Advancements and challenges in developing *in vivo* CAR T cell therapies for cancer treatment

Thuy Anh Bui,[a,b,e] Haoqi Mei,[a] Rui Sang,[c] David Gallego Ortega,[a,d,e] and Wei Deng[a,c,*]

[a]School of Biomedical Engineering, University of Technology Sydney, Ultimo, NSW 2007, Australia
[b]Whitlam Orthopaedic Research Centre, Ingham Institute for Applied Medical Research, Liverpool, NSW 2170, Australia
[c]Graduate School of Biomedical Engineering, ARC Centre of Excellence in Nanoscale Biophotonics, Faculty of Engineering, UNSW Sydney, NSW 2052, Australia
[d]Garvan Institute of Medical Research, The Kinghorn Cancer Centre, Darlinghurst, NSW 2010, Australia
[e]School of Clinical Medicine, Faculty of Medicine, University of New South Wales Sydney, Kensington, NSW 2052, Australia

eBioMedicine
2024;106: 105266
Published Online 1 August 2024
https://doi.org/10.1016/j.ebiom.2024.105266

Summary

The Chimeric Antigen Receptor (CAR) T cell therapy has emerged as a ground-breaking immunotherapeutic approach in cancer treatment. To overcome the complexity and high manufacturing cost associated with current *ex vivo* CAR T cell therapy products, alternative strategies to produce CAR T cells directly in the body have been developed in recent years. These strategies involve the direct infusion of CAR genes via engineered nanocarriers or viral vectors to generate CAR T cells *in situ*. This review offers a comprehensive overview of recent advancements in the development of T cell-targeted CAR generation *in situ*. Additionally, it identifies the challenges associated with *in vivo* CAR T method and potential strategies