International Journal of Biology and Life Sciences
ISSN: 2957-9511 | Vol. 17, No. 1, 2026

Research Progress on Challenges in In Vivo CAR-T Cell Therapy

Jialing Liu *

College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, Jiangsu, 211816, China
*** Corresponding author Email:** 202321166006@njtech.edu.cn

Abstract: The emergence of chimeric antigen receptor (CAR) T-cell therapy heralds a paradigm shift in cancer immunotherapy. Different from in-vitro CAR-T products, in-vivo CAR-T engineering accomplishes in-situ reprogramming of endogenous T cells, making possible long-lasting anti-tumor cytotoxic activity, which is cost-effective in production and has immediate accessibility as an off-the-shelf product. However, challenges like low target specificity, immune suppression in the tumor microenvironment, treatment resistance, and safety concerns do impede its clinical translational application. This review summarizes the main CAR delivery vectors representative preclinical and clinical studies as well as new strategies to overcome current limitations with the purpose of facilitating the clinical development of this emerging therapy.

Keywords: CAR-T Cell Therapy; Approved Gene Therapy; In Vivo CAR-T Cells; Gene Delivery.

1. Introduction

Despite considerable research efforts and substantial resources having been invested, cancer still continues to be a formidable health burden globally, with several million new cases being detected each year. Driven by population growth and aging, the increase in global cancer incidence has brought about severe socioeconomic and healthcare burdens. Although traditional treatment methods including surgery, chemotherapy, and radiotherapy have made considerable progress, their efficacy remains limited, especially for advanced, drug-resistant, and intractable malignant tumors. Over the past ten years, cancer immunotherapy has come into being as a revolutionary treatment method. By activating and readjusting the host's own immune mechanisms, immunotherapy can arouse targeted anti-tumor effects, together with reduced toxicity, lasting clinical results, and the capacity to get over the drug resistance of traditional pharmacology.

Chimeric antigen receptor (CAR) T-cell therapy is a vital branch in adoptive cell therapy. By means of gene reprogramming, the T lymphocytes that are genetically engineered and bear CAR constructs targeting tumors are made to be able to depend on antigen recognition and then eliminate malignant cells. Generally speaking, CAR-T cells are usually generated via in-vitro or in-vivo engineering strategies. However, the traditional ex vivo CAR-T therapy is beset by the complicated manufacturing process, the lengthy preparation cycle and the extremely high cost, which severely restricts the possibility of its wide clinical application. To surmount these obstacles, the in vivo CAR-T technology has developed rapidly in the past decade. This innovative strategy, which has applied the targeted gene delivery vectors, directly transduces the endogenous T lymphocytes in situ, so when compared with the traditional in vitro protocols, it simplifies the treatment process, shortens the treatment timeline, and also indeed greatly reduces the costs.

2. Limitations of Ex Vivo CAR-T cell therapy

Currently, CAR-T cell therapies are divided into two types, namely in vitro and in vivo. The in vitro CAR-T cell therapy, before re-inputting T lymphocytes into the recipient, includes genetic engineering manipulation as well as in-vitro expansion of them. This technology has already become mature and there is a standardized manufacturing process that accords with the Good Manufacturing Practice for drugs. By the year 2026, more than a dozen autologous or allogeneic CAR-T therapies have been given approvals by regulatory authorities all over the world. Although they are mainly targeted at hematological malignancies (like B-lymphocytic leukemia, plasma cell myeloma, and diffuse large B-cell non-Hodgkin lymphoma), these treatment methods in phase I/II clinical studies have also shown therapeutic potential for some solid malignant tumors, including gastric cancer, glioblastoma, hepatocellular carcinoma, pancreatic adenocarcinoma.

However, the inherent limitations of ex vivo CAR-T therapy do harm to its safe and scalable conditions. The long preparation period makes this method not practical for patients with rapid disease progression. Additionally, the customized characteristic of these living cell therapies has led to high costs, which has obstructed their extensive application. This technology also obviously depends on the quality of T-cells; if the patient's T-cell quantity is insufficient or the function is impaired (T-cell exhaustion), production often just cannot work. In addition, allogeneic cell immunotherapy products are easily rejected by the host immune system and may also trigger graft-versus-host reaction. Concerns about safety still remain as crucial barriers, like cytokine release syndrome (CRS) and immune effector cell-related neurotoxicity syndrome (ICANS), which in extreme cases may lead to severe organ dysfunction. Eventually, long-term efficacy is often restricted by antigen escape, T-lymphocyte depletion and immunosuppressive tumor microenvironments, and as a result, some patients have disease recurrence.

3. In Vivo CAR-T Cell Therapy

In order to relieve the complicated procedures and the overly high costs connected with traditional ex vivo CAR-T platforms, in vivo CAR-T therapy has come into existence, becoming an economically feasible and potentially standardized alternative. Different from the ex vivo mode, in vivo CAR-T therapy makes use of targeted gene delivery vectors and carries out gene redirection on host T lymphocytes through in-situ transduction. By avoiding large-scale in-vitro cell expansion and using systematic vector delivery instead of the infusion of manufactured cell products, this strategy greatly simplifies the therapeutic work process, reduces medical costs, and also eases infusion-related toxicity. However, because the newly generated in-vivo CAR-T cells are not subject to in-vitro quality verification, only the delivery vectors can carry out standardized quality control. Therefore, the combined targeting specificity, transduction efficiency and genomic editing accuracy of these vectors will determine the therapeutic effect and the biosafety status. At present, the virus-based vectors as well as the modified nanocarriers can indeed be considered as the main platforms for in vivo CAR-T targeted delivery.

