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REVIEW

Advances in strategies to improve the immunotherapeutic efficacy of chimeric antigen receptor-T cell therapy for lymphoma

Tianshu Yu[1,2], Xianhuo Wang[3], Ou Bai[4], Huilai Zhang[3], Wenbin Qian[1,2]

[1]Department of Hematology, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China; [2]Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Key Laboratory of Molecular Biology in Medical Sciences, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China; [3]Department of Lymphoma/State Key Laboratory of Druggability Evaluation and Systematic Translational Medicine, Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Tianjin's Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin, the Sino-US Center for Lymphoma and Leukemia Research, Tianjin 300060, China; [4]Department of Hematology, the First Hospital of Jilin University, Changchun 130015, China

ABSTRACT Chimeric antigen receptor-T (CAR-T) cell therapy is a precise immunotherapy for lymphoma. However, its long-term efficacy faces many challenges related to tumor cell heterogeneity, interference from immunosuppressive microenvironments, CAR-T cell exhaustion, and unmanageable adverse events. Diverse modifications have been introduced into conventional CAR-T cells to overcome these obstacles; examples include addition of recognition sites to prevent immune escape, coupling of cytokine domains to enhance killing ability, blocking of immune checkpoint signals to resist tumor microenvironments, and inclusion of suicide systems or safety switches to improve safety and flexibility. With increasing understanding of the importance of metabolism and epigenetics in cancer and cytotherapy, glycolysis, methylation, and acetylation have become crucial CAR-T cell therapeutic targets. Universal and *in situ* CAR-T cells are also expected to be used in clinical applications, thus providing hope to patients with relapsed/refractory lymphomas.

KEYWORDS Chimeric antigen receptor-T cell; lymphoma; cytotherapy; immunotherapy; epigenetics; metabolism

Introduction

Chimeric antigen receptor-T (CAR-T) cells, which are genetically programmed to specifically recognize and kill target cells, have altered the therapeutic landscape of lymphoma. In CAR-T cell therapy, T cells from the peripheral blood of patients who have undergone lymphodepletion management are sorted, purified, and activated by anti-CD3/CD28 antibodies. The CAR is then constructed and integrated into T cells with viral vectors or non-viral methods such as transposons. The CAR-T cells are subsequently amplified *in vitro* to the desired quantity before reinfusion into patients[1]. The basic structure of a CAR typically includes the following: 1) an extracellular single-chain variable fragment (scFv) responsible for antigen recognition in a major histocompatibility complex (MHC)-unrestricted manner; 2) a hinge area composed of the IgG1 constant domain; 3) a transmembrane domain that serves as a connector, usually derived from CD8; 4) an intracellular co-stimulatory domain with a crucial role in generating the second signal of T cell activation; and 5) a signal transduction domain responsible for signal cascade activation, often CD3ζ containing immunoreceptor tyrosine-based activation motifs (**Figure 1A**)[2]. After the surface antigens are identified by scFv, CAR-T cells exert anti-tumor activity through granzyme and perforin secretion, thus inducing cell apoptosis in a Fas-Fas ligand-dependent

Correspondence to: Huilai Zhang and Wenbin Qian
E-mail: zhlwgq@126.com and qianwb@zju.edu.cn
ORCID ID: https://orcid.org/0009-0004-7141-9236 and https://orcid.org/0000-0002-6138-5616
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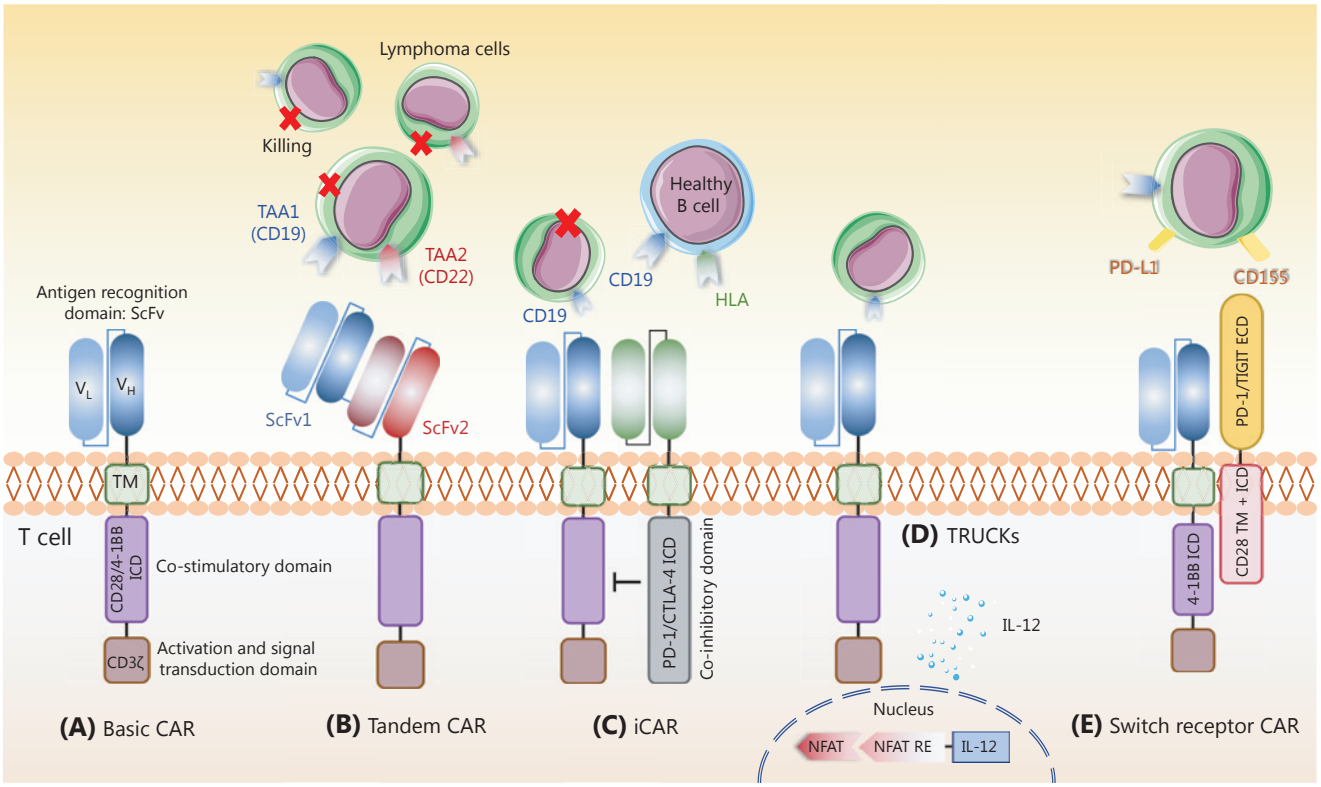


Figure 1 Structures and functions of various CARs. (A) The basic second-generation CAR consists of an antigen recognition domain (typically an scFv composed of hinge-linked V_L and V_H chains), a TM domain, a co-stimulatory domain (usually derived from the intracellular domain of CD28 or 4-1BB), and an activation and signal transduction domain (commonly CD3ζ). After target antigen recognition, the signal transduction domain stimulates T-cell activation (e.g., immunoreceptor tyrosine-based activation motif phosphorylation in CD3ζ) with the assistance of the co-stimulatory domain. (B) The tandem CAR, compared with the basic CAR, has an additional antigen recognition domain with 2 scFvs arranged in tandem. Either TAA1 (e.g., CD19) or TAA2 (e.g., CD22) can activate CAR signaling, thereby forming an OR gate preventing immune escape. (C) The iCAR, compared with the basic CAR, has an additional CAR structure that recognizes antigens expressed exclusively on healthy cells (usually a subtype of HLA) and transmits inhibitory signals. After simultaneous binding to CD19 and HLA, the immunosuppressive signals transmitted by the co-inhibitory domain (usually derived from the intracellular domain of PD-1 or CTLA-4) prevent conventional CARs from exerting their killing function, thereby forming an AND-NOT gate that protects normal cells from being killed, while targeting malignant B cells for elimination. (D) TRUCKs enable conditional cytokine secretion through the incorporation of NFAT response elements (NFAT RE) into cytokine genes (e.g., cDNA of IL-12). After activation of the CAR signal by the target antigen, the NFAT transcription factor is activated, binds the NFAT RE, and initiates the transcription of cytokine genes. This approach enhances the cytotoxic activity of CAR-T cells while minimizing systemic toxicity. (E) The immune checkpoint ligands expressed on lymphoma cells (e.g., PD-L1 and CD155) promote CAR-T cell exhaustion by transmitting immunosuppressive signals. The switch receptor consists of a truncated inhibitory receptor (usually derived from the extracellular domain of PD-1 or TIGIT) and a truncated stimulatory receptor (usually derived from the transmembrane and intracellular domains of CD28). After interacting with immune inhibitory ligands, the switch receptor delivers a CD28-mediated positive signal instead of a negative signal, which in turn enhances CAR-T cell cytotoxicity and overcomes exhaustion. CAR, chimeric antigen receptor; CTLA-4, cytotoxic T lymphocyte associated protein 4; ECD, extracellular domain; HLA, human leukocyte antigen; iCAR, inhibitory chimeric antigen receptor; ICD, intracellular domain; NFAT, nuclear factor of activated T cells; NFAT RE, NFAT response element; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; scFv, single-chain variable fragment; TAA, tumor-associated antigen; TIGIT, T-cell immune receptor with Ig and immunoreceptor tyrosine-based inhibition motif domains; TM, transmembrane; TME, tumor microenvironment; TRUCKs, T cells redirected for universal cytokine-mediated killing; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

pathway and producing inflammatory cytokines that antagonize the immunosuppressive tumor microenvironment (TME) and induce host immune responses[3].

