Clinical relevance

Although CD19 CAR-T cell therapy has achieved amazing overall CR rates in patients with R/R B-ALL and B-cell lymphoma (Larson and Maus, 2021), 40%–60% of CR patients eventually experience relapse. At the 2021 American Society of Hematology (ASH) annual meeting, real-world outcomes of tisa-cel treatment of pediatric and young adult patients with R/R B-ALL showed that, after a median follow-up of 21.5 months (11.9–37.2 months), the median event-free survival (EFS) was 14.0 months (95% confidence interval (CI), 9.8–24.8) and the median relapse-free survival (RFS) was 23.9 months (13.0–not estimated (NE)). The 12-month EFS rate was 54.3% and the RFS rate was 62.3% (John et al., 2021). In axi-cel-treated patients with R/R large B-cell lymphoma (LBCL), a 12-month EFS rate of 43% (95% CI, 33%–52%) and a 24-month EFS rate of 38% (95% CI, 28%–47%) were reported (Jacobson et al., 2021).

The relapse after CAR-T cell therapy in B-cell malignancies is defined as the recurrence of tumor cells after achieving CR by target-specific CAR-T cell infusion, such as CD19 CAR-T. Generally, the relapse can be categorized as the bone marrow relapse, the extramedullary relapse (such as in the central nervous system (CNS) or testis), or a combined relapse according to anatomical sites (Tallen et al., 2010). More specifically, the relapse can also be classified as antigen-positive or antigen-negative based on the expression profile of initially targeted antigens of tumor cells. The prognosis in each type of relapse is historically diverse. The pattern of relapse is influenced by various factors, including the nature of tumor cell itself, the properties of the CAR-T cells, and interaction with the specific TME (Gaudichon et al., 2019).

3 Challenges of relapse after CAR-T cell therapy in B-cell malignancies

Tumor cells, CAR-T cells, and the TME can each greatly affect the long-term clinical outcomes after CAR-T cell therapy (Fig. 1).

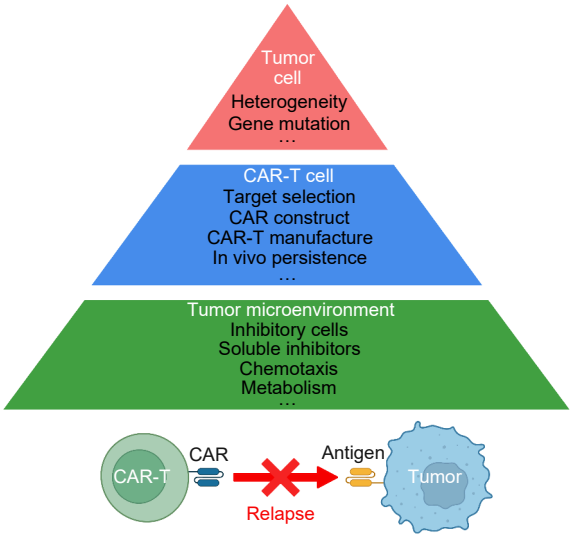

Fig. 1 Landscape behind relapse after CAR-T cell therapy. The tumor cells, CAR-T cells, and the tumor microenvironment can each affect relapse after CAR-T cell therapy. CAR-T: chimeric antigen receptor-T. Created with BioRender.com.

3.1 Tumor cell

The conceptualization of hallmarks of cancer depicts the complexity of tumor cells, and this also applies to B-cell malignancies (Hanahan, 2022). Tumor cells with native heterogeneity and superior adaptability commonly resist eradication by CAR-T cells, leading to eventual relapse, as seen in both antigen-positive relapse and antigen-negative relapse (Fig. 2).

3.1.1 Heterogeneity

Tumor heterogeneity is widely recognized. The generation of intra-tumoral phenotypic diversity lays a foundation for the proliferative expansion and selective survival of the fittest tumor cell, which promotes clonal evolution and malignant progression (Mcgranahan and Swanton, 2017). The heterogeneity can be partly achieved by stochastic mutations in individual tumor cells that generate the most adaptative subclones in response to the alteration of the TME or specific cancer therapies. In addition, a selective differentiation

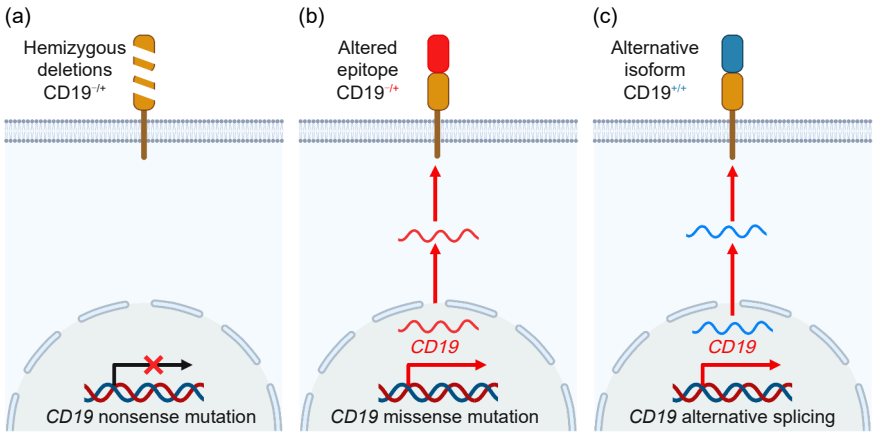

Fig. 2 Mechanisms of CD19 antigen escape during relapse after CAR-T cell therapy. (a) Nonsense mutation with loss-of-heterozygosity CD19 expression; (b) Missense mutation produces CD19 with altered epitope; (c) Alternative splicing produces alternative CD19 isoform. CD19: cluster of differentiation 19. Created with BioRender.com.

hierarchy inherited from a cancer stem cell (CSC) with indefinite self-renewal ability also contributes to the distinct cell types (Prasetyanti and Medema, 2017). The hierarchy can be plastic and be reversed by terminally differentiated cells acquiring a mutation and dedifferentiating into CSCs. Phenotypic heterogeneity is well reflected by the pattern of expression of antigens on tumor cells. Indeed, in B-cell malignancies, heterogenous antigen density of CD19, CD20, or CD22 has been be detected from the same patient-derived tumor cells (Kailayangiri et al., 2020). Those tumor cells with low or negative antigen expression levels before CAR-T cell infusion may have a chance to escape and undergo secondary clonal expansion. Surprisingly, sequential loss of tumor surface antigens in lymphoma was reported even after multiple infusions of CAR-T cells with different targets, which might have been related to the pre-existence of double-negative subclones caused by mutation-mediated deficiency of DNA repair (Yu et al., 2017; Shalabi et al., 2018).