3.1. Viral Vectors

The viral vector makes use of the innate infectivity of the virus to deliver the CAR transgene into T cells, showing an excellent transduction efficiency. The virus-based systems for in-vivo CAR-T engineering mainly consist of retroviral vectors, lentiviral vectors (LVs) and adeno-associated viral vectors (AAVs). Generally speaking, recombinant virus particles are typically produced by transiently co-transfecting packaging cell lines and multiple plasmids, which promotes the establishment of standardized vector formulations.

Lentiviral Vectors. Based on the genome of human immunodeficiency virus type 1 (HIV-1), the lentiviral vector which has the pathogenic and replication-related genes deleted and retains the efficient cell infection and genome integration ability. As a non-replicable viral vector, lentiviruses (LVs) which can make exogenous transgenic genes be expressed in host cells for a long time. In order to reduce the complexity and cost of the CAR-T therapy for B-cell malignancies, Nicolai et al. constructed a retroviral vector that carries T-cell activation and costimulatory signal cassettes, which is named VivoVec particles, and can directly generate functional CAR-T cells in vivo. In non-human primates that do not have lymphodepletion, the systemic administration of these particles can effectively induce a strong anti-CD20 CAR-T response and lead to B-cell depletion that lasts for more than 10 weeks[1]. Similarly, in the treatment of solid tumors that are brought about by Fanconi anemia (FA), Andrea et al. developed a second-generation lentiviral vector that encodes T1E CAR. This second-generation lentiviral vector is able to effectively generate CAR-T lymphocytes that target pan-ErbB. And these cells come from both healthy donors (HDs) and those who have FA. These genetically engineered CAR-T lymphocytes have shown considerable cytotoxic activity in clinical research and also have an acceptable safety profile and almost negligible treatment-related toxicity as well[2]. Furthermore, numerous investigations have verified the long-term biosafety of LV-modified T cells just like Julie et al. By means of lentiviral vectors (LVs) to deliver gene-modified antigen receptors for addressing human immunodeficiency virus-1 (HIV-1) infection and malignant tumors, after vector integration, no signs of T lymphocyte tumor transformation are detected. The integration site analysis involving 176 patients further confirms that there is no carcinogenic insertion mutagenesis related to secondary malignant tumors [3].

Adeno-associated Virus (AAV). The adeno-associated virus (AAV), which is a non-replicating single-stranded DNA parvovirus, has turned into one of the safest and most widely utilized viral vectors for in-vivo gene therapy since it is non-toxic, has a very low immunogenic potential, has a broad tissue tropism, and can also continuously express transgenes[4]. For example, in the prior research, as Teresa and others demonstrated, the optimization of gene delivery in primary human trabecular meshwork cells is carried out using the transduction property that is inherent in AAV, thereby offering new insights into glaucoma treatment [5].

Retroviral Vectors. The retroviral vector is a replication-defective particle that is genetically engineered from the genome of natural retroviruses. At present, γ-retroviruses (γ-RVs), especially Moloney murine leukemia virus (MLV), function as the main retroviral platforms for CAR-T cell engineering[6].

3.2. Nanoparticle Carriers

Based on nanoparticles, the carriers that are promising non-viral delivery platforms composed of synthetic or biocompatible materials will achieve targeted delivery of cargos and temporal-spatial release through nanoscale biological effects and interface interactions. Compared to viral vectors, nanocarriers that have lower immunogenicity, fairly good biosafety, and wider cell applicability are present there. Specifically, such nanoparticles are able to penetrate quiescent and extremely hard-to-transfect primary cells via endocytosis, membrane fusion or other endocytic mechanisms. Moreover, they are also capable of regulating the half-life of therapeutic payloads in the body and optimizing programmable release kinetics. Nowadays the mainstream non-viral nanocarriers mainly consist of polymer nanoparticles lipid nanoparticles as well as extracellular vesicles which are exosomes.

Polymer Nanocarriers. Polymer nanocarriers which are delivery systems having matrix structures and having particle size ranges from 1 to 1000 nanometers, which are made from polymers of natural origin or through chemical synthesis via self-assembly, emulsification or ion-mediated gelation, support diverse chemical functionalizations as well as controlled payload release[7].Additionally, the manufacturing of thermal-responsive nanocarriers which have the characteristics of lower critical solution temperature (LCST) and higher critical solution temperature (UCST) has further enhanced the precision of drug release regulation as well[8]. Additionally, the reversible deactivation radical polymerization (RDRP) technology can precisely craft the macromolecular structures of the customized polymer prodrug nanocarriers, quite effectively enhancing the delivery efficiency and therapeutic performance indeed[9].

Lipid Nanoparticles (LNPs). LNPs that are those spherical self-assembled nanoparticles are composed of ionizable lipids, auxiliary phospholipids, cholesterol, and polyethylene glycol-lipid conjugates[10]. Because of having a uniform lipid core structure, LNPs have high encapsulation efficiency, expandable manufacturability, and low material cost and so on[7]. Functionally, lipid nanoparticles (LNPs)

which act as protective shells for nucleic acid cargos so as to prevent enzyme degradation, ensure endosomal escape, and then deliver intact cargos to the cytoplasm of target cells are present as per[8]. It is worthy of noting that the delivery based on LNP has turned into a core technology in modern vaccine research and development. Specifically, the LNP-mRNA vaccine has a swift production process and has made significant contributions in the research and clinical transformation of COVID-19 vaccines.

Extracellular Vesicles (EVs, Exosomes). EVs that are those endogenous vesicles which almost all types of cells actively secrete[11] are as natural intercellular delivery entities that cause biological signal transduction to occur by horizontally transferring bioactive molecules. This process which shows high target precision and stability and does not depend on direct cell-to-cell contact is presented. EVs that carry various bioactive substances such as antigens, MHC molecules, immune-related proteins, and functional RNAs, thereby bring about two-way regulating effects on innate and adaptive immune responses. Significantly, neoplasm-generated extracellular vesicles can result in the formation of immunosuppressive neoplastic microenvironments and help tumors evade immune attack.