Before the advent of CAR-T cells, patients with chemotherapy-resistant B-cell lymphoma (BCL) had poor prognosis, with a median overall survival (OS) of only 6 months.

The emergence of CAR-T cell therapy has not only provided progression-free survival and OS benefits, but also improved quality of life for patients with relapsed/refractory (R/R) B-cell non-Hodgkin lymphoma and led to the establishment of a sustainable standard of care[4]. However, CAR-T cell therapy faces many challenges arising from tumor cell heterogeneity; interference from the TME; T cell exhaustion; and severe adverse events, such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome. In recent years, advances in tumor immunology and genetic engineering have driven CAR evolution. New generations of CARs armed with diverse molecular weapons have achieved substantial progress in enhancing the accuracy of recognition, stimulating endogenous immune responses, strengthening killing activity, resisting TME and exhaustion, and improving safety and flexibility, thus gradually overcoming the limitations of CAR-T cell therapy. This review focuses on advances in CAR-T cell treatments for lymphoma, and additionally summarizes new CAR modification strategies and their potential prospects.

Improving antigen recognition through Boolean logic gates

Preventing tumor escape with OR gates

CD19 CAR-T cells are the most widely used CAR-T cells for the treatment of B cell malignancies. However, lymphoma cells may lose CD19 expression because of pre-existing CD19-negative cells, antigen maturation failure, transport barriers, epitope masking, or lineage switching, thereby resulting in ineffective treatment or recurrence[5]. Bispecific CAR-T cells not only compensate for the shortcomings of a single target but also avoid survival competition when several CAR-T products are mixed. The 3 main types of bispecific CAR-T cells are co-transduced CAR-T cells with a high expression density of CARs and product heterogeneity; bicistronic CAR-T cells with 1:1 co-expression of 2 types of CARs but diminished viral transduction efficiency; and tandem CAR-T cells containing 1 CAR structure with 2 scFvs, which address vector length issues[5].

CD20 and CD22, highly expressed antigens in mature B cells, are the most commonly applied alternatives (**Figure 1B**). Zah et al.[6] have constructed CD19/20 tandem CAR-T cells by incorporating CD19 and CD20 scFvs *via* modified G4S and EAAAK linkers, and achieved superior degranulation and cytokine secretion. CD19/20 tandem CAR-T cells kill wild-type Raji cells and CD19-negative Raji cells with equal efficiency[7]. Loop CD19/20 CAR-T cells have been developed and demonstrated to be more effective than their tandem counterparts, possibly because the loop structure facilitates the formation of superior immune synapses and enhanced stability of interactions with antigens[8]. CD19/22 bispecific CAR-T cells have been validated to outperform sequential management of CD19 and CD22 CAR-T cells in antigen loss-relapse models of B-cell acute lymphoblastic leukemia (B-ALL)[9]. Ma et al.[10] have found that the EAAAK linker is more effective than the G4S linker in enhancing cytotoxicity and secretion ability. Clinical trials of CD19/22 CAR-T cells for R/R non-Hodgkin lymphoma (NHL) have attained an overall response rate (ORR) exceeding 60%[11]. Trispecific CD19/20/22 CAR-T cells coupling a CD19/20 tandem CAR with a CD22 CAR by using P2A have been found to eradicate antigen-heterogeneous tumor cells *in vitro* and *in vivo*[12]. Clinical applications of trispecific CAR-T cells are soon expected.

CD79b, a component of BCR signaling, is a key receptor for maintaining B cell function. CD79b/CD19 bispecific CAR-T cells have been found to effectively eliminate heterogeneous lymphoma composed of both CD19(−) and CD19(+) cells *in vivo*[13]. Combinations of CD37 or B-cell maturation antigen with CD19 have also been tested for NHL[14,15]. Li et al.[16] have demonstrated the superior efficacy of CD38/LMP1 bispecific CAR-T cells to monospecific CAR-T cells in treating NK/T-cell lymphoma. Beyond CD4 and CD5, CD7 is another important immunotherapy target for T-cell lymphoma (TCL). CD5/CD7 bispecific CAR-T cells have been demonstrated to mitigate tumor antigen escape in preclinical studies on T-cell malignancies[17]. Nanobody-derived CD30/CD5 CAR-T cells have recently been shown to exhibit enhanced targeting and cytotoxic potency compared to single-target and single scFv-derived bispecific CAR-T cells in TCL[18]. Nanobody-derived bispecific CARs may have promising synergistic effects, because of their relatively small size and low immunogenicity.

Decreasing on-target, off-tumor toxicities with AND-NOT gates

CAR-T cells target primarily tumor associated antigens (TAAs) that are also expressed in normal tissues, thus leading to on-target, off-tumor toxicities. Current targets of BCL are abundant in healthy B cells and inevitably cause B cell aplasia.

The AND-NOT gate consists of 2 CAR structures. One structure is responsible for recognizing TAAs expressed in both tumor and normal tissues, and transmits cytotoxic signals. The other structure is responsible for identifying antigens expressed only in normal cells, and transmits robust inhibitory signals that antagonize the activation of conventional CAR signals, and therefore is called an inhibitory CAR (iCAR). Cells containing both CAR- and iCAR-targeted antigens are retained (**Figure 1C**). A PD-1- and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)-based iCAR has been reported to limit cell behavior induced by the activation of CAR and endogenous TCR[19]. One driver of inactivation of tumor suppressor genes is loss of heterozygosity; the presence of this allelic deletion in more than 90% of human cancers provides a basis for distinguishing between tumors and healthy tissues[20]. Class I human leukocyte antigen (HLA) molecules exhibit high expression in healthy cells but are largely absent in cancer cells. Hamburger et al.[21] have used CD19 as an activator, HLA-A*02 as a blocker, and NK-cell inhibitory receptor LIR-1 as the intracellular domain of iCAR. These engineered T cells killed CD19(+) HLA-A*02(−) Raji cells but preserved CD19(+)HLA-A*02(+) cells in a mixed system. More importantly, the AND-NOT module operated reversibly through the integration of different signals and changing states. Similarly, Tao et al.[22] have invented a killer inhibitory receptor- and PD-1-based iCAR that recognizes CD19 and HLA-C1 (iKP CAR-T). These iKP CAR-T cells eradicated CD19(+)HLA-C1(−) Daudi cells while exhibiting low cytotoxicity toward CD19(+)HLA-C1(+) normal B cells. The efficiency of this strategy depends on the equilibrium between CAR and iCAR signal strength. Another strategy, the AND gate, is represented by synthetic Notch receptor (synNotch CAR), which was designed as a multi-antigen priming circuit. Unfortunately, because almost all TAAs of lymphomas are also expressed in normal lymphocytes, this strategy is currently challenging to apply in lymphoma treatments.

Inhibiting immune checkpoint signals to overcome the TME

The expression of inhibitory receptors/ligands in tumors promotes tumor progression and resistance to treatment by decreasing the contact between T cells and antigen-presenting cells, promoting regulatory T cell (Treg) differentiation, and inhibiting pro-inflammatory cytokine production. PD-L1 is associated with B symptoms and high-risk categories in the International Prognostic Index for lymphoma. The OS of patients with diffuse large B-cell lymphoma (DLBCL) with PD-L1-positive lymphoma cells is shorter than that of patients with PD-L1-negative cells[23]. In DLBCL, CTLA-4 promotes the invasion of lymphoma cells and Treg differentiation[24]. LAG-3 and TIM-3 are widely expressed in the TME in classical Hodgkin lymphoma[25]. Therefore, curbing the sensitization of immunoinhibitory signals is essential for successful CAR-T cell therapy.

Blocking interactions between immunosuppressive receptors and ligands

Targeting the PD-1/PD-L1 pathway is a classic strategy for cancer treatment. Insertion of anti-PD-1 scFv into the CAR vector enhances the anti-tumor activity of CAR-T cells. The expansion and IFN-γ secretion of anti-PD-1 scFv-producing CAR-T cells, compared with conventional CAR-T cells, increases under continuous antigen stimulation, whereas the expression of the exhaustion biomarkers PD-1 and LAG-3 is restricted and is accompanied by limited apoptosis of tumor-infiltrating lymphocytes (TILs)[26,27]. CAR-T cells fused with PD-L1 scFv also exhibit strong proliferation and cytotoxicity[28]. Huang and colleagues[29] have found that silencing PD-1 in CD19 CAR-T cells (PD1-19bbz) leads to a decline in Treg quantity and exhaustion marker expression, an increase in central memory phenotype, and enhanced oxidative phosphorylation (OXPHOS) and metabolic adaptability. A phase I clinical trial of PD1-19bbz has achieved remarkable results: all 21 patients with R/R BCL achieved objective remission, and more than 80% achieved CR[30].