3.1.2 Gene mutation

To some extent, CAR-T treatment is also an artificial selective pressure on tumor cells that magnifies the effects of adaptive capacity. Under CAR-T cell cytotoxicity, a small proportion of tumor cells may undergo secondary gene mutations that lead to antigenic epitope transformation, disturbance of antigen translocation onto the cell membrane, or antigen endocytosis, which helps tumor cells to avoid recognition by CAR-T cells (Figs. 2a and 2b). For the first time, scientists have identified acquired mutations in exons 2 and 4 of the *CD19* gene accounting for CD19 protein loss, with hemizygous deletions in four patients resistant to CD19-directed CAR-T cell therapy (CART-19) (Sotillo et al., 2015). Also, genetic mutations in *CD19* exons 2 to 5 were found to induce loss-of-heterozygosity that produced a truncated protein with a nonfunctional or absent transmembrane domain. This consequently induced an irreversible loss of surface antigen and failure of CAR-T treatment in 12 patients with CD19-negative relapse (Orlando et al., 2018). Furthermore, the *CD19* exon 2 was confirmed to be essential for the integrity of the *CD19* transcript, and the frameshift mutation in exon 2 exhibited robust retention of intron 2 with a nonsense codon 40 amino acids downstream of the exon 2–intron 2 junction. This was consistent with findings in the same sample with no evidence of exon 2 skipping (Asnani et al., 2020). Moreover, point mutations in *CD19* exon 3 can affect the epitope recognized by CD19 FMC63 CAR-T cells and induce CD19-positive relapse in patients with high-grade B-cell lymphoma. However, the same mutated tumor cells were able to be eradicated by the CD19 21D4 CAR-T cells with an alternative recognition epitope (Zhang Z et al., 2020). Homozygous B-cell maturation antigen (*BCMA*) gene deletion was also recognized theoretically as a risk factor in patients with multiple myeloma (MM) relapsing after anti-BCMA CAR-T cell therapy (da Vià et al., 2021). In this regard, a novel method involving detecting circulating tumor DNA (ctDNA) to simultaneously track multiple somatic mutations may facilitate our

understanding of the distinct biological subtypes and genomic evolution patterns in relapsed tumor cells (Scherer et al., 2016). Moreover, ctDNA measurement can be used as a disease-monitoring tool following CAR-T cell treatment, as ctDNA was detectable at, or before, radiographic relapse in 94% of patients with disease progression (Frank et al., 2021).

3.1.3 Alternative splicing

Alternative splicing is another common intrinsic factor which does not affect genome integrity during antigen-negative relapse (Fig. 2c). Although genetic mutations in the exons of the *CD19* gene influenced its protein expression and recognition by specific CAR-T cells, alternative splicing also contributed in nearly half of the patients with CD19 antigen-negative relapse from the same group (Sotillo et al., 2015). Alternatively spliced *CD19* messenger RNA (mRNA) species were found to produce a compromised CD19 epitope by splicing factor serine/arginine-rich splicing factor 3 (SRSF3)-mediated exon 2 skipping, which also generated a truncated CD19 variant that failed to trigger cytotoxicity by CD19 CAR-T cells (Sotillo et al., 2015). Those isoforms pre-existed at diagnosis, again reflecting tumor heterogeneity (Fischer et al., 2017).

3.1.4 Lineage switch

During the hematopoiesis, diverse cell types are generated from hematopoietic stem cells (HSCs) through the process of fate determination. Under intrinsic factors and extrinsic signals, the lineage of cells is gradually restricted by a subsequent step of highly regulated differentiation, which is normally irreversible. However, leukemia cells can hijack this regulatory system and take advantage of lineage plasticity to avoid lineage-specific antigen targeting by CAR-T cells, resulting in resistance and relapse (Thankamony et al., 2021). Lineage switch is defined as a process by which leukemia cells convert into different lineages at relapse compared to the initial diagnosis, with a transformation of cell morphology and immune types. This is most commonly seen in pre-B leukemia (Thoms et al., 2021). Lineage-specific transcription factors, of which the most well-known is the mixed lineage leukemia (MLL) transcription factor, along with microenvironment signals participate in the lineage conversion of leukemia (Hu et al., 2017). The acquisition of the CD19-negative myeloid phenotype was frequently seen in B-ALL patients with the *MLL* gene rearrangement (Gardner et al., 2016; Jacoby et al., 2016). In addition, a patient with R/R chronic lymphocytic leukemia (CLL) relapsed after CD19 CAR-T treatment, with a clonally related plasmablastic lymphoma that evaded lineage-specific targeting by means of multiple independent molecular genetic evolution events (Evans et al., 2015).

3.1.5 Resistance to cell death

Resistance to cell death as a basic hallmark of cancer protects tumor cells from targeted eradication by the endogenous immune system. CAR-T cells exert cytotoxicity on tumor cells mainly in a death receptor-dependent manner. With the help of the innovative clustered regularly interspaced short palindromic repeats (CRISPR)-based genome-wide loss-of-function screening, scientists have discovered inherent resistance to CAR-T cell cytotoxicity by impaired cell death signaling, even with positive antigen expression (Singh et al., 2020). Deficiency in apoptosis of tumor cells by loss of NADPH oxidase activator (NOXA), a B-cell lymphoma 2 (BCL2) family protein, was also reported in resistance to CAR-T cell therapy. Pharmacological augmentation of NOXA expression by histone deacetylase (HDAC) inhibitors dramatically sensitized tumor cells to CAR-T cell-mediated clearance (Yan X et al., 2022).

3.2 CAR-T cells

The CAR-T cell is a genetically engineered T cell coupled with a synthetic antigen-specific receptor that recognizes cell surface antigens on tumor cells in a major histocompatibility complex (MHC)-independent manner. Tumor relapse is closely associated with the compromised function and persistence of CAR-T cells in vivo. Long-term follow-up revealed higher peak blood levels of CAR-T cells to be positively correlated with a longer duration of response (DOR) (Cappell et al., 2020). Surprisingly, two patients with CLL sustained CR with CAR-T cells remaining detectable for a decade after infusion. This was attributed to a persistent highly-activated $CD4^+$ CAR-T cell population with cytotoxic characteristics. This suggests that the long-term potential and clonal stability of CAR-T cells play a vital role in relapse-free durable remission (Melenhorst et al., 2022). However, by combining single-cell RNA sequencing (scRNA-seq) and cellular

indexing of transcriptomes and epitopes by sequencing (CITE-seq), a deficiency of T helper 2 function was predictable and associated with CD19-positive relapse in CD19 CAR-T cell products from 12 R/R ALL patients (Bai et al., 2022). Therefore, CAR-T cell functional characteristics determined by the CAR design and manufacturing process can significantly affect the risk of relapse and long-term outcome.