4. Challenges and Research Advances of In Vivo CAR-T Cell Therapy

In order to attain sufficient therapeutic effectiveness and biocompatibility of in-vivo CAR-T therapy, according to its basic mode of action, there are several key technical pre-requisites that must be fulfilled: (1) The delivery vector has to be precisely combined with endogenous T lymphocytes, with almost no off-target genomic alterations, and at the same time avoid the reprogramming of non-T cell populations; (2) The vector system should endow the body with a relatively higher transduction efficiency, promote the rapid generation of functional CAR-T effector lymphocytes, and keep long-term anti-tumor activity; (3) In the immunosuppressive tumor microenvironment (TME), the CAR-T cells produced in the body need to resist local immunosuppressive signals, like the activation of the PD-1/PD-L1 and CTLA-4/CD28 axes, endure the exhaustion of T lymphocytes, and maintain a stable metabolic homeostasis; (4) The cytokine release and systemic immune response brought about by the proliferation of in-vivo CAR-T need to be strictly controllable, and also have almost no off-target toxicity and minimal vector immunogenicity.

As an emerging therapeutic modality, the in-vivo CAR-T technology still meets with multiple obstacles in regard to transformation. But in recent years it has been developing rapidly technically and has achieved transformative breakthroughs in multiple key research directions.

4.1. Targeting Precision Optimization Strategies

Strict supervision is needed in three key aspects for the precise targeting of the in-vivo CAR-T engineering: vector tropism, antigen recognition, and genome integration. To get over the problems of low transfection efficiency and insufficient cell type selectivity in mouse T cell transfection, a CD8-specific DARPin molecule is screened by the researchers and is presented on the surface of lentiviral and adeno-associated viral vectors respectively generating mCD8-LV and mCD8-AAV[12]. Within the whole mouse blood, there is a transduction specificity of more than 99% for CD8+ T cells realized by mCD8-LV, with the CD19 CAR expression cassette that is effectively delivered and the resulting strong tumor-killing activity induced.Meanwhile, compared with the traditional AAV2, the mCD8-AAV has more than twenty times the increased transduction efficiency and also retains the high-targeting accuracy, thus providing a reliable tool for the mouse T cell-specific CAR gene delivery[12].

In order to solve the problems of low targeting efficiency and poor scalability during CAR-T cell manufacturing, Dai et al. came up with the CLASH high-throughput site-specific insertion platform[13]. This system combines AAV-mediated transduction with Cas12a/Cpf1 mRNA delivery to facilitate accurate, extensive genomic modification in primary human T lymphocytes while minimizing stochastic genomic incorporation, resulting in CAR insertion efficiencies spanning 37.4%–51%[13]. For antigen recognition optimization, Meng et al. replaced conventional single-chain variable fragment (scFv) domains with disulfide-stabilized polycyclic peptides as novel recognition moieties [14]. Such a redesign leads to chimeric antigen receptors (CARs) possessing moderate antigen affinity and rapid binding-dissociation kinetics, making possible antigen density-dependent cytotoxicity. This strategy, which selectively eliminates tumor cells having high antigens while keeping normal tissues having low antigens, rather effectively alleviates the excessive release of cytokines as well[14].

In order to further improve the specificity and safety of treatment, the CAR-T lymphocytes with TCR fuzzy logic gating are designed by Kondo et al. by integrating the distinguishing function of endogenous TCR into the CAR-mediated signal network[15]. The strong TCR-self-antigen binding can strengthen the CAR-mediated activation, whereas the weakened physiological TCR signals can suppress the excessive activation, thereby distinguishing the malignant cells from the normal tissues. For the bispecific TCR/CAR engineered T lymphocytes that target innovative tumor antigens and HER2, in the humanized mouse model, compared with the conventional CAR-T lymphocytes, they demonstrate a stronger therapeutic effect on solid malignant tumors, and the toxicity both inside and outside the tumor is negligible[15].

In the process of improving non-viral delivery, a cationizable lipid nanoparticle (LNP) library was constructed by Margaret and her team, and then the optimal C14-4 formulation was screened out, which is used to solve the problem of the cytotoxicity limitations of traditional electroporation and viral delivery[16]. The LNP system that can efficiently transfer CAR-encoded mRNA into primary human T lymphocytes, with its CAR expression being similar to that of electroporation yet having a significantly reduced cytotoxicity, supports the low-toxicity non-viral CAR-T cell engineering[16]. In order to avoid carrying out antibody binding in targeted delivery, researchers have come up with lipid nanoparticles (LNPs) which are based on cardiolipin-like diphosphamide lipid (CAMP)[17]. The unique topological and mechanical properties of CAMP-LNPs allow T cells to efficiently drill in, and the encapsulated circular mRNA can make transgenic expression last for a long time without additional antibody modification[17].

Jiame and the others further designed the double CD3/CD7-targeted NCtx lipid nanoparticles (LNPs) that at that time encapsulated the microcircular DNA (mcDNA)

carrying the CAR encoding as well as the SB100x transposase messenger ribonucleic acid (mRNA)[18]. The combination system has achieved efficient T-cell targeting and stable CAR genome integration. To enhance the targeting based on extracellular vesicles in lung cancer treatment, Zhu et al. constructed the hybrid nanovesicle (Lip-CExo@PTX) by combining the bispecific MSLN/PD-L1-targeted CAR-T exosomes, the lung-targeting liposomes, and the ones loaded with paclitaxel (PTX) . These bispecific targeting vesicles that can achieve over 95% lung tissue enrichment and that trigger anti-tumor effects through paclitaxel-induced immunogenic cell death, antagonism of programmed death ligand 1-mediated immune suppression as well as improvement of T lymphocyte deficiency exist[19].