However, opposing opinions have also been expressed. Although PD-1 silencing in CAR-T cells results in resistance to PD-L1-mediated immunosuppression, no significant improvements in anti-tumor function have been observed. Moreover, the survival, proliferation, and differentiation of CAR-T cells into effector memory T cells are hindered[31]. Agarwal et al.[32] have found that CTLA-4 deletion is more effective than PD-1 deletion in invigorating CAR-T cells. These controversies suggest that different modification methods might lead to different outcomes or that blocking a single target might not achieve optimal effects. CAR-T cells with concomitant deletion of PD-1 and T cell immune receptor with Ig and ITIM domains (TIGIT) have been found to enhance the anti-cancer effects in B-cell acute lymphoblastic leukemia (B-ALL) mouse models[33]. PD-1 inhibition is primarily responsible for cytotoxicity, and

TIGIT inhibition contributes mainly to phenotype optimization. The synergistic effects produced by double-KO CAR-T cells make them superior to their single-KO counterparts.

Switching immunosuppressive signals to co-stimulatory signals

The switch receptor usually consists of the truncated extracellular domain of an inhibitory receptor and the transmembrane and intracellular domains of a co-stimulatory receptor (**Figure 1D**). The PD-1/CD28 switch CAR was first constructed by Liu et al.[34] PD-1/CD28 CAR-T cells have been found to outperform their prototype when co-cultured with cells overexpressing PD-L1. Xenograft mouse models treated with PD1/CD28 CAR-T cells showed stronger tumor lysis, higher number of TILs, and restricted PD-1 and LAG-3 expression compared to conventional CAR-T cells treated with pembrolizumab. Our team has confirmed the superiority of switch receptor CAR-T cells over traditional CAR-T cells in lymphoma[35]. CD19-PD-1/CD28 CAR-T cells, compared with normal CD19 CAR-T cells, exhibited better anti-tumor potency in co-culture with PD-L1(+) Raji cells, as demonstrated by higher cytotoxicity and production of pro-inflammatory factors (IL-12, IFN-γ, and TNF-α), elevated expression of activation markers (CD69, CD27, and NKG2D), and lower TIM-3/LAG-3 double-positive cells. In mouse models established with PD-L1(+) Raji cells, CD19-PD-1/CD28 CAR-T cell treatment resulted in a lower tumor burden than treatment with CD19 CAR-T cells combined with anti-PD-1 monoclonal antibodies. In a subsequent phase Ib clinical trial of R/R BCL, the ORR in 17 patients was 58.8%, and 7 patients achieved CR[35]. We have also transfused CD19-PD-1/CD28 CAR-T cells in 6 patients with R/R DLBCL in whom CD19 CAR-T cell therapy had failed. Half the patients achieved CR, and the longest response duration exceeded 2 years[36], thus suggesting that switch receptor CAR-T cells might serve as a salvage measure after failure of traditional CAR-T cell therapy. The T3/28 CAR-T cells integrating TIM-3 and CD28 showed more powerful Burkitt's lymphoma cell killing ability than their prototype[37]. Abundant granzyme B and perforin, lower expression of exhaustion indicators, and higher levels of CD25 and CD27 have been found in T3/28 CAR-T cells compared to conventional CAR-T cells. In animal models based on Daudi cells, CD19-T3/28 CAR-T cells with a less differentiated phenotype have been found to be more persistent than CD19 CAR-T cells, and to prolong survival in mice. Another inhibitory receptor, TIGIT, competitively inhibits CD226 signaling through interaction with CD155, and mediates inhibition of NK and T cell immune responses. CD19-TIGIT/CD28 CAR-T cells have been found to maintain sufficient release of IFN-γ and IL-2, and to lead to up-regulation of pERK and Bcl-xl. Furthermore, the switch from TIGIT to CD28 enhances the affinity of TCR and prevents T cell dysfunction under repetitive antigen exposure[38].

Transforming growth factor-β1 (TGF-β1) phosphorylates SMADs by sequentially binding TGF-β receptor II (TRII) and recruiting TRI, thus mediating tumor invasion and metastasis[39]. Reversal of TGF-β signaling is typically achieved by combining it with cytokine receptors. Noh et al.[40] have combined TRII with IL-7R to generate a TGF-β/IL-7 switch CAR. These TGF-β/IL-7 CAR-T cells have been found to maintain high cytotoxicity and mRNA expression of TNF-α and IFN-γ in a TGF-β-rich milieu. In a study in Burkitt's lymphoma mouse models, all mice treated with CD19-TGF-β/IL-7 CAR-T cells survived and remained tumor-free until the experimental endpoint. A study on prostate cancer has linked TRI and TRII to IL-12 receptors, with an aim of converting TGF-β signals into IL-12 and IL-15-mediated activation signals. TGF-β/IL-12/15 CAR-T cells preserved antigen-specific cytotoxicity and expansion potential in the presence of TGF-β[41]. With a recently created "double-switch" CAR, TIGIT-PD1/CD28 CAR-T cells have been found to overcome dual immunosuppressive microenvironments in a colorectal cancer model[42]. The development of "multiple-switch" CARs is anticipated, given that TME formation is not driven by a single immune checkpoint. Beyond the canonical patterns described above, combinations of other immunosuppressive molecules (such as LAG-3 and CTLA4) and co-stimulatory molecules (such as ICOS, OX40, and CD27) are also expected to enhance the efficacy of CAR-T cells—a possibility requiring further research.

Enhancing killing capacity *via* cytokine intensification

The enhancement of anti-tumor activity might also require a third signal beyond the second signal provided by the co-stimulatory domain. The combined use of pro-inflammatory factors has been found to enhance CAR-T cell cytotoxicity and maintain their viability, mobilize the host immune system to assist in killing tumor cells, and reprogram the immunosuppressive environment. However, systemic adverse events

caused by direct administration of cytokines limits clinical feasibility. T cells redirected for universal cytokine-mediated killing (TRUCKs) can optimally use cytokines and have dual roles: CAR-T cells attack after local accumulation of bioactive substances, thereby improving anti-tumor effects while minimizing systemic exposure (**Figure 1E**)[43].

IL-12

IL-12 increases the proliferation of NK cells and T cells; promotes type 1 T helper cell (Th1) and macrophage (M1) polarization; enhances dendritic cell (DC) antigen presentation; and induces IFN-γ and TNF-α secretion[44]. Kueberuwa et al.[45] have inserted the 2A linker sequence downstream of the CAR, followed by IL-12 cDNA, to establish CD19-IL-12 CAR-T cells with cytotoxicity more than two-fold stronger than that of their non-IL-12 secreting counterparts. These CD19-IL-12 CAR-T cells have been found to eradicate BCL in mice without lymphodepletion pre-treatment through inducing epitope spreading and a robust memory immune response, thus achieving a four-fold longer median survival than observed in naive mice. Notably, although no evidence of persistent CAR-T cells was observed in long-term surviving mice, the mice were protected against tumor re-challenge; these findings indicated successful induction of the host anti-tumor response. To further decrease the potential risks of constitutive cytokine secretion, an additional nuclear factor of activated T-cell (NFAT) response element can be included for CAR, which induces IL-12 expression only when triggered by specific antigen binding[46]. Similarly, hypoxia inducible IL-12 CAR-T cells have recently emerged, given the hypoxic TME. The special domain was constructed by fusing the IL-12p70 subunit with the oxygen dependent degradation (ODD) domain of hypoxia inducible factor-1α. Only under hypoxic stimulation, CAR19/hIL12ODD-T cells secrete IL-12, which is accompanied by a surge in proliferation and IFN-γ secretion, as well as enrichment in the central memory phenotype. CAR19/hIL12ODD-T cells have been found to cure DLBCL in mouse models and to elicit fewer treatment-associated adverse effects than normal IL-12 CAR-T cells[47].

IL-15

IL-15, another cytokine with similar biological functions to IL-12, increases CTL and NK cell activity, and induces B cell proliferation and differentiation by activating the JAK-STAT and PI3K-MAPK pathways[48]. IL-15 CAR-T cells up-regulate the anti-apoptotic protein BCL-2 and down-regulate PD-1 expression, thus presenting a more adaptive central memory phenotype than their IL-15-non-expressing counterparts[49]. Although IL-15 alone has a short half-life, IL-15/IL-15Rα complex formation prolongs its half-life by nearly 20 fold. Consequently, a more stable IL-15/IL-15sushi CAR has been constructed by coupling IL-15 and the sushi domain of IL-15Rα[50]. Feng et al.[51] have assessed the anti-tumor efficacy of CD4 IL-15/IL-15sushi CAR-T cells in preclinical studies. The almost complete lysis of the tumor cell lines and significantly diminished tumor burden in mice demonstrated the superiority of these cells to conventional CD4 CAR-T cells. A phase I clinical trial conducted in TCL has demonstrated promising efficacy[51]. Feng's team[52] has also administered CD5 IL-15/IL-15sushi CAR-T cell therapy to a patient with R/R T-cell lymphoblastic leukemia/lymphoma, who achieved a good clinical response. New carriers can load adjuvants and enable CAR-T cells to release them at the proper time, thus expanding the window of cytokine-assisted treatments. Loading of reduction-responsive nanogels containing IL-15 agonists onto CAR-T cells has enabled expansion by more than ten-fold at tumor sites in mice without triggering systemic toxicity[53]. High serum IL-15 levels have been associated with high remission rates in BCL[54], and the IL-15 agonist polymer NKTR-255 has been shown to induce the proliferation and accumulation of CD19 CAR-T cells in a dose-dependent manner[55]. Therefore, the application of IL-15 CAR-T cell therapy in BCL is soon expected.