3.2.1 CAR design

The CAR structure consists of an antigen-recognition single-chain variable fragment (scFv), a linker molecule, a transmembrane domain, and a costimulatory domain. The components integrated in the CAR design have fundamental effects on CAR-T cell function. Further refinement and manipulation of biophysical parameters in CAR design would enhance the quality of signaling and improve the precise tuning of CAR-T cell activity.

3.2.1.1 Antigen target design

Appropriate selection of the antigen target is crucial to the safety and efficacy of CAR-T cell therapy. The tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) are limited in B-cell malignancies. Widely used suitable targets for B-cell leukemia and lymphoma currently include only CD19, CD20, and CD22. For patients with antigen-negative relapse after several rounds of CAR-T treatment, more rigorous therapies are needed. Combinatorial autocrine-based selection to rapidly identify specific ligands for B-cell receptors on the surface of tumor cell may realize personalized treatment and prevent relapse (Stepanov et al., 2018). The powerful high-throughput genome-wide CRISPR-screening technology may facilitate the discovery of more suitable and effective antigen targets for CAR-T cell therapy (Ancos-Pintado et al., 2022).

3.2.1.2 Recognition pattern

CAR is a receptor empirically designed to mimic and simplify natural T cell receptor (TCR) function. However, CAR may also have some shortcomings compared to traditional TCR in some respects. TCR recognizes antigens in an MHC-restricted mechanism, but with at least 100-fold more sensitivity to an antigen than CAR (Salter et al., 2021). Moreover, natural TCR bears intact signaling domains, whereas CAR typically incorporates a single TCRζ in the signaling element (Majzner and Mackall, 2018). Therefore, the density of target antigens may affect the transduction strength of activation signaling in CAR-T cells. The low density of either antigen on tumor cells or CAR on CAR-T cells significantly impairs the anti-tumor efficacy, suggesting that the stoichiometric relationship affects the functionality of CAR (Walker et al., 2017). Up-regulating CD22 expression rather than increasing CAR affinity contributes to improved CAR-T cell functionality and persistence in B-cell malignancies (Ramakrishna et al., 2019; Yang et al., 2021). In addition, the divergent antigen-binding affinity between CAR and TCR contributes to the variant formation and quality of immune synapse during antigen recognition (Harris et al., 2018). The CAR-T cell immune synapse in lack of lymphocyte function-associated antigen-1 (LFA-1) adhesion rings leads to more rapid induction and faster detachment from the target cell, which may explain the more intensive cytotoxicity exerted by CAR-T cells (Davenport et al., 2018). In conclusion, CAR-T cells exhibit more rapid and vigorous cytotoxicity by means of MHC-unrestricted recognition, but can target only cell surface antigens, and their functionality is affected by the surface density on both sides. On the other hand, TCR-T cells with MHC-associated specificity and sensitivity to tumor antigens are safer and less influenced by antigen modulation or variation, but it is hard to develop a universal product. Which type of receptor is superior in the control of tumor relapse is currently unknown.

3.2.1.3 Signaling transduction domains

The costimulatory domain in the CAR construct is another fundamental element that dominates transduction of activation signaling and greatly influences CAR-T cell function. CD28 or 4-1BB costimulatory domains are most widely used in CAR-T cell therapy (Ying et al., 2019). CAR-T cells with a CD28 domain exert a swifter and stronger cytotoxic response, but with less persistence due to apparent exhaustion (Savoldo et al., 2011). In contrast, CAR-T cells with a 4-1BB domain exhibit moderate functionality while maintaining long-term sustainability (Long et al., 2015; Guedan et al., 2018). The non-signaling extracellular spacer domain, also known as the linker molecule, is also important for CAR-T cell anti-tumor activity in vivo (Hudecek et al., 2015; Alabanza et al., 2017). The length and composition of immunoglobulin G (IgG)-derived extracellular spacer domains may influence the interaction between the Fc domain within the

spacer and the Fc receptor-bearing host myeloid cells, which brings activation-induced T-cell death (AICD) and CAR-T cell clearance. By modifying distinct regions in the CH2 domain to abrogate binding to Fc receptors, in vivo persistence and anti-tumor effects of CAR-T-cells can be improved. Finally, the immunogenicity of scFv in CAR can cause host immunological rejection by anti-CAR antibody production, leading to the eventual failure of CAR-T cell therapy (Maus et al., 2013).

3.2.2 CAR-T cell manufacture

A favorable clinical response to CAR-T cell therapy requires both well-functioning autologous or donor-derived T cells and a manufacturing process with quality control. Patients with B-cell malignancies already have a compromised or dysregulated immune system. An impaired immune system can result in a decrease in immunological diversity, and an increase of senescent T cells may promote cancer progression. Most patients receive multiple lines of chemotherapy before CAR-T cell apheresis. These factors may profoundly affect autologous T cells, leading to worse CAR-T cell function.

Moreover, CAR-T cells are usually cultured in a medium with complete nutrition and additional cytokines and growth factors to improve ex vivo expansion before reinfusing into patients. However, such culture systems are so specialized that CAR-T cells may not be well-adapted to the in vivo hostile microenvironment shaped by tumor cells. Tumor cells compete to uptake glucose, amino acids, glutamine, fatty acids, and other growth factors, thereby interfering the normal energy production and protein synthesis in CAR-T cells. In the meantime, the metabolic production of lactase and reactive oxygen species would dampen the function and persistence of CAR-T cells (Xia et al., 2021). Therefore, the lack of metabolic rewiring in CAR-T cells during ex vivo culture compromises their capacity to meet their energetic and anabolic demands when countering the TME, which fails to exert durable anti-tumor efficacy in patients.

Even worse, the contamination of leukemia cells during the manufacturing process of CAR-T cells may result in accidental transfection of CAR genes into leukemia cells, which increases the risk of tumor resistance and eventual relapse (Ruella et al., 2018). In addition, clonal expansion of CAR-T cells due to accidental lentivector integration in the Casitas B-lineage lymphoma (*CBL*) gene has raised the risk of carcinogenic insertional mutagenesis (Shah et al., 2019). Therefore, a safer and more efficient process for manufacturing CAR-T cells is urgently needed to ensure better clinical efficacy.