4.2. Immunosuppression and Drug Resistance

Overcoming treatment resistance and the immunosuppression mediated by the tumor microenvironment (TME) are two key bottlenecks restricting the efficacy of current CAR-T therapies. Although CAR-T products have shown strong efficacy against hematological malignancies, their therapeutic performance in solid tumors is still rather sub-optimal, mainly because of the inhibitory signals generated by the TME. To improve the durability of the treatment and turn the transient regression of tumors into long-term cures, which is of the greatest significance for promoting the clinical application and translational value of CAR-T therapy.

In the recent research concerning resistance, it was verified by Baldacci et al. that TROP2-targeted CAR-T cells possess the ability to eliminate the residual tumor cells which are resistant to EGFR inhibitors, thus creating a rather promising strategy to enhance clinical prognosis and prevent tumor recurrence as well[20]. From the perspective of mechanism, as demonstrated by Zhong et al., a mechanism of solid tumor resistance mediated by small extracellular vesicles (sEVs) is further described. sEVs secreted by solid tumors carry tumor-specific antigens and PD-L1 which directly interact with CAR-engineered T lymphocytes and then significantly impair the proliferation ability, migration ability as well as the cytolytic activity of CAR-T cells. This tumor defense axis based on sEV has significantly reduced the effectiveness of CAR-T, thereby creating a new intervention target for optimizing the immunotherapy for solid tumors as stated in [21].

Apart from the immune suppression mediated by sEV the tumor cells can also bring about the situation of CAR-T functional impairment through the intercellular protein phagocytosis. The research by Zhai et al. shows that the CAR molecules from the chimeric antigen receptor (CAR)-engineered T lymphocytes will be taken up by the solid tumor cells, and finally lead to the masked tumor antigens and the functional resistance to the treatment achieved. This phenotypic transition is positively related to tumor antigen density and CAR signal sensitivity and depends on the intact cholesterol metabolism pathway. The pharmacological inhibition of cholesterol metabolism by atorvastatin can effectively reverse this resistant phenotype. Moreover that kind of personalized CAR sensitivity matching strategy namely using low sensitivity CAR for high antigen tumors and high sensitivity CAR for low antigen tumors, can greatly enhance the persistence of anti-tumor and also inhibit tumor recurrence as stated in the literature[22].

In order to cope with the immune escape and recurrence of solid tumors, various novel combinations and structural optimization strategies are put forward. There is a study that constructs an extracellular vesicle platform (Exo-OVA-aCD3/aEGFR) derived from tumor antigen-activated dendritic cells.These modified extracellular vesicles that display MHC-antigen complexes and CD86 costimulatory molecules as well as conjugated anti-CD3 and anti-EGFR antibodies can effectively trigger the activation of endogenous T lymphocytes and mediate the communication between tumor-immune cells.The progression, metastasis and recurrence of solid tumors are notably inhibited by this system, and it can also synergize with the anti-PD-L1 immune checkpoint blockade, further enhancing the anti-tumor immunity[23].

Regarding the CAR structure remodeling, Koichi and the others have developed a dual-antigen responsive CAR-T platform strategy that is targeted at GD2/B7-H3 or MSLN/CSPG4.This type of architectural structure separates the CD28 and 4-1BB costimulatory domains while retaining the common CD3ζ signaling chain and uses the CD8α stalk to promote CAR dimerization.Such a framework can ensure the exclusive CAR activation when the dual antigens bind, keep the TCR signal mechanism continuously activated, and balance the glycolysis and oxidative phosphorylation metabolisms. Both in vitro and in vivo experimental models have confirmed that this bispecific targeted CAR-T platform can enhance the anti-tumor efficacy, prolong the treatment persistence, and effectively suppress the tumor escape mediated by antigen loss[24].

4.3. Tumor Microenvironment Remodeling and Metabolic Reprogramming Strategies

Although the in-body CAR-T therapy has the potential to subvert the tradition, most of the contemporary methods used for tumor microenvironment (TME) reconstruction are based on the in-vitro CAR-T engineering framework. However, these pioneering optimization strategies which are for gradually enhancing the in-situ CAR-T treatment method provide the necessary theoretical and technical basis. In order to reverse the TME-driven immune suppression and enhance the intratumoral T cell infiltration, Tian et al. mined The Cancer Genome Atlas (TCGA) dataset and identified key chemokines related to the recruitment of T lymphocytes in solid malignant tumors. With the increasing expression gradient of CCL4/CCL5 within the tumor, the CAR-T lymphocytes that have been modified by CCR5 show significantly enhanced migration ability towards the tumor. Meanwhile, the genetically engineered CAR-T lymphocytes that have the ability to secrete interleukin-12 (IL-12) can effectively repolarize the immunoinhibitory M2-polarized tumor-associated macrophages into proinflammatory M1 phenotypes, thereby greatly reducing the immunoinhibition that is mediated by the local TME[25].

In order to simultaneously eliminate the stromal barriers of the tumor microenvironment and optimize the stemness of CAR-T cells, a dual-functional FAP/IL-15 CAR-T cell constructed by Pang et al.[26] is presented.These cells, by identifying fibroblast activation protein, precisely target and eliminate cancer-associated fibroblasts, thus successfully dismantling the immunosuppressive stromal barrier of solid tumors indeed. Moreover, the autocrine IL-15 signal makes the CAR-T lymphocytes differentiate into stem cell memory T lymphocyte subsets, which greatly increases the cell's survival ability, proliferation potential, continuous activation

ability, and also the anti-tumor efficacy against solid malignant tumors[26].