IL-7 and combination with chemokines

IL-7 suppresses the exhaustion phenotype, preserves stemness, and inhibits apoptosis in T cells[56]. Transgenic expression of IL-7 in CAR-T cells maintains low metabolic activity during the resting state but rapidly exerts anti-lymphoma effects after tumor antigen exposure, thus demonstrating their adaptability[57]. The combination of chemokines or chemokine receptors with cytokines can synergistically enhance therapeutic efficacy, potentially doubling the desired effects while reducing the required dosage. IL-7 and CCL19 are crucial for the formation of T cell zones in lymphoid organs. IL-7-IL-7R signaling promotes the survival and proliferation of T cells, whereas the CCL19-CCR7 interaction maintains chemotactic activity[58]. Moreover, 7 × 19 CAR-T cells expressing IL-7 and CCL19 in tandem have been found to mimic the roles

of reticular fibroblasts, and facilitate CAR-T cell and host immune cell migration and infiltration into tumor sites. These 7 × 19 CAR-T cells have achieved excellent anti-cancer effects through inducing co-localization of T cells and DCs, and stimulating the memory response of CAR-T cells and host T cells, thus outperforming a 1:1 mixture of IL-7 CAR-T cells and CCL19 CAR-T cells[58]. Moreover, 7 × 19 CAR-T cells produce higher IFN-γ, TNF-α, and granzyme B levels while expressing lower levels of PD-1, LAG-3, and TIGIT compared to conventional CAR-T cells, thus exhibiting a more suitable phenotype[58,59]. We have demonstrated the outstanding efficacy and safety of inducible 7 × 19 CD19 CAR-T cells in a phase I trial and expansion phase trials in R/R LBCL. After a single infusion, the ORR at 3 months was nearly 80%, with more than half of the patients achieving CR[60]. The combination of IL-7 with CXCR5 or CCR2b has been reported to have potential in treating solid tumors, and the selection of chemokines or chemokine receptors may depend on the cancer type or TME[61,62].

Other cytokines

IL-2 is essential for T cell proliferation and activation, whereas prolonged exposure to IL-2 is not beneficial to CAR-T cells, by driving effector phenotypes without promoting memory formation. Correspondingly, IL-2 concentration and exposure time must be limited to be suitable for CAR-T cells[63]. Curran et al.[64] have found that CD19 CAR-T cells expressing CD40L show promotion of Th1 polarization, up-regulation of the expression of co-stimulatory molecules and death receptors, and induction of DC maturation, thereby improving long-term survival in DLBCL mouse models. IL-18 and IL-21 CD19 CAR-T cells have been demonstrated to enhance anti-tumor efficacy with stronger proliferation, less apoptosis, and greater imperviousness to the TME compared to traditional CAR-T cells[65,66]. Studies have demonstrated that IL-15/IL-18 × CXCR2 CAR-T cells and IL-15 × CCL19 CAR-T cells increase the chemotaxis and infiltration of T cells toward target sites in solid tumors, thus providing insights for CAR-T research in lymphomas[67,68]. Engineered CAR-T cells with the IL23-p40 subunit might surpass IL-15 and IL-18 in driving T cell proliferation and survival in an autocrine manner[69]. Inverted cytokine receptor (ICR), which is similar to the switch receptor in converting inhibitory cytokine signals to inflammation-stimulating signals, has shown high potency in solid tumors. The Th2-associated cytokine IL-4 is currently the most frequently used target for inducing anti-inflammatory responses. IL-4R/IL-7R ICR and IL-4R/IL-21R ICR support tumor rejection by promoting Th1 and Th17 polarization, respectively[70,71]. The IL-4/IL-15 ICR has been shown to enhance the activation, degranulation, and amplification of CAR-T cells, while restraining excessive differentiation[72]. Other immunomodulatory factors such as IL-10 and IL-35 are additional potential candidates for lymphoma.

Decreasing adverse events with safety switches

CAR-T cells, as "living drugs," are difficult to control. Abundant release of pro-inflammatory cytokines during the anti-tumor process can lead to widespread organ dysfunction. Adverse events may escalate and become fatal, despite the enhancement of anti-tumor efficacy. Although the activity of the transfused CAR-T cells cannot be directly manipulated *in vitro*, the introduction of safety switches or inducible suicide systems might potentially be used to regulate CAR-T cells more precisely.

Suicide systems

The herpes simplex virus-thymidine kinase initiated by ganciclovir is a classical suicide system. However, its application is somewhat limited by its conflict with antiviral therapy and slow onset. In contrast, antibody-dependent cell-mediated cytotoxicity (ADCC) is a practical strategy (**Figure 2A**). The truncated epidermal growth factor receptor (EGFRt) peptide is specifically recognized by cetuximab and cleared through ADCC. EGFRt-CD19 CAR-HSCs have been found to improve survival in Raji-based models and to achieve long-lasting ablation exceeding 80% after cetuximab injection[73]. The interaction between CD20 and rituximab results in rapid self-killing through both ADCC and complement-dependent cytotoxicity (CDC) without affecting T-cell characteristics[74]. A more compact marker/suicide gene, RQR8, has been developed on the basis of the CD8 stalk, followed by CD20 mimotopes and a CD34 fragment recognized by the monoclonal antibody QBend10. The expression of RQR8 enables selection and tracking of CAR-T cells through a CD34 system, as well as specific depletion by rituximab but not ofatumumab. Therefore, ofatumumab can be included in the pre-treatment regimen

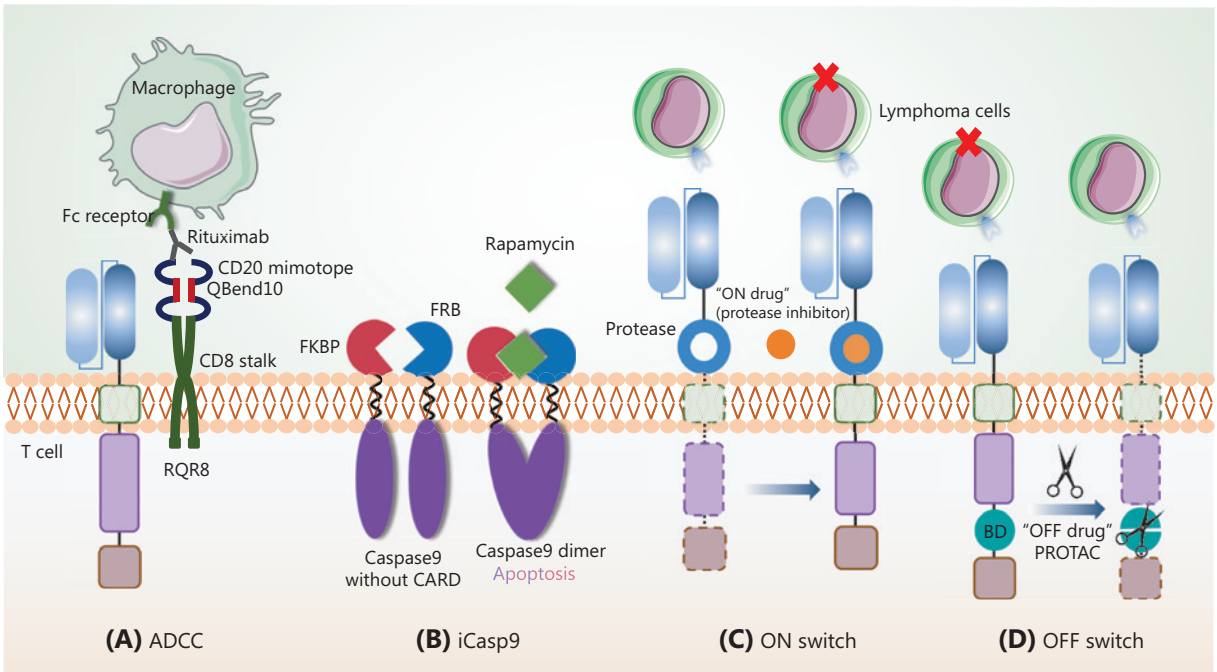


Figure 2 Inducible suicide system and safety switch. (A) Monoclonal antibodies induce cell death *via* ADCC. The RQR8, which contains CD20 mimotopes, is recognized by the anti-CD20 monoclonal antibody rituximab. CAR-T cells expressing the RQR8 domain retain normal function in the absence of rituximab. After rituximab addition, macrophages bind the Fc segment of rituximab *via* Fc receptors, thus leading to the destruction of CAR-T cells. (B) The iCasp9 consists of a modified caspase9 (typically with deletion of the CARD domain) and dimerization inducer binding domains (usually FKBP and FRB). Caspase9 lacking the CARD domain cannot achieve self-dimerization and induce apoptosis; consequently, the CAR-T cells function normally. The addition of rapamycin induces binding of FK506 binding protein (FKBP) to the FKBP-rapamycin binding domain (FRB), and subsequent formation of the caspase9 homodimer and apoptotic cascade activation. (C) A protease with autoproteolytic activity (e.g., HCV-NS3), inserted between the scFv and the signal transduction domain, leads to degradation of the CAR signal. The addition of an ON drug (e.g., HCV-NS3 inhibitor ASV) inactivates the protease and allows for complete CAR signal transmission. (D) The CAR structure is modified to include a bromodomain (BD), which does not interfere with CAR function. PROTAC compounds degrade BD-tagged proteins, thereby inactivating the CAR signal. Removal of the OFF drug restores CAR function. Safety switches enable a reversible gain or loss of function in CAR-T cells in a dose-titratable manner, without compromising cell viability. The CAR structure outlined with a dashed box indicates the failure of complete signal transduction in the CAR pathway. The red cross represents the killing of lymphoma cells. ADCC, antibody-dependent cell-mediated cytotoxicity; BD, bromodomain; FKBP, FK506 binding protein; FRB, FKBP-rapamycin binding domain; iCasp9, inducible caspase9 suicide system; PROTAC, proteolysis-targeting chimaera.