3.2.3 CAR-T cell functional defects

CAR-T cells have unique biological functions compared to conventional T cells due to the presence of the CAR. Although CAR-T cells overcome the restriction of MHC-dependent antigen recognition, most biological behaviors related to CAR signaling remain unknown. It is believed that CAR-T cell exhaustion due to antigen stimulation or self-clustering tonic signaling contributes most to the impaired function and shortened persistence of CAR-T cells in vivo, and epigenetic reprogramming actively reshapes its differentiation fate.

3.2.3.1 CAR-T cell exhaustion

Constitutive ligand-dependent CAR activation induces an exhaustive phenotype in CAR-T cells, manifested as decreased proliferation, impaired cytotoxicity, shortened survival, terminal differentiation, and reduced persistence. In parallel, ligand-independent self-clustering of CAR scFv triggers CAR CD3-z phosphorylation (Long et al., 2015) and downstream tonic signaling that accelerates CAR-T cell exhaustion, and impairs anti-tumor effects in the absence of antigen exposure (Calderon et al., 2020). This effect is more obvious in CAR-T cells with a high density of CARs on the surface (Walker et al., 2017). Recently, an extraordinary behavior was discovered: CAR-T cells transferred the target antigen from tumor cells to themselves by trogocytosis, leading to fratricide CAR-T cell killing and exhaustion, and facilitating immune escape through reversible antigen loss on tumor cells and inducible inhibitory receptors on CAR-T cells (Hamieh et al., 2019).

Much effort has been devoted to understanding the molecular characteristics associated with CAR-T cell exhaustion (Ajina and Maher, 2018). The transcription factor, nuclear factor of activated T cell (NFAT), is important for CAR-T cell activation downstream of the CD28 costimulatory domain (Macian, 2005). $CD8^+$ CAR-T cells exhibited high expression of inhibitory receptors programmed cell death protein-1 (PD-1) and T cell immunoglobulin and mucin domain-containing

protein 3 (TIM3) associated with secondary NFAT-initiated activation of the nuclear receptor transcription factor nuclear receptor 4 group A (NR4A) family, similar to endogenous tumor-infiltrating $CD8^+$ lymphocytes (Chen J et al., 2019). Further investigation discovered that epigenetic thymocyte selection-associated high mobility group box (TOX) transcription factors downstream of NFAT cooperatively contributed to T cell exhaustion with NR4A (Khan et al., 2019; Seo et al., 2019).

3.2.3.2 Epigenetic reprogramming

Studies have shown that epigenetic reprogramming is involved in regulating the transcription and expression of T cell depletion and memory-related genes (Pauken et al., 2016; Sen et al., 2016). Under continuous antigen stimulation, T cells will gradually start DNA methylation, reducing their memory potential and killing ability (Ghoneim et al., 2017). To investigate epigenetic effects on CAR-T cell depletion and function, researchers performed longitudinal genome-wide DNA methylation analysis on CD19 CAR-T cells in patients with B-ALL. CAR-T cells were found to undergo repression of genes associated with memory potential and exhaustion DNA methylation programming, including TOX (Zebley et al., 2021). As the treatment went on, CAR-T cells showed a decreased expansion ability, increased methylation levels of memory-related genes, and gradually demethylated exhaustion-related genes, leading to the overall transition to CAR-T cell depletion. In addition, transient inhibition of CAR signaling reversed CAR-T cell depletion through epigenetic remodeling (Weber et al., 2021). These studies indicated a complex regulatory network involved in CAR-T cell functional defects at the both the genetic and epigenetic levels.

3.3 Tumor microenvironment

Setting aside the interplay between CAR-T cells and tumor cells alone, a complex interactive network in the TME greatly affects the behavior of both types of cells. Various inhibitory immune or stromal cells within the TME antagonize CAR-T cell cytotoxicity, but support the senescence and survival of tumor cells. Aberrant chemotaxis attracts tumor cell migration and retention, but blocks CAR-T cell trafficking. A hypoxic and nutrition-deprived metabolic environment favors tumor cell behavior, while threatening CAR-T cell function and survival. Accumulating evidence suggests that the immunosuppressive milieu of the TME contributes enormously to relapse after CAR-T cell therapy (Gaudichon et al., 2019; Yan et al., 2019).

3.3.1 Inhibitory cellular components

The heterogenous population of inhibitory cells in the TME affecting tumor relapse consists mainly of tumor-associated macrophages (TAMs), mesenchymal stem cells (MSCs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) (Sterner and Sterner, 2021). These inhibitory cells may share several similar mechanisms of immunosuppression of CAR-T cells.

Macrophages can be functionally classified into subtypes M1 and M2, depending on their localization and surrounding environment, with the ability to perform mutual conversion (Chen C et al., 2020). In brief, M1 macrophage is involved in immune defense against infection and cancer through phagocytosis and secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6. In contrast, M2 macrophage takes part in tissue repair and immune tolerance (Kadomoto et al., 2021). Increased TAM infiltration was found positively related to a short remission period and high relapse risk, with CD68 expression indicating an M2 phenotype (Yan et al., 2019). Tumor interferon (IFN) signaling is correlated with macrophage content in the TME while exerting suppression on CAR-T cells by up-regulating programmed cell death-ligand 1 (PD-L1) and MHC-II on the tumor cell surface (Jain et al., 2021). Therefore, TAMs commonly associated with the M2 subtype contribute to tumor relapse after CAR-T cell therapy.

MSCs are multipotent stem cells found mainly in the bone marrow where they serve an immunomodulation function (Li and Hua, 2017). MSCs can inhibit T cell proliferation, induce apoptosis, and promote polarization into Tregs under stimulation by pro-inflammatory cytokines (Regmi et al., 2019). MSCs also influence the CAR-T cell subpopulation and cytokine release through the galectin family, IL-10, and transforming growth factor-β (TGF-β) (Guerrouahen et al., 2019). However, a recent study reported that bone marrow MSCs from pediatric patients with B-ALL highly suppressed T-cell responses, but did not compromise CD19 CAR-T cell activity (Zanetti et al., 2020). Further investigations are needed to determine the precise effect of MSCs on CAR-T cell efficacy.