Another crucial approach which enhances the adaptability of CAR-T cells in the metabolically unfavorable tumor microenvironment is metabolic reprogramming according to Niu et al. An innovative CAR-T lymphocyte is created by simultaneously expressing Foxp3 and the third-generation CAR expression cassette[27]. Mechanistically Foxp3 combines with Drp1 to inhibit mitochondrial fission which will inhibit glycolysis and oxidative phosphorylation while promoting lipid metabolism. In this way, the re-adjusted metabolism makes CAR-T cells have a metabolic advantage similar to that of regulatory T cells greatly enhancing their adaptability in the in vivo tumor microenvironment and the durability of long-term anti-tumor activity. It is worth noting that CAR-T_{Foxp3} cells do not exert an inhibitory immune activity but instead significantly reduce the expressions of exhausted markers including PD-1, LAG-3 and TIM-3. This metabolically optimized strategy effectively enhances the survival ability of CAR-T lymphocytes in the inhibitory tumor microenvironment while retaining the intrinsic anti-tumor efficacy[27].

4.4. CRISPR Gene Editing-Mediated Technical Optimization

It is worth noting that CRISPR gene editing technology, as a highly precise genome engineering tool kit, has significantly facilitated the technological iteration and performance optimization of the current CAR-T therapy. Wu et al. established an ultrasonic-triggered CRISPR, which acts as an innovative pre-treatment method for directed CAR-T therapy with respect to solid tumors[28]. This system, by employing AAV vectors to deliver FUS-inducible CRISPR components in vivo, when focused ultrasound (FUS) is activated, will mediate targeted telomere disruption in tumor cells. This will lead some tumor cells to express a clinically validated antigen, turning them into in-situ immune "training centers". These training hubs identify the induced antigens through their synthesized Notch receptors to activate synNotch CAR-T cells, and then induce the expression of CARs targeting a universal tumor antigen, thereby being able to eliminate adjacent tumor cells. This strategy can regulate the tumor's genome and epigenome in a non-invasive way in both spatial and temporal aspects, providing a method of paradigm shift for solid tumor immunotherapy[28].

In order to further enhance the biosafety and consistency of the in-vivo CAR genome engineering, Nyberg et al. developed a dual-vector co-delivery system for precise T-cell engineering modification[29]. This platform has utilized the encapsulated vectors to deliver the CRISPR-Cas9 ribonucleoprotein complex (RNP) and has also been paired with the directionally evolved AAV-hT7 vectors that have the enhanced T-cell tropism and serum resistance so as to provide the homologous recombination templates. The design with bidirectional vectors optimizes the specificity of T cell targeting and greatly reduces the risks of insertional mutagenesis and tumor antigen escape. Notably, this system achieves the efficient site-specific knock-in of the CAR cassette into the TRAC locus of human T lymphocytes at the in-vivo scale for the first time.The integrated CAR expression driven by the natural endogenous TRAC promoter ensures the uniformity and physiological nature of CAR transcription. Such a design effectively avoids the expression heterogeneity and potential tumorigenicity caused by random virus integration in conventional CAR-T engineering as mentioned in [29].

4.5. CRS and Off-target Toxicity

The cytokine release syndrome (CRS) together with the off-target toxicity has always been the main biosafety obstacle restricting the clinical application of in-vivo CAR-T therapy. To relieve such adverse events, a variety of innovative engineering and nanomaterial-based strategies have been put forward. A cell surface polyethylene glycol (PEG) shielding strategy for in-vivo CAR-T regulation has been established [30]. PEG covalently grafted onto the surface of CAR-T cell membranes forms spatial hindrance that is used to block the abnormal binding between CAR-engineered T lymphocytes and mononuclear phagocytes, thereby inhibiting the excessive secretion of pro-inflammatory cytokines. As the CAR-T cells in the body multiply, the PEG on the surface is gradually diluted which leads to the CAR-engineered T lymphocytes being able to specifically recognize and eliminate tumor cells before the immune activation driven by mononuclear cells. The time-based masking mechanism successfully constructs a controllable and safe therapeutic window[30].

In order to carry out CRS intervention precisely and as required, the TCR signal-responsive cetuximab nanogels (NGs) were developed by Li et al.[31]. The nanogels that are formed through NHS-S-S-NHS cross-linking are non-covalently bound to CAR-T cells by means of anti-CD45, which not only notably prolongs the in-vivo retention time of the nanogels but also keeps the anti-tumor activity of CAR-T. During the period of tumor treatment when CAR-T is overly activated, the increased reducing environment on the surface of T cells will result in the disintegration of nanogels and the release of cetuximab monomers to inhibit cytokine release syndrome (CRS) in-situ. In preclinical models, this system effectively relieves CRS-related symptoms such as high fever, weight loss, vascular leakage, coagulation dysfunction and neurotoxicity and causes no organ damage as well [31].

In addition, to reduce off-target toxicity and systemic inflammation at the same time, a nanophotogenetics platform was developed by Ruan et al. which integrates light-induced CAR (LiCAR) T cells and upconversion nanoparticles (UCNPs)[32]. The LiCAR system can only be fully activated via the dual stimulation of tumor antigens and optical signals, while UCNPs can transform penetrating near-infrared light into functional blue light, thereby enabling the optical regulation of deep tissues. The in vitro and in vivo experimental evaluations confirm that this platform will selectively eliminate CD19-positive hematological and solid tumor cells under near-infrared irradiation, while retaining the non-irradiated tumor tissues and normal parenchymal cells. This strategy effectively reduces off-target tissue damage and can also relieve the cytokine surge related to CRS, providing an innovative safe regulation method for in vivo CAR-T therapy[32].