before CAR-T cell therapy[75]. Alemtuzumab-mediated ADCC, which targets CD52 on CAR-T cells, is suitable for TCL[76]. Antibody-drug conjugate-mediated killing is independent of ADCC and CDC. The ADC CH12-monomethylauristatin F (MMAF) is internalized by FR806-expressing cells and inhibits mitosis without targeting wild-type EGFR in normal tissues. More than 90% of FR806-CD19-CAR-T cells have been found to be eliminated from Daudi-based mouse models after a single dose of CH12-MMAF[77].

Caspase9-mediated apoptosis is also widely used. Straathof et al.[78] have removed the CARD domain responsible for the physiological homodimerization of caspase9 and replaced it with the mutant FK506 binding protein (FKBP12-F36V) to establish an inducible caspase9 suicide system (iCasp9). The inert prodrug rimiducid induces dimerization of caspase9 and initiates the apoptotic cascade response. An inducer concentration of 1 nM has been found to achieve nearly 99% clearance. However, high concentrations of rimiducid have not been found to result in significant toxicity toward non-transduced T cells[79]. After exposure to rimiducid, the significantly decreased levels of IL-6, TNF-α, and IFN-γ in the serum in a Burkitt's lymphoma mouse model suggest effective control of CRS[80]. Similarly, replacing CARD with FKBP12 and the FKBP-rapamycin binding (FRB) domain of mTOR enables the formation of a rapamycin-induced heterodimer (**Figure 2B**)[81]. The feasibility of clinical application of iCasp9-CAR-T cells

in NHL continues to be investigated. Multiple mechanisms hinder the achievement of 100% elimination, regardless of the method used. Combinatorial suicide systems, such as iCasp9 + iCasp8 or iCasp9 + RQR8, are expected to improve clearance and decrease cell regeneration, given that each system is independently activated by its specific inducer[82].

ON-OFF switches

In contrast to direct cell death, regulation of the expression density or stability of CAR is a non-lethal strategy that can achieve reversible loss-of-function in CAR-T cells (**Figure 2C and D**). The device activating CAR-T cell function in response to exogenous stimuli is termed an ON switch. The inducible dimerization system formed by FKBP12 and FRB has been embedded into the hinge area to separate scFv from the cell membrane, thereby enabling complete CAR signal transmission after rapamycin analog-induced dimerization[83]. In Sakemura's studies[84], an all-in-one, third-generation tetracycline-inducible vector (Tet-on) was selected as the core element. The Tet-on protein associated with a tetracycline response element promoter was fused with the CD19 CAR and consequently induced CAR expression in the presence of doxycycline. Toll-like receptor (TLR) and CD40 signaling are crucial for maintaining T-cell activity. The inducible Myd88/CD40 system (iMC) consists of a truncated Myd88, CD40, and a tandem FKBP12-F36V domain[85]. After exposure to rimiducid, the iMC system triggers phosphorylation of extensive signaling networks and up-regulation of genes associated with the NF-κB pathway, and consequently increases the potency of CAR-T cells. The hepatitis C virus NS3 protease (HCV-NS3), together with its inhibitor asunaprevir (ASV), successfully forms a chemogenetic switch[86]. HCV-NS3 undergoes autoproteolysis at both the N- and C-termini, and consequently dissociates the intact CAR after integration between the scFv and hinge. ASV repeatedly switches the complete CAR signal on and off in a dose-dependent manner. The orthogonal cytokine-cytokine receptor system also adjusts the behaviors of CAR-T cells as needed. Pairing of the PEGylated mutant IL-2 (STK-009) with its companion mutant IL-2Rβ (hoRb) allows for selective transmission of positive signals to CAR-T cells without disturbing the native IL-2/IL-2R system. STK-009 dose-dependently increases CD19-hoRb CAR-T cell expansion, cytotoxicity, and tumor infiltration in bulky lymphoma models. After withdrawal of STK-009, CAR-T cells contract, thereby limiting CRS[87].

Juillerat et al.[88] have built an OFF switch by also using HCV-NS3 and ASV, but adding a protease target site and a degron. HCV-NS3 segregates the degron from CAR in the absence of ASV and therefore allows CAR-T cells to function normally. ASV prevents HCV-NS3 from cleaving the degron and directs CAR into the degradation pathway. Importantly, CAR expression and CAR-T cell function are restored by ASN elution. Similarly, a CAR bearing a bromodomain can be targeted and degraded by a proteolysis-targeting chimaera (PROTAC)[89]. Anti-tumor small molecules with kinase inhibitory effects have also been used as switch triggers. Dasatinib prevents the phosphorylation of multiple components in CAR signaling, including Lck, ZAP70, and CD3ξ chains[90]. However, as a non-specific inhibitor, dasatinib also interferes with other kinase families and affects the anti-tumor efficacy of endogenous T cells. Lenalidomide degrades multiple proteins and proteases by mediating the interaction between E3 ubiquitin ligase and a zinc finger degradation motif, thus enabling either an ON or OFF switch to be built[91]. Simultaneous use of ON and OFF switches may enable more timely regulation. For instance, a rimiducid-stimulated iMC activation system combined with a rapamycin-induced iCasp9 suicide system forms an orthogonal switch enabling separate control and enhanced precision of regulation[92].

The suicide system usually requires only 1 or 2 doses of medications to rapidly clear transgenic T cells within hours. In contrast, ON switches require long-term administration to maintain CAR-T cell activity, and discontinuation results in delayed clearance of small-molecules. OFF switches also require a duration of drug exposure to inactivate CAR-T cells. Safety switches provide reversibility and flexibility, in contrast to irreversible apoptosis, and different switches can be co-expressed in a single cell and independently regulated by distinct drugs.

Epigenetic regulation in CAR-T cells

Targeting DNA methylation

Epigenetics regulates gene transcription without altering DNA sequences and plays a major role in tumor occurrence. Epigenetic dysregulation caused by gene mutations has been found in many cases of lymphoma, and epigenetic agents have become a cornerstone of cancer treatments. DNA methylation usually inhibits gene transcription by blocking the binding of promoters to transcription factors or recruiting

transcriptional repressors. DNA methylation in tumor cells mediates their immune escape through decreases or even loss of tumor antigens[93]. The DNA methyltransferase (DNMT) inhibitor decitabine up-regulates the expression of CD19 in lymphoma cell lines, thereby enhancing their sensitivity to CD19 CAR-T cell therapy[94]. In human studies on R/R NHL, inclusion of decitabine in pre-treatment regimens has achieved favorable therapeutic effects[94]. The process of T cell differentiation from naïve to effector stage can lead to exhaustion, which is further exacerbated by antigen-independent tonic signaling. T cell exhaustion not only manifests as the overexpression of immunosuppressive receptors but also is fundamentally regulated by epigenetic mechanisms[95]. Prolonged *in vitro* culture of CAR-T cells increases methylation levels, thus down-regulating stemness-maintaining genes *via* hypermethylation and potentially affecting long-term post-infusion survival[96]. CAR-T cells also experience DNA methylation reprogramming after infusion into patients' bodies[97]. Wang et al.[98] have demonstrated that, compared with untreated CD19 CAR-T cells, CD19 CAR-T cells primed with decitabine (dCAR-T) show decreased expression of exhaustion and immune suppression-associated genes, but increased expression of memory and proliferation-associated genes (e.g., *TCF7*, *LEF1*, and *IL-7R*). Consequently, dCAR-T cells are superior in terms of proliferation, cytotoxicity, central memory phenotype, and cytokine/chemokine secretion, even after repeated antigen stimulation. In Raji-based models, dCAR-T cells rapidly eliminate the tumor burden, improve the survival rate, and display more favorable expansion and homing ability than conventional CAR-T cells[98]. Prinzing et al.[99] have constructed DNMT3A-KO CAR-T cells by using CRISPR/Cas9, and demonstrated their superiority in both solid tumors and leukemia. Their findings have indicated that DNMT3A-regulated genes are enriched in TCR signaling, lymphocyte migration, and apoptosis related pathways. The deficiency in DNMT3A impeded *de novo* methylation, thereby restricting the terminal differentiation of T cells and maintaining their stem cell-like memory phenotype (**Figure 3A**). The deletion of other DNMT subtypes and the use of other knockout methods are worthy of further exploration in lymphomas.