MDSCs consist of heterogeneous myeloid progenitors, including immature macrophages, granulocytes, and dendritic cells (DCs). MDSCs are known to suppress cytotoxic T lymphocyte (CTL) proliferation and function through up-regulated expression of the inhibitory molecule PD-L1 (Wang et al., 2017). They are accumulated in tumors in response to various cytokines and growth factors (Lindo et al., 2021). Intriguingly, MDSCs may originate and remain in the same place where leukemia and lymphoma reside, and exert antigen-independent suppression on T cells (Kumar et al., 2016). Whether MDSCs contribute to tumor relapse after CAR-T cell therapy needs further validation.

Tregs are $CD4^+CD25^+$ T cells suppressing autoreactive T cells to prevent an excessive immune response and maintain homeostasis (Sakaguchi et al., 2006; Langier et al., 2010). This function relies on the master transcription factor forkhead box P3 (FOXP3). TGF-β is also a critical factor for the generation of Tregs (Yang et al., 2008). Increased frequencies of Tregs in circulating peripheral blood were observed in leukemia patients, consistent with their reduced remission rates following chemotherapy. Therefore, in regard to the prominent role of Tregs in immunosuppression and tumor escape, routine lymphodepletion regimens, including fludarabine and cyclophosphamide, are given before CAR-T cell infusion to eliminate Tregs and other inhibitory cells. The exact role of Tregs in affecting the long-term outcomes after CAR-T cell therapy is still unclear.

3.3.2 Chemotaxis

Despite direct immunosuppression of CAR-T cell function, the same inhibitory cells along with other stromal cells also create a conditional chemotaxis that favors tumor cell migration, retention, and resistance, while compromising CAR-T cell trafficking and approach. This aspect is most apparent in leukemia relapse in the bone marrow, testis, and CNS where the CXC chemokine ligand 12 (CXCL12)/CXC chemokine receptor 4 (CXCR4) axis contributes most.

CXCL12 was first discovered as an important chemokine in the bone marrow for HSC quiescence, retention, and homeostasis (Pinho and Frenette, 2019). Later, the CXCL12/CXCR4 axis was found to be involved in the pathogenesis of B-cell leukemia, promoting leukemia cell homing, survival, and proliferation in the bone marrow (Colmone et al., 2008; Peled et al., 2018). A similar chemotaxis also plays an important role in the CNS infiltration of leukemia cells (Williams et al., 2016). CAR-T cells lacking CXCR4 exhibit poor migration and retention in the bone marrow, let alone trafficking into the CNS with its physical barrier, the blood–brain barrier (BBB). However, recent clinical trials proved the safety and efficacy of CD19 CAR-T cell therapy with a high response rate in patients with CNS leukemia and lymphoma (Siddiqi et al., 2021; Tan et al., 2021; Qi et al., 2022). This suggests that there may be other possible mechanisms for the CNS infiltration of CAR-T cells.

3.3.3 Metabolism

Hypoxic and nutrition-deprived metabolic environments, especially in the bone marrow and CNS, compromise CAR-T cell function and contribute to tumor resistance and relapse (Wilson and Hay, 2011). Under hypoxia, tumor cells adapt by progressing transcriptional reprogramming through the master regulator hypoxia-inducible factor-1α (HIF-1α) (Jing et al., 2019). However, oxygen is essential for CAR-T cell proliferation and cytotoxicity through oxidative phosphorylation in mitochondria (van Bruggen et al., 2019). Apart from the oxygen deficiency in the TME, hematological tumor cells catabolize the semi-essential amino acid arginine to drive cell proliferation. CAR-T cells are susceptible to the low arginine microenvironment due to the low expression of the arginine resynthesis enzymes argininosuccinate synthase (ASS) and ornithine transcarbamylase (OTC) (Fultang et al., 2020). Recent application of scRNA-seq technology will help bring insights into the heterogeneity and dynamics of immune cells in the TME (Ren et al., 2021), as well as context-dependent metabolic divergence during CAR-T cell therapy and other immunotherapies (Artyomov and van den Bossche, 2020).

4 Future approach to surmount relapse after CAR-T cell therapy in B-cell malignancies

Future approaches to address or prevent relapse after CAR-T cell therapy in B-cell malignancies are destined to involve multiple aspects in accordance with the nature of tumor cells themselves, the properties of CAR-T cells, and the landscape within the specific

TME. Developing suitable preclinical evaluation models will provide deep insights into the mechanisms behind tumor relapse and indicate CAR-T cell therapeutic potency in the clinic (Si et al., 2022).

4.1 Optimized antigen targeting

The optimization of antigen targeting is always a focus of CAR-T cell therapy, to achieve better tumor recognition and reduce adverse effects.

4.1.1 Novel target

Discovering novel surface antigens during lineage-specific commitment of B cells will compensate for the limited selection of TSAs and TAAs available for CAR-T cell therapy (Huang et al., 2020). Targeting CD22, another surface protein involved in the regulation of B-cell antigen receptor signaling, has induced CR in patients with LBCL who relapsed after CD19 CAR-T cell therapy (Fry et al., 2018; Baird et al., 2021). CD30 in Hodgkin lymphoma is also under investigation as a promising candidate for CAR-T cell therapy (Ramos et al., 2020). Surface antigen CD37 expressed in B-cell non-Hodgkin lymphomas, CLL, and even cutaneous or peripheral T-cell lymphoma is a suitable target. CD37 CAR-T cells demonstrated antigen-specific activation, cytokine production, and cytotoxic activity against two different lymphoid lineages, without evidence of significant T-cell fratricide (Scarfò et al., 2018). Moreover, CD70 and CD123 are both indicated during relapse with lineage switch after traditional CAR-T cell therapy, suggesting that they may have value as novel targets (Muñoz et al., 2001; Shaffer et al., 2011). Although a novel target-directed CAR-T cell therapy may not work in the clinic or work only in certain types of B-cell malignancies, promoting clinical translation and discovering more potential targets are still of great potential benefit to patients.