5. Future Perspectives

Generally speaking, the in-vivo CAR-T therapy is a paradigm-shifting approach that gets over the inherent limitations of traditional ex-vivo manufacturing and has a simplified production process and enhanced economic feasibility. Nevertheless multiple obstacles in terms of transformation still prevent its clinical application, including obstacles such as insufficient in-vivo delivery efficiency,

suboptimal T-cell targeting precision, tumor microenvironment-mediated immune suppression and potential long-term biosafety risks. With the continuous development of vector optimization, the in-vivo CAR-T technology, together with the precise gene editing technology, the multi-antigen cooperative CAR design, and the tumor microenvironment remodeling strategy, is expected to attain stronger therapeutic effects, precise controllability, and higher biosafety. Such technological improvements will further optimize the current immunotherapy regimens and offer a promising clinical alternative for treating patients with refractory malignant tumors, especially those with stubborn solid tumors.

References

[1] Nicolai, C. J., Parker, M. H., Qin, J., Tang, W., Ulrich-Lewis, J. T., Gottschalk, R. J., Cooper, S. E., Hernandez Lopez, S. A., Parrilla, D., Mangio, R. S., Ericson, N. G., Brandes, A. H., Umuhoza, S., Michels, K. R., McDonnell, M. M., Park, L. Y., Shin, S., Leung, W. H., Scharenberg, A. M., Kiem, H. P., Larson, R. P., Beitz, L. O., & Ryu, B. Y. (2024). In vivo CAR T-cell generation in nonhuman primates using lentiviral vectors displaying a multidomain fusion ligand. Blood, 144(9), 977–987. https://doi.org/10.1182/blood.2024024523.

[2] López, A., Charbonnier, D., Vela, P., Díez, B., Río, P., Sánchez, R., Alberquilla, O., Martín-Antonio, B., Minguillón, J., Esquinas, E., García-Escudero, R., Errazquin, R., Del Marro, S., Pascual, A., Lorz, C., Juarranz, Á., Barahona, A., Balmaña, J., Maher, J., Bueren, J. A., & Casado, J. A. (2025). Lentiviral-mediated panErbB CAR-T cell therapy against head and neck squamous cell carcinomas for patients with Fanconi anemia. Molecular Therapy – Oncology, 33(4), 201060. https://doi.org/10.1016/j.omton.2025.201060.

[3] Jadlowsky, J. K., Hexner, E. O., Marshall, A., Grupp, S. A., Frey, N. V., Riley, J. L., Veloso, E., McConville, H., Rogal, W., Czuczman, C., Hwang, W. T., Li, Y., Leskowitz, R. M., Farrelly, O., Karar, J., Christensen, S., Barber-Rotenberg, J., Gaymon, A., Aronson, N., Bernstein, W., Melenhorst, J. J., Roche, A. M., Everett, J. K., Zolnoski, S. A., McFarland, A. G., Reddy, S., Petrichenko, A., Cook, E. J., Lee, C., Gonzalez, V. E., Alexander, K., Kulikovskaya, I., Ramírez-Fernández, Á., Minehart, J. C., Ruella, M., Gill, S. I., Schuster, S. J., Cohen, A. D., Garfall, A. L., Shah, P. D., Porter, D. L., Maude, S. L., Levine, B. L., Siegel, D. L., Chew, A., McKenna, S., Lledo, L., Davis, M. M., Plesa, G., Herbst, F., Stadtmauer, E. A., Tebas, P., DiNofia, A., Haas, A., Haas, N. B., Myers, R., O'Rourke, D. M., Svoboda, J., Tanyi, J. L., Aplenc, R., Jacobson, J. M., Ko, A. H., Cohen, R. B., June, C. H., Bushman, F. D., & Fraietta, J. A. (2025). Long-term safety of lentiviral or gammaretroviral gene-modified T cell therapies. Nature Medicine, 31(4), 1134–1144. https://doi.org/10.1038/s41591-024-03478-6.

[4] Naso, M. F., Tomkowicz, B., Perry, W. L., 3rd, & Strohl, W. R. (2017). Adeno-Associated Virus (AAV) as a vector for gene therapy. BioDrugs, 31(4), 317–334. https://doi.org/10.1007/s40259-017-0234-5.

[5] Borrás, T., Xue, W., Choi, V. W., Bartlett, J. S., Li, G., Samulski, R. J., & Chisolm, S. S. (2006). Mechanisms of AAV transduction in glaucoma-associated human trabecular meshwork cells. The Journal of Gene Medicine, 8(5), 589–602. https://doi.org/10.1002/jgm.886.

[6] Lech, P., & Somia, N. V. (2008). Retrovirus vectors. Contributions to Nephrology, 159, 30–46. https://doi.org/10.1159/000125574.

[7] Date, T., Nimbalkar, V., Kamat, J., Mittal, A., Mahato, R. I., & Chitkara, D. (2018). Lipid-polymer hybrid nanocarriers for delivering cancer therapeutics. Journal of Controlled Release, 271, 60–73. https://doi.org/10.1016/j.jconrel.2017.12.016.

[8] Bordat, A., Boissenot, T., Nicolas, J., & Tsapis, N. (2019). Thermoresponsive polymer nanocarriers for biomedical applications. Advanced Drug Delivery Reviews, 138, 167–192. https://doi.org/10.1016/j.addr.2018.10.005.

[9] Guerassimoff, L., Ferrere, M., Bossion, A., & Nicolas, J. (2024). Stimuli-sensitive polymer prodrug nanocarriers by reversible-deactivation radical polymerization. Chemical Society Reviews, 53(12), 6511–6567. https://doi.org/10.1039/d2cs01060g.