Targeting histone acetylation

Histones can be epigenetically modified in a more diverse manner than DNA, for example through methylation, acetylation, and ubiquitination. Histone acetylation is inversely regulated by histone acetyltransferases and histone deacetylases (HDACs). The addition of acetyl groups weakens the interaction between histones and DNA, and leads to a loosened chromatin structure that facilitates gene transcription. HDAC inhibitor (HDACi) treatment upregulates tumor antigen and CAR expression at the post-transcriptional level, and increases CAR-T cell recognition and killing of tumor cells, potentially *via* activated protein transportation signaling pathways[100,101]. Silencing of HDAC11 with shRNA enhances the proliferation and cytolysis of CAR-T cells, thereby maintaining the central memory phenotype and decreasing the proportion of PD-1-TIM-3 double-positive cells after exposure to tumor antigens and ultimately enable a successful recall response (**Figure 3B**)[102]. HDAC1 expression is significantly higher in CAR-T cells than conventional T cells, and this expression further increases during antigen stimulation-induced exhaustion. Class I HDACi treatment decreases HDAC1 expression and enhances H3K27ac activity, while preserving the differentiation and memory potential of CAR-T cells by activating the Wnt/β-catenin pathway[103]. The ubiquitin ligase Cbl-b has also been found to be up-regulated in exhausted TILs, whereas knockout of Cbl-b enhances the anti-tumor capability of CAR-T cells[104]. Many targets remain to be explored in epigenetic modifications of histones in CAR-T cell therapy.

Metabolism and CAR-T cells

Generally, lymphoma cells show enhanced uptake of glucose and glutamine, highly efficient *de novo* synthesis of fatty acids, and preferential utilization of glycolysis as an energy source. The high metabolic rate adapted to high proliferation leads to formation of a TME with low oxygen, nutrient deficiency, and high concentrations of lactate, and ultimately up-regulated expression of immune checkpoints and functional impairment of TILs. Mechanistically, this response is attributed to MYC expression and activation of the PI3K-Akt-mTOR pathway[105]. High metabolic status of tumors is associated with poor prognosis and enhanced severity of adverse events after CAR-T cell infusion[106]. Therefore, modifications targeting metabolism are indispensable for CAR-T cell therapy. Appropriate nutrient restriction, relatively high levels of IL-7, IL-15, and IL-21, and 4-1BB co-stimulation improve the metabolic fitness of CAR-T cells[107]. In this section, we discuss the effects of nutrient metabolism on CAR-T cells and corresponding improvement strategies, particularly focusing on redirecting glycolysis to OXPHOS.

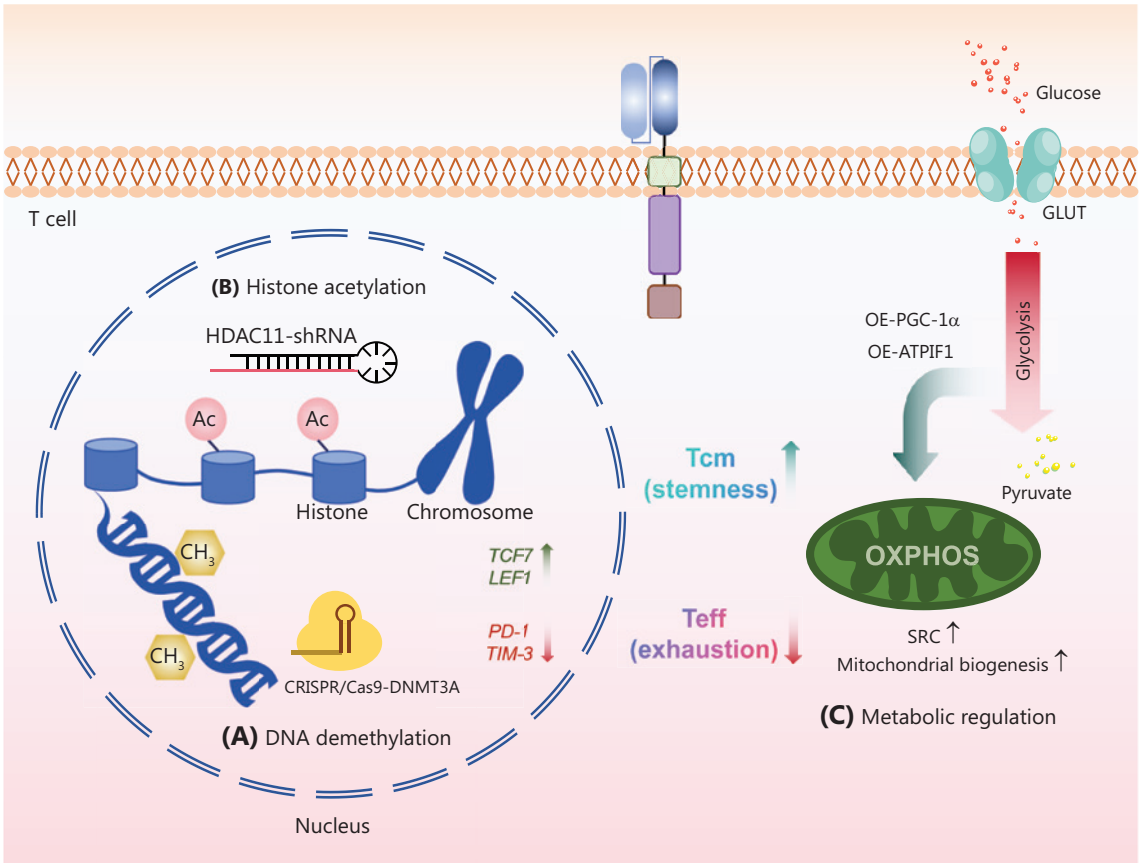


Figure 3 Epigenetics and metabolism in the regulation of CAR-T cells. (A) DNA methylation contributes to tumor immune escape by down-regulating tumor antigen expression and CAR expression, while also impairing the stemness of CAR-T cells. DNA demethylation (e.g., DNMT3A deletion) promotes cell memory and stemness by up-regulating stemness-associated genes (e.g., TCF7 and LEF1) and down-regulating exhaustion-associated genes (e.g., PD-1 and TIM-3). (B) Histone acetylation (e.g., HDAC11 silencing) also promotes the central memory phenotype and resistance to cell exhaustion by increasing stemness-associated gene expression while inhibiting exhaustion-associated gene expression. (C) Effector T cells, which are prone to exhaustion, favor glycolysis for supplying energy. Long-lived central memory T cells, which are advantageous in cancer therapy, tend to use OXPHOS for their energy supply. Interfering with key enzymes or intermediates in glucose metabolism (e.g., overexpression of PGC-1α and ATPIF1) can redirect the metabolic preference from glycolysis to OXPHOS, thereby enhancing mitochondrial function, stemness maintenance, and exhaustion resistance. Metabolism regulates cell differentiation and exhaustion through epigenetics, and epigenetics in turn affects metabolic processes. Ac, acetyl group; ATPIF1, adenosine triphosphate synthase inhibitory factor 1; CH_3, methyl group; DNMT3A, DNA methyltransferase 3A; GLUT, glucose transporter; HDAC11, histone deacetylase 11; LEF1, lymphoid enhancer binding factor 1; OE, overexpression; OXPHOS, oxidative phosphorylation; PD-1, programmed cell death-1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; SRC, spare respiratory capacity; TCF7, transcription factor 7; Tcm, central memory T cell; Teff, effector T cell; TIM-3, T cell immunoglobulin and mucin-domain-containing-3.

Targeting glucose metabolism

During the differentiation of naïve T cells into effector T cells (Teffs), glycolysis is used to meet the energy demands of rapid activation. In contrast, memory T cells are relatively stable and tend to use OXPHOS for their energy supply[107]. Teffs are prone to exhaustion, whereas T cells with a central memory phenotype are favored in cancer therapy, because of their stemness. Accordingly, the modification and cultivation strategies for CAR-T cells are aimed at inhibiting glycolysis, and improving mitochondrial biosynthesis and OXPHOS (**Figure 3C**). The oncometabolite D-2-hydroxyglutarate (D2HG) inhibits T cell expansion and effector cytokine production, increases glucose uptake, and decreases the proportion of central memory subpopulations. D2HGDH-overexpressing (OE) CD19 CAR-T cells catabolize D2HG into intermediate metabolites

in the tricarboxylic acid cycle, thus enhancing the persistence of CAR-T cells and the survival rate in xenografted mice[108]. The transcriptional coactivator PPAR-γ coactivator 1α (PGC-1α) coordinates mitochondrial biogenesis and antioxidant activity. PGC-1α-OE CAR-T cells have enhanced respiratory capacity, diminished reactive oxygen species damage, and a metabolic profile resembling that of long-lived memory T cells[109]. Single-cell RNA sequencing has identified ATP synthase inhibitory factor 1 (ATPIF1) as a necessary anti-tumor factor. The ATPIF1-OE CD19 CAR-T cells shift glycolysis toward OXPHOS, promote the secretion of IFN-γ and granzyme B, and enhance *in vitro* and *in vivo* anti-tumor efficacy[110]. S-nitrosoglutathione reductase (GSNOR)-OE CAR-T cells alleviate the excessive production of nitric oxide caused by continuous CAR signals, and facilitate memory T cell differentiation and resistance to mitochondrial dysfunction[111]. Other molecules that impair mitochondrial biogenesis and morphology, and decrease spare respiratory capacity, such as NR4A, MCJ, and C1QBP, need to be downregulated in CAR-T cells[112-114].