4.1.2 Multiple targeting

Sequential infusion of CAR-T cells with alternative targets or using dual-target and multi-target CAR-T products in the beginning may overcome antigen-negative relapse (Fig. 3). A dual targeting strategy can be achieved through either a bicistronic vector to allow expression of two different CARs on the same cell, co-transduction to encode two CAR constructs via transduction with multiple vectors, or tandem transduction

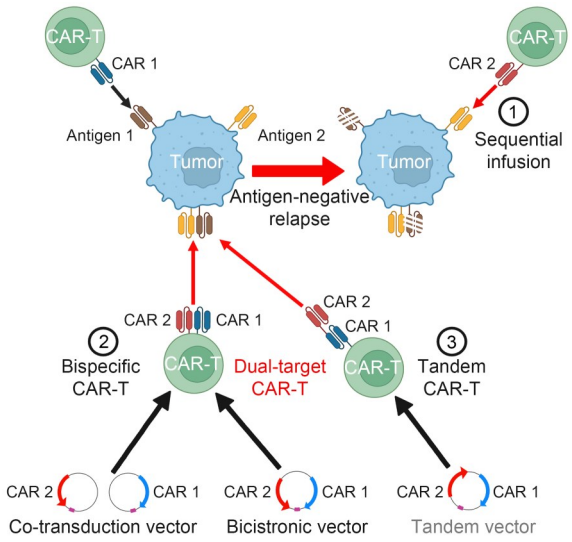

Fig. 3 Treatment strategies for relapse after CAR-T cell therapy. 1: sequential infusion of CAR-T cells with different targets; 2: application of bispecific CAR-T cells produced by transduction of a co-transduction or bicistronic vector; 3: application of tandem CAR-T cells produced by transduction of a tandem vector. CAR-T: chimeric antigen receptor-T. Created with BioRender.com.

to encode two CARs on the same chimeric protein using a single vector. As tumor cells may progress into antigen-negative relapse following treatment with a single-target CAR-T cell product, these methods providing alternative targets combined with the original would reduce the chance of immune escape to a great extent. In particular, a tandem CAR (TanCAR) with a proof-of-concept of Boolean "OR"-gated signal computation can recognize either of the targets and sufficiently trigger CAR-T cell activation, thereby overcoming antigen escape-mediated relapse after single target-directed therapy (Tong et al., 2020). A CD19/CD20-bispecific CAR efficiently prevents antigen escape by malignant B cells (Zah et al., 2016). A tandem CD19/CD20 CAR effectively lessens the chance of leukemic escape mutations and prevents relapse, with minimal toxic effects (Schneider et al., 2017). A CD19/CD22-bispecific or cocktail CAR-T cell therapy exhibited high durable CR rates and favorable safety profiles in both R/R B-ALL and B-cell lymphoma (Dai et al., 2020; Cao et al., 2021; Yan N et al., 2022). Interestingly, a recent tri-specific CD19×CD20×CD22 variable domain of heavy chain of heavy-chain antibody (VHH) CAR-T cell (LCAR-AIO) therapy efficiently eradicated antigen-heterogeneous B-cell tumors, with enhanced expansion and prolonged persistence

in preclinical in vivo models. This indicates a potential capacity to treat patients with prior relapse after CD19 CAR-T cell therapy (Zhou Z et al., 2021). Targeting markers associated with lineage switch, such as CD70 or CD123, combined with a conventional CD19 target, is a promising compound strategy to overcome antigen-negative relapse (Ruella et al., 2016; Tu et al., 2019; Yan et al., 2020).

4.2 Enhanced CAR-T cell function

Although currently approved commercial CAR-T products with second-generation CARs have shown great clinical efficacy, limitations still exist. Therefore, the engineering of CAR-T cells based on precise modulation of CAR design and enhancement on multiple levels represents a new strategy to reduce CAR-T cell functional defects and overcome relapse.

4.2.1 CAR construct

The selection and induction of additional optimal costimulatory domains, such as OX40, inducible T-cell co-stimulator (ICOS), and CD27 in third-generation CARs, have greatly enhanced cytotoxicity and prolonged persistence compared with those of the second generation (Enblad et al., 2018; Roselli et al., 2021). Furthermore, the production of fourth-generation CARs, involving fusion of extra cytokines or antibodies into the CAR construct, may enhance CAR-T cell activation, proliferation, and anti-tumor activity by modulating the specific TME (Adachi et al., 2018; Chen YH et al., 2019; Hong et al., 2020; Webster et al., 2021). CAR-T cells overexpressing IL-15 showed strong proliferation, persistence, and antitumor activity in patients with B-ALL (Sun et al., 2021). Co-expression of IL-7 and C-C motif chemokine ligand 19 (CCL19) induces the infiltration of peripheral T cells and DCs into the tumor, and improves the memory responses of recipient T cells and administrated CAR-T cells against tumors (Adachi et al., 2018). The autocrine signaling of checkpoint inhibitors (Li et al., 2017; Rafiq et al., 2018) and the intrinsic knockout of inhibitory receptors by CRISPR/CRISPR-associated protein 9 (Cas9) gene editing (Hu et al., 2019) are promising approaches to resist the immunosuppressive tumor milieu. Spatiotemporal regulation of the magnitude of CAR expression is functionally beneficial, as constitutive CAR activation signaling leads to the exhaustion of CAR-T cells. Antigen-regulated self-driving CD19 CARs with additional dominant negative TGF-β expression under transcriptional control of synthetic activating protein-1 (AP-1), nuclear factor-κB (NF-κB), and signal transducer and activator of transcription 5 (STAT5) promoters enhance tumor-dependent activation and limit exhaustion in B-cell lymphoma models (Webster et al., 2021).

4.2.2 CAR-T persistence

Multi-dimensional assessment of CAR-T cell infusion products may identify potential mechanisms related to durable remission or relapse. Molecular factors in the pre-manufacture of T cells also provide insights into the long-term persistence of CAR-T cell therapy (Chen et al., 2021). Comprehensive evaluation by performing bulk RNA-seq or scRNA-seq assay for transposase-accessible chromatin sequencing (ATAC-seq) and CITE-seq helps unveil genomic, epigenomic, and functional characteristics that determine therapeutic responses and clinical outcomes (Fraietta et al., 2018a; Deng et al., 2020). Alterations in chromatin accessibility and conformation reveal a dynamic regulatory landscape in CAR-T cell exhaustion during ex vivo expansion (Gennert et al., 2021). Joint profiling of chromatin accessibility and random integration sites of CAR lentivirus may discover essential gene regulation associated with durable treatment (Wang et al., 2020). Targeting the epigenome improves the memory phenotype and persistence of CAR-T cells. Sequencing results of CAR-T cells at the stage of peak response showed that the insertion of a CAR gene into the ten-eleven translocation 2 (*TET2*) gene induced unfunctional methylcytosine dioxygenase and inhibition of exhaustion (Fraietta et al., 2018b). CAR-T cells with *TET2* knockdown showed similar epigenetic characteristics to those at peak response, with a high memory phenotype and strong killing ability. DNA methyltransferase 3α (DNMT3A) is another important epigenome target that drives CAR-T depletion. DNMT3A knockout in CAR-T cells enhanced proliferation and maintained long-term memory, which improved efficacy during CLL treatment (Prinzing et al., 2021). In addition, continuous expression of c-Jun in T cells enhanced CAR-T cell function (Lynn et al., 2019). More recently, inhibition of overactivated calcium signaling involved in tonic signaling rendered CAR-T cells resistant to exhaustion, thereby enhancing anti-tumor efficacy (Shao et al., 2022). Histone