[10] Eygeris, Y., Gupta, M., Kim, J., & Sahay, G. (2022). Chemistry of lipid nanoparticles for RNA delivery. Accounts of Chemical Research, 55(1), 2–12. https://doi.org/10.1021/acs.accounts.1c00544.

[11] Ibrahim, S. A., & Khan, Y. S. (2023). Histology, extracellular vesicles. In StatPearls. StatPearls Publishing.

[12] Michels, A., Frank, A. M., Günther, D. M., Mataei, M., Börner, K., Grimm, D., Hartmann, J., & Buchholz, C. J. (2021). Lentiviral and adeno-associated vectors efficiently transduce mouse T lymphocytes when targeted to murine CD8. Molecular Therapy – Methods & Clinical Development, 23, 334–347. https://doi.org/10.1016/j.omtm.2021.09.014.

[13] Dai, X., Park, J. J., Du, Y., Na, Z., Lam, S. Z., Chow, R. D., Renauer, P. A., Gu, J., Xin, S., Chu, Z., Liao, C., Clark, P., Zhao, H., Slavoff, S., & Chen, S. (2023). Massively parallel knock-in engineering of human T cells. Nature Biotechnology, 41(9), 1239–1255. https://doi.org/10.1038/s41587-022-01639-x.

[14] Meng, X., Fu, K., Liu, Y., Liu, Y., Zhang, J. Z., Kang, X., Wu, C., & Tsai, Y. H. (2026). Disulfide-directed multicyclic peptides for chimeric antigen receptors targeting solid tumors. Journal of the American Chemical Society, 148(1), 400–413. https://doi.org/10.1021/jacs.5c13642.

[15] Kondo, T., Bourassa, F. X. P., Achar, S., DuSold, J., Céspedes, P. F., Ando, M., Dwivedi, A., Moraly, J., Chien, C., Majdoul, S., Kenet, A. L., Wahlsten, M., Kvalvaag, A., Jenkins, E., Kim, S. P., Ade, C. M., Yu, Z., Gaud, G., Davila, M., Love, P., Yang, J. C., Dustin, M. L., Altan-Bonnet, G., François, P., Taylor, N. (2025). Engineering TCR-controlled fuzzy logic into CAR T cells enhances therapeutic specificity. Cell, 188(9), 2372–2389.e35. https://doi.org/10.1016/j.cell.2025.03.017.

[16] Billingsley, M. M., Singh, N., Ravikumar, P., Zhang, R., June, C. H., & Mitchell, M. J. (2020). Ionizable lipid nanoparticle-mediated mRNA delivery for human CAR T cell engineering. Nano Letters, 20(3), 1578–1589. https://doi.org/10.1021/acs.nanolett.9b04246.

[17] Zhang, Z., Ma, B., Li, B., Li, Z., Gao, M., Zhao, H., Peng, R., Hu, J., Wang, Y., You, W., Gui, X., Wang, R., Hu, X., Chen, B., Zhang, Y., Hao, Y., Sun, X., Rao, P., Zhang, L., Lu, M., Zhou, D., Yang, Y., Deng, M., & Miao, L. (2025). Cardiolipin-mimic lipid nanoparticles without antibody modification delivered senolytic in vivo CAR-T therapy for inflamm-aging. Cell Reports Medicine, 6(7), 102209. https://doi.org/10.1016/j.xcrm.2025.102209.

[18] Bimbo, J. F., van Diest, E., Murphy, D. E., Ashoti, A., Evers, M. J. W., Narayanavari, S. A., Vaz, D. P., Rijssemus, H., Zotou, C., Saber, N., Lei, Z., Mayrhofer, P., Geerlings, M., Schiffelers, R., & Lubelski, J. (2025). T cell-specific non-viral DNA delivery and in vivo CAR-T generation using targeted lipid nanoparticles. Journal for Immunotherapy of Cancer, 13(7), e011759. https://doi.org/10.1136/jitc-2025-011759.

[19] Zhu, T., Chen, Z., Jiang, G., & Huang, X. (2023). Sequential targeting hybrid nanovesicles composed of chimeric antigen receptor T-cell-derived exosomes and liposomes for enhanced cancer immunochemotherapy. ACS Nano, 17(17), 16770–16786. https://doi.org/10.1021/acsnano.3c03456.

[20] Baldacci, S., Brea, E. J., Facchinetti, F., Li, Z., Ngo, K., Malhotra, S., Booker, M. A., Tolstorukov, M. Y., Chakravarti,

S., Hinchey, C., Mahadevan, N. R., Lococo, F., D'Agnelli, S., Gnetti, L., Campanini, N., Leonetti, A., Feng, W. W., Tsai, J. A., Hartley, A. V., Locquet, M. A., Fournel, L., Alifano, M., Mansuet-Lupo, A., Saldanha, A., Haller, W., Zasadil, L. M., Zielinska, M., Bui, K., Tuladhar, B., Lizotte, P. H., Ivanova, E. V., Sequist, L. V., Gokhale, P. C., Paweletz, C. P., Smith, E. L., Jänne, P. A., & Barbie, D. A. (2025). Eradicating drug-tolerant persister cells in EGFR-mutated non-small cell lung cancer by targeting TROP2 with CAR-T cellular therapy. Cancer Discovery, 15(11), 2235–2250. https://doi.org/10.1158/2159-8290.CD-24-1515.