Beyond direct modification of CAR genes, a more widely used approach involves altering culture conditions or combining small-molecule inhibitors during CAR-T cell manufacturing. Replacing glucose with galactose as a carbon source in the medium enhances mitochondrial activity[115]. Inhibiting the monocarboxylate transporter MCT-1 improves CD19 CAR-T cell efficacy by preventing lactate accumulation[116]. Isocitrate dehydrogenase 2 (IDH2) restricts the pentose phosphate pathway and cytoplasmic citrate availability, thereby decreasing acetyl-CoA levels. Treatment with IDH2 inhibitors decreases the expression of multiple genes in the glycolytic pathway by increasing histone acetylation, and leads to maintenance of chromatin accessibility for genes essential for memory T cell differentiation[117]. Down-regulation of CD38-mediated signaling pathways, inosine supplementation, and inhibition of mitochondrial pyruvate carriers have also been shown to inhibit glycolysis, increase mitochondrial activity, and induce the transcription of stem cell-associated genes *via* epigenetic regulation[118-120]. Metabolism and epigenetics are closely intertwined and together influence the biological processes of cells.

Targeting amino acid and fatty acid metabolism

T cells have low expression of arginine resynthesis enzymes and are sensitive to arginine concentrations. Argininosuccinate synthase (ASS)/ornithine transcarbamylase (OTC)-OE CAR-T cells significantly improve cell proliferation in low arginine TME[121]. Supplementation with branched-chain amino acids enhances the anti-tumor efficacy of CAR-T cells by promoting their proliferation, as well as their secretion of TNF-α and IFN-γ. Yang et al.[122] have constructed a branched-chain α-keto acid dehydrogenase kinase (BCKDK)-OE CAR. These BCKDK-OE CD19 CAR-T cells display intensified cytotoxicity, decreased exhaustion markers, elevated central memory subsets, and diminished apoptosis compare to conventional CD19 CAR-T cells. Some amino acids are unfavorable to CAR-T cells because of their immunosuppressive effects and therefore require accelerated catabolism. Lymphoma cells often express indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenine. Kynurenine up-regulates exhaustion markers such as PD-1 and consequently diminishes T-cell anti-tumor ability[123]. Kynureninase-modified CD19 CAR-T cells enhance the effector memory phenotype and cytokine secretion by blocking kynurenine buildup, and achieve enhanced killing of IDO1-positive cancer cells[124]. A gain-of-function CRISPR screen in CD8(+) T cells has identified proline dehydrogenase 2 (PRODH2) as a booster in CAR-T cell therapy; this enzyme catalyzes the first step of proline catabolism. PRODH2 knock-in has been found to reprogram CAR-T cell metabolism and immunity; decrease glycolysis; boost mitochondrial biosynthesis and OXPHOS; and upregulate memory-associated genes. These PRODH2-OE CAR-T cells show stronger proliferation and killing capacity than their wild-type counterparts in models of both solid tumors and hematological malignancies[125].

Inhibition of acyl-CoA: cholesterol acyltransferase 1 (ACAT1), a key cholesterol esterification enzyme, enhances TCR clustering in CD8(+) T cells and consequently facilitates the formation of immune synapses. In contrast, silencing ACAT1 does not affect OXPHOS, glycolysis, immune checkpoint expression, and the Treg ratio, and therefore is an ideal therapeutic target[126]. Knockdown of ACAT1 by shRNA or pharmacological inhibition with avasimibe in CD19 CAR-T cells increases CD69 and CD107a expression, promotes cell proliferation and secretion of IFN-γ and granzyme B, and enhances anti-tumor efficacy in BCL mouse models[127,128].

Universal CAR-T cell therapy

Gene-edited allogeneic T cells

Patients with lymphoma who require CAR-T cell treatment generally have undergone multiple lines of chemotherapy,

thus resulting in a significant decrease in the number of lymphocytes, insufficient T cell proliferation capability, susceptibility to exhaustion, and a risk of contamination with tumor cells. "Off-the-shelf" universal CAR-T cells from healthy donors (HD) can overcome the deficient quantity and quality of patients' T cells, while providing time and cost savings (**Figure 4A**). However, graft-*vs.*-host disease (GVHD) caused by allogeneic MHC and TCR poses an obstacle[129]. Removing exclusion factors and enhancing tolerance to allogeneic CAR-T products are required. Common gene editing tools include zinc finger nucleases; transcription activator-like effector nucleases (TALENs); and CRISPR and CRISPR-associated (Cas) proteins. Among these, the CRISPR/Cas9 system is the most frequently used method, because of its simplicity, flexibility, and potential for multiplex editing[129].

TCR-negative HD-derived CAR-T cells obtained by knocking out the TCRα constant (TRAC) locus by using TALEN® mRNA exhibit similar oncolytic activity to wild-type CAR-T

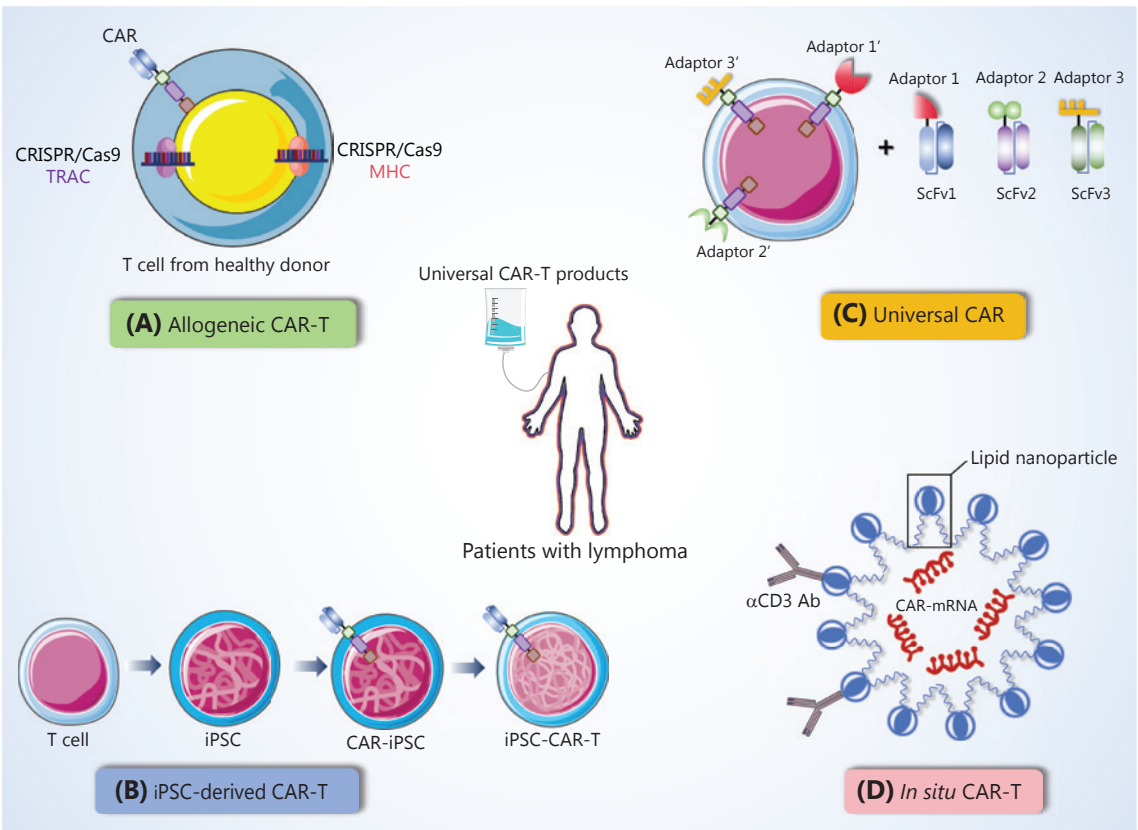


Figure 4 Universal CAR-T cells. (A) Allogeneic CAR-T cells derived from healthy donors address deficiencies in both the quantity and quality of patients' T cells, whereas GVHD caused by allogeneic MHC and TCR is an obstacle. T cells from healthy donors with TRAC and MHC knockout achieved through CRISPR/Cas9 technology demonstrate improved efficacy and diminished risk of GVHD. (B) iPSC-derived CAR-T cells exhibit diminished exhaustion, because of their self-renewal ability and unlimited expansion. The preparation of iPSC-derived CAR-T cells involves several key steps: (1) isolation of T cells, (2) reprogramming of T cells into iPSCs, (3) introduction of the CAR construct into iPSCs, and (4) differentiation of CAR-iPSCs into functional CAR-T cells. (C) Adaptor-dependent universal CARs enable T cells to recognize a wide range of modified antigen-specific molecules, thus enhancing the flexibility and targeting range. In this system, the recognition domain and the signal transduction domain of a CAR are split into 2 distinct parts, each associated with an adaptor. These adaptor-mediated lock-and-key mechanisms allow CARs to recognize multiple antigens either simultaneously or sequentially. (D) *In vivo* CAR-T cell technology enables *in situ* programming of CAR-T cells within the human body, thus eliminating the need for T cells to be removed from their physiological environments. Nanoparticles, such as lipid nanoparticles, carrying CAR-encoding mRNA and anti-CD3 antibodies can be delivered into patients and specifically taken up by T cells, thereby generating CAR-T cells *in situ*. CAR-T, chimeric antigen receptor T-cell; CRISPR, clustered regularly interspaced short palindromic repeats; GVHD, graft-*vs.*-host disease; iPSC, induced pluripotent stem cell; MHC, major histocompatibility complex; TRAC, T-cell receptor α constant.