acetylation, histone methylation, and DNA-binding proteins in epigenetic genomes also affect CAR-T cell function. Moreover, specific knockout of transcription factors, such as transducin-like enhancer of split 4 (TLE4) and IKAROS family zinc finger 2 (IKZF2) based on genome-wide CRISPR screening, promoted CAR-T cell effector function and inhibited CAR-T cell exhaustion (Wang DR et al., 2021). Equally, novel modulator genes and biomarkers of malignant transformation in tumor cells could be identified and used as new therapeutic targets in combination with CAR-T cell therapy to prevent tumor relapse (Ancos-Pintado et al., 2022).

4.2.3 CAR-T manufacture

Optimization of the manufacturing process of CAR-T cells is essential for improving anti-tumor efficacy in vivo. Resolving the immunomodulatory metabolic landscape based on the stable isotope-resolved metabolomics (SIRM) (Fan et al., 2016) and metabolomics-edited transcriptomic analysis (META) (Fan et al., 2005) may improve metabolic complements during the manufacturing process of CAR-T cells (Xu et al., 2019). This may help ameliorate CAR-T cell metabolic fitness and overcome metabolic derangement and stress in the TME. Developing ex vivo models to simulate the TME for drug screening helps improve the therapeutic effect of CAR-T cells after infusion. A three-dimensional (3D) TME-mimicry culture was developed to study TAM modulation of T-cell-based cancer immunotherapy within the TME, and validated the efficacy of PD-1/PD-L1 and monoamine oxidase-A (MAO-A) blockade therapies (Li et al., 2022). In addition, organoid systems also provide a novel strategy for high-throughput drug screening by simulating the TME. Patient-derived breast tumor samples were isolated into single cells to generate tumor organoids, which were further co-cultured with $CD8^+$ T cells to study the effects of epigenetic inhibitors on the anti-tumor activity of T cells (Zhou ZL et al., 2021).

To avoid rejection by the host immune system, replacement of murine-origin CAR scFv with a fully humanized form reduced immunogenicity and prolonged CAR-T cell persistence. Inventing universal CAR-T cells by transcription activator-like effector nuclease (TALEN) or CRISPR/Cas9-mediated knockout of TCR, CD52, and human leukocyte antigen (HLA) in T cells from healthy donors has hugely improved product quality for high tumor-burden patients with urgent need (Hu et al., 2021). This may help overcome the functional deficiency of pre-manufactured autologous T cells. Discovering alternative cell sources for the sufficient production of CAR-T cells may help shorten the period of manufacture and improve clinical efficacy. Exploring CAR-T cell production from induced pluripotent stem cells (iPSCs) or replacing chassis cells may enhance leukemia cell clearance in vivo (Zhang L et al., 2020; Rafei et al., 2021; Sadeqi Nezhad et al., 2021). Developing split, universal, and programmable (SUPRA) CAR-T cells capable of target switch and response control to enhance recognition and diminish side effects is a promising approach for the future.

4.3 Combinational treatment strategies

We may also look ahead to resolving relapse after CAR-T cell therapy by combining treatment strategies, including application of checkpoint inhibitors and small molecule compounds, disruption of tumor cell chemotaxis or enhancement of CAR-T cell trafficking, and stimulation of the endogenous anti-tumor immune response (Fig. 4).

4.3.1 Immune checkpoint inhibitor

The combination of a monoclonal antibody (mAb)-based immune checkpoint blockade (ICB) and CAR-T cell therapy has proven to be safe and effective in treating hematologic malignancies (Wang et al., 2019). The traditional PD-1-blocking antibody, pembrolizumab, ameliorated tumor progression, induced a clinically significant anti-tumor response, and significantly prolonged progression-free survival (PFS) in patients with diffusive LBCL and patients with transformed follicular lymphoma (tFL) (Chong et al., 2017; Zheng et al., 2021). CAR-T cells armored with secreting PD-1-blocking scFv in both a paracrine and autocrine manner improved efficacy in clinically relevant syngeneic and xenogeneic mouse models of $PD\text{-}L1^+$ hematologic and solid tumors (Rafiq et al., 2018).

4.3.2 Small molecule compounds

In recent years, the enhancement of CAR-T cell function by epigenetic small molecule inhibitors has continued to attract attention. Decitabine, a DNA methylase inhibitor, reversed exhaustion-related DNA

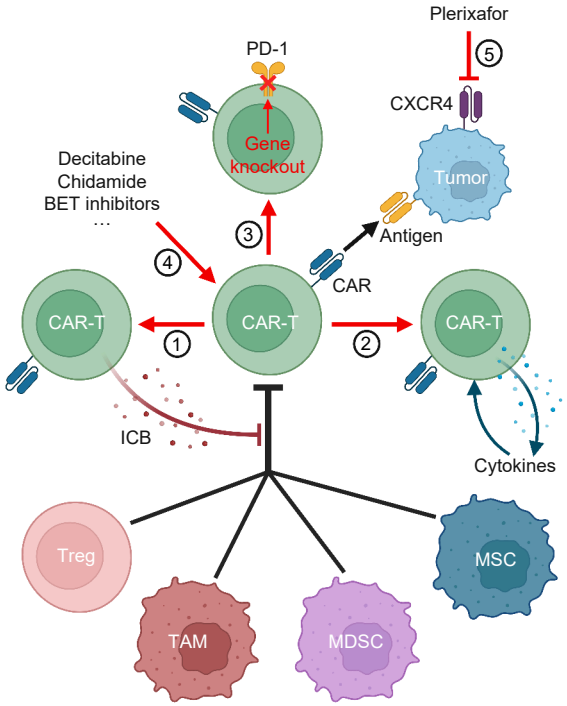

Fig. 4 Combinational treatment strategies indicated in relapse after CAR-T cell therapy. 1: CAR-T cells with self-secreting ICB, such as anti-PD-1 scFv; 2: CAR-T cells with self-secreting cytokines, such as IL-7, IL-12, IL-15, and CCL19; 3: CAR-T cells with intrinsic PD-1 knockout; 4: the application of small-molecule drugs to enhance CAR-T cell functions; 5: antagonist effect of plerixafor on CXCR4 to inhibit tumor cell homing. CAR-T: chimeric antigen receptor-T; PD-1: programmed cell death protein-1; BET: bromodomain and extra-terminal; ICB: immune checkpoint blockade; Treg: regulatory T cell; TAM: tumor-associated macrophage; MDSC: myeloid-derived suppressor cell; MSC: mesenchymal stem cell; scFv: single-chain variable fragment; IL: interleukin; CCL19: C-C motif chemokine ligand 19; CXCR4: CXC chemokine receptor 4. Created with BioRender.com.