[21] Zhong, W., Xiao, Z., Qin, Z., Yang, J., Wen, Y., Yu, Z., Li, Y., Sheppard, N. C., Fuchs, S. Y., Xu, X., Herlyn, M., June, C. H., Puré, E., & Guo, W. (2023). Tumor-derived small extracellular vesicles inhibit the efficacy of CAR T cells against solid tumors. Cancer Research, 83(16), 2790–2806. https://doi.org/10.1158/0008-5472.CAN-22-2220.

[22] Zhai, Y., Du, Y., Li, G., Yu, M., Hu, H., Pan, C., Wang, D., Shi, Z., Yan, X., Li, X., Jiang, T., & Zhang, W. (2023). Trogocytosis of CAR molecule regulates CAR-T cell dysfunction and tumor antigen escape. Signal Transduction and Targeted Therapy, 8(1), 457. https://doi.org/10.1038/s41392-023-01708-w.

[23] Fan, M., Liu, H., Yan, H., Che, R., Jin, Y., Yang, X., Zhou, X., Yang, H., Ge, K., Liang, X. J., Zhang, J., & Li, Z. (2022). A CAR T-inspiring platform based on antibody-engineered exosomes from antigen-feeding dendritic cells for precise solid tumor therapy. Biomaterials, 282, 121424. https://doi.org/10.1016/j.biomaterials.2022.121424.

[24] Hirabayashi, K., Du, H., Xu, Y., Shou, P., Zhou, X., Fucá, G., Landoni, E., Sun, C., Chen, Y., Savoldo, B., & Dotti, G. (2021). Dual targeting CAR-T cells with optimal costimulation and metabolic fitness enhance antitumor activity and prevent escape in solid tumors. Nature Cancer, 2(9), 904–918. https://doi.org/10.1038/s43018-021-00244-2.

[25] Tian, Y., Zhang, L., Ping, Y., Zhang, Z., Yao, C., Shen, C., Li, F., Wen, C., & Zhang, Y. (2025). CCR5 and IL-12 co-expression in CAR T cells improves antitumor efficacy by reprogramming tumor microenvironment in solid tumors. Cancer Immunology, Immunotherapy, 74(2), 55. https://doi.org/10.1007/s00262-024-03909-w.

[26] Pang, Y., Chen, L., Sun, Q., He, N., Lai, Z., Wang, X., Xie, Z., Yang, W., & Wang, W. (2025). A new strategy for CAR-T therapy in solid tumors: IL-15-autocrine signaling augments tumor stroma depletion and promotes a TSCM subset in the TME. Cell Death & Disease, 17(1), 39. https://doi.org/10.1038/s41419-025-08405-2.

[27] Niu, C., Wei, H., Pan, X., Wang, Y., Song, H., Li, C., Qie, J., Qian, J., Mo, S., Zheng, W., Zhuma, K., Lv, Z., Gao, Y., Zhang, D., Yang, H., Liu, R., Wang, L., Tu, W., Liu, J., Chu, Y., & Luo, F. (2025). Foxp3 confers long-term efficacy of chimeric antigen receptor-T cells via metabolic reprogramming. Cell Metabolism, 37(6), 1426–1441.e7. https://doi.org/10.1016/j.cmet.2025.04.008.

[28] Wu, Y., Huang, Z., Liu, Y., He, P., Wang, Y., Yan, L., Wang, X., Gao, S., Zhou, X., Yoon, C. W., Sun, K., Situ, Y., Ho, P., Zeng, Y., Yuan, Z., Zhu, L., Zhou, Q., Zhao, Y., Liu, T., Kwong, G. A., Chien, S., Liu, L., & Wang, Y. (2024). Ultrasound control of genomic regulatory toolboxes for cancer immunotherapy. Nature Communications, 15(1), 10444. https://doi.org/10.1038/s41467-024-54477-7.

[29] Nyberg, W. A., Bernard, P. L., Ngo, W., Wang, C. H., Ark, J., Rothrock, A., Borgo, G. M., Kimmerly, G. R., Jung, J. H., Allain, V., Hamilton, J. R., Baldwin, A., Stickels, R., Wyman, S., Khan, S. H., Lang, S., Marsh, D., Almudhfar, N., Novick, C., Mortazavi, Y., Zhang, S., AbdElwakil, M. M., Sandoval, L. R., Hwang, S., Chu, S. N., Jung, H., Liu, C., Sharma, D., McCreary, T., Li, Z., Satpathy, A. T., Carnevale, J., Rutishauser, R. L., Cromer, M. K., Roybal, K. T., Dodgson, S. E., Doudna, J. A., Asokan, A., & Eyquem, J. (2026). In vivo site-specific engineering to reprogram T cells. Nature, 652(8110), 712–721. https://doi.org/10.1038/s41586-026-10235-x.

[30] Gong, N., Han, X., Xue, L., El-Mayta, R., Metzloff, A. E., Billingsley, M. M., Hamilton, A. G., & Mitchell, M. J. (2023). In situ PEGylation of CAR T cells alleviates cytokine release syndrome and neurotoxicity. Nature Materials, 22(12), 1571–1580. https://doi.org/10.1038/s41563-023-01646-6.

[31] Li, H. M., Wang, Z. C., Huang, L., et al. (2025). Suppression of cytokine release syndrome by conjugating TCR signaling-responsive siltuximab nanogels with CAR-T cells. Nano Research, 18, 94908075. https://doi.org/10.26599/NR.2025.94908075.

[32] Nguyen, N. T., Huang, K., Zeng, H., Jing, J., Wang, R., Fang, S., Chen, J., Liu, X., Huang, Z., You, M. J., Rao, A., Huang, Y., Han, G., & Zhou, Y. (2021). Nano-optogenetic engineering of CAR T cells for precision immunotherapy with enhanced safety. Nature Nanotechnology, 16(12), 1424–1434. https://doi.org/10.1038/s41565-021-00982-5.