cells when co-cultured with Raji cells. HD-derived CAR-T cells have been found to contribute to more rapid tumor control and higher survival rates in lymphoma mouse models compared to CAR-T cells derived from patients[130]. TRAC-KO CAR-T cells obtained with CRISPR/Cas9 are essentially the same as wild-type CAR-T cells in terms of phenotype, antitumor ability, and mitochondrial activity, and have been found to improve the survival rate without GVHD incidence in a mouse model of Burkitt's lymphoma[131]. Another source of immunogenicity is allogeneic MHC. Preclinical studies have demonstrated that TCR-MHCI double-KO CAR-T cells constructed by simultaneous removal of β-2 microglobulin and TRAC have killing ability equal to that of wild-type CAR-T cells[132]. A phase I clinical trial of the corresponding product CTX110 is currently ongoing, but the preliminary results have reassuringly indicated an ORR > 50% and no occurrence of GVHD[133]. Furthermore, TCR-MHCI-MHCII triple-KO CAR-T cells have been demonstrated to be more persistent in the allogeneic environments with successful handling of tumor rechallenge compared to single- and double-KO CAR-T cells[134]. However, gene-editing methods pose a potential threat to genome stability. Off-target mutations and complex chromatin rearrangements may pose a risk of carcinogenesis[33,35]. The protein expression blocker (PEBL), a non-gene editing approach to down-regulation of endogenous protein expression, is usually applied in TCL to prevent "fratricide." The CD3ε-PEBL includes a CD3ε-scFv and a retention sequence, which anchors the CD3/TCRαβ complex in the endoplasmic reticulum or Golgi apparatus, and subsequently blocks its transportation to the CAR-T cell membrane surface[135]. TCR retention in the cytoplasm abrogates TCR-mediated signaling without affecting the immunophenotype and function of CAR-T cells, or *de novo* TCR synthesis, thereby decreasing the risk of T cell developmental disorders.

Induced pluripotent stem cells

Another source of "off-the shelf" CAR-T cells is induced pluripotent stem cells (iPSCs), which can be reprogrammed from somatic cells by transduction of Yamanaka 4 factors (*OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*). CAR-T cells derived from primary cells, whether autologous or allogeneic, are prone to exhaustion after *in vitro* culture and multiplex editing, thus decreasing production and increasing genetic toxicity. In contrast, iPSCs have a theoretically unlimited expansion ability and are suitable for gene manipulation, and consequently can achieve a variety of functional enhancements and maintain homogeneity (**Figure 4B**)[136]. These iPSC CD19 CAR-T cells have RNA signatures similar to those of conventional CAR-T cells and exhibit comparable degranulation, cytotoxicity, and cytokine secretion abilities. Moreover, iPSC CD19 CAR-T cells preserve the TCR from the initial clones and are more resistant to exhaustion compared to conventional CD19 CAR-T cells because of weaker tonic CAR signaling[137]. Harada et al.[138] have established dual-antigen receptor-rejuvenated CAR-T cells (DRrejTs) targeting LMP in EBV infection-associated lymphoma. iPSCs were first reprogrammed from LMP2-specific CTLs and then inserted into the LMP1-CAR. iPSC-derived DRrejTs show prolonged persistence and induce robust tumor repression in mice. However, iPSC-derived CAR-T cells have several drawbacks, such as the risk of teratoma formation and the need for protocol standardization[139]. The lineage selection of iPSCs and *in vitro* differentiation and amplification systems must be further optimized and homogenized.

Adaptor-dependent universal CARs

To increase flexibility and expand the identification range, a "third-party" intermediary system, which divides the antigen targeting domain and the T cell signaling transduction domains, has been introduced into the CAR. This "lock-and-key" module endows CAR-T cells with nearly unlimited antigen specificity and precise controllability, and enables CAR-T cells to recognize and bind various modified antigen-specific molecules simultaneously or sequentially (**Figure 4C**)[140]. The biotin binding immune receptor system uses avidin as the extracellular moiety of T cells to recognize biotinylated antigen specific molecules[141]. A split, universal, and programmable (SUPRA) CAR consists of a receptor with a leucine zipper on T cells and a separate scFv with a leucine zipper adaptor, thereby enabling the adjustment of binding affinity through leucine zipper configuration, and the modulation of the activation threshold and potency of CAR-T cells[142]. Other tags such as FITC, peptide neo-epitope, inert NKG2D, the spyTag-spyCatcher system, and an enzymatic self-labeling SNAPtag have notably been applied in cancer cytotherapy[143]. Switch-mediated universal CARs have enabled replacement and expansion of targeted antigens without CAR-T cell modification, as well as fine-tuning of CAR-T cell activity in a dose-titratable manner. Preclinical and clinical studies on these universal CARs in lymphoma remain limited. The added exogenous sequences or epitopes might require complicated

engineering; moreover, the transient interactions and immunogenicity of the tags might result in poor engraftment.

In situ CAR-T cells

Current studies have indicated that the generation of CAR-T cells is no longer limited to separation, *in vitro* manufacturing, and reinfusion. *In vivo* CAR-T cell technology enables *in situ* programming of CAR-T cells in the human body, thereby allowing T cells to remain in their physiological environments, and saving time and costs. Lentiviral vectors (LVs) and nanocarrier delivery systems are frequently used tools for CAR delivery. Antibodies targeting pan-T cell markers are packaged with the carriers to facilitate specific CAR recognition and internalization by T cells. These CD8-targeted CD4CAR-LVs successfully form CD8(+) CAR-T cells in mice with angioimmunoblastic T-cell lymphoma and almost completely eliminate CD4(+) tumor cells, thus improving the survival rate[144]. LVs pseudotyped with cocal glycoproteins (VivoVec platform) have enhanced stability and efficiency in delivering CARs. CD3-targeted CD19CAR-VivoVec have been found to kill Nalm6 cells while retaining CD19(−) cells, thus dose-dependently decreasing tumor burden in B-ALL mice[145]. Currently, lipid nanoparticles (LNPs) are central to the development of *in vivo* CAR-T cell therapies (**Figure 4D**), because of their substantial loading capacity and lower immunogenicity than viral vectors[146]. Smith et al.[147] have used nanoparticles to deliver CD19 CAR-encoding plasmid DNA to ALL mice and successfully generated CAR-T cells with anti-leukemia efficacy. The CAR modification strategies described earlier can also be used for *in vivo* CAR-T cell therapy. For example, CD3-targeted LNPs containing mRNAs encoding both CAR and IL-7 have been found to significantly improve anti-melanoma efficacy[148], and CD3-targeted CD19CAR-LNPs carrying IL-6-shRNA have been developed to avoid the risk of CRS[149]. Several aspects require further attention, such as LNP stability, optimization of mRNA sequences, multi-dose administration, and the standardization of many processes before clinical application.

Perspectives

Some studies have integrated strategies to construct CAR-T cells with more comprehensive capabilities. CD19/CD20 bispecific CAR-T cells equipped with IL-7 and IL-15 have achieved 95% ORR and 55% CR in patients with R/R NHL[150]. Moreover, CD19 CAR-T cells simultaneously armed with IL-15 and the iC9 suicide gene have shown strong and rapid tumor eradication[151]. Recently, researchers have focused on trogocytosis, in which CAR-T cells and lymphoma cells can act as both donors and recipients. TAA exchange through trogocytosis causes CAR-T cell "fratricide" and consequently facilitates immune escape of tumor cells. Activating transcription factor 3 (ATF3) has been found to promote trogocytosis, and approaches targeting this gene have been developed[152]. Superior CAR-T cells must have characteristics of accurate identification; strong killing ability, stability and persistence; high safety and flexibility; and easy accessibility. More sophisticated modifications might introduce greater operational difficulties and higher genetic risks. Contradictions may exist among the variety of strategies, and a balance must be struck between efficacy and memory, as well as between lethality and safety.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Wenbin Qian, Huilai Zhang.
Collected the data: Tianshu Yu, Xianhuo Wang, Ou Bai.
Wrote the paper: Tianshu Yu, Wenbin Qian.

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