methylation, improved CD123 CAR-T cell proliferation and memory phenotype, and enhanced CD123 CAR-T cell cytotoxicity (You et al., 2020). Low-dose decitabine-treated CD19 CAR-T cells maintained high expression of memory-related genes and low expression of exhaustion-related genes. Acquired enhanced proliferation and increased pro-inflammatory cytokine secretion all enhanced CAR-T cell anti-tumor activity (Wang Y et al., 2021). Chidamide, an HDAC inhibitor, has been reported to promote CD22 expression on the surface of B-cell lymphoma, thereby enhancing CD22 CAR-T cell targeting in vivo (Yang et al., 2021). Bromodomain and extra-terminal (BET) inhibitor treatment with CAR-T cells also promoted proliferation, reversed exhaustion, and enhanced efficacy in B-cell lymphoma (Kong et al., 2021). The use of bioactive materials during CAR-T cell culture and the combination of small molecular compounds to reverse the exhaustion state and prolong persistence are also applicable (Bao et al., 2021).

4.3.3 Chemotaxis

Targeting the chemotaxis landscape of the TME within the most favorable relapse sites by remodeling the intracellular signaling transduction of chemokine receptors on CAR-T cells or by the combinational use of chemokine receptor blockers to leukemia cells improves CAR-T cell migration and restrains leukemia cell retention, extending immune surveillance and preventing leukemia relapse. As the CXCL12/CXCR4 axis plays an essential role in the bone marrow and CNS relapse of leukemia, it offers a potential therapeutic target. High expression of CXCR4 on ALL blasts has been shown to be a predictor of poor prognosis (Cancilla et al., 2020). However, CXCR4 expression is drastically down-regulated in cytokine-induced killer (CIK) cells. Over-expression of CXCR4 in CD33 CAR-T cells facilitated the homing of CAR-CIKs to the bone marrow and subsequent leukemia eradication (Biondi et al., 2021). Although a B-ALL xenografted mouse model treated with CXCR4 antagonist plerixafor reduced engraftment into the bone marrow and liver, it failed to prevent the infiltration of tumor cells into the CNS, which suggested that other chemotaxis or mechanisms might mediate such behavior (Williams et al., 2016). Further exploration of the role of the CXCL12/CXCR4 axis, as well as other chemotaxis, in B-cell malignancy relapse after CAR-T cell therapy would broaden our therapeutic strategies to simultaneously target the TME.

4.3.4 Endogenous immune activation

Although few studies have comprehensively examined the characteristics of the intact TME due to a lack of analysis technologies, a recent study involving multiplex immunostaining and in-situ hybridization (ISH) assays found activation of CAR and non-CAR-T cells within the TME following CAR-T cell therapy (Chen PH et al., 2020). Another study using scRNA-seq revealed substantial modification of the TME during CAR-T cell therapy. IFN-γ produced by CAR-T cells not only enhanced endogenous T and natural

killer cell activity, but was also essential for sustaining CAR-T cell cytotoxicity. CAR-T cell-derived IFN-γ facilitated host IL-12 production, supporting both the host immune and CAR-T cell responses (Boulch et al., 2021). It is possible that specific CAR-T cell cytotoxicity induced epitope spreading of TCR, which activated recruitment of DCs and endogenous T cells into the TME. These findings suggest a cross-talk between CAR-T cell subsets and the TME is essential for sustained cytotoxic activity. Potential strategies to activate the anti-tumor response of the endogenous immune system combined with CAR-T cell therapy may help overcome relapse and maintain long-term remission.

5 Future perspectives

CAR-T cell therapy is a novel and rapidly evolving cellular immunotherapy that has achieved significant long-term durable remission in cancer patients compared to traditional cancer therapies. Although CAR-T cell therapy shows remarkable clinical efficacy in the treatment of B-cell malignancies, tumor relapse remains a major obstacle that hinders its broad application.

Intratumor heterogeneity as a natural intrinsic factor fosters tumor evolution under selective pressure. Metabolic reprograming and dynamic interplay between tumor cells and the microenvironment provide tumor cells with superior adaptability for survival under stress by specific therapy. CAR-T cells with flaws in CAR design and functional defects may reduce anti-tumor efficacy and durable remission. Moreover, a hostile metabolic and immunoinhibitory landscape in the TME compromises CAR-T cell function while favoring tumor cell survival, escape, and relapse. To confront immune escape and tumor relapse, upgraded CAR-T cells with optimized antigen recognition and enhanced function and combinational treatment strategies would facilitate precise targeting and overcome an immunosuppressive milieu. Note that "while the priest climbs a post, the devil climbs ten": every promising therapy might also drive tumor evolution, as suggested by Darwin's theory of evolution. Potential strategies to activate the anti-tumor response of the endogenous immune system combined with CAR-T cell therapy may overcome relapse and maintain long-term remission in the future.

With the accumulation of knowledge about the underlying mechanisms of tumor relapse after CAR-T cell therapy, better treatment approaches to induce prolonged RFS in patients are destined to emerge. Though current progress has unraveled only a surface of mystery, by comprehensively studying the cellular interactions and molecular biology from different perspectives and novel angles we will learn much more in the future.

6 Conclusions

Studies of relapse after CAR-T cell therapy in B-cell malignancies have revealed deep insights into the underlying mechanisms, and inspired us to come up with various CAR optimization and innovative treatment strategies. As new approaches are tested and implemented, CAR-T cell therapy is destined to bring success to cellular immunotherapy.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81730008).

Author contributions

Tianning GU and Meng ZHU wrote the original manuscript. Tianning GU drafted the figures. Yongxian HU and He HUANG were responsible for conceptualization and manuscript revision. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Tianning GU, Meng ZHU, He HUANG, and Yongxian HU declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this review.

This review does not contain any studies with human or animal subjects performed by any of the authors.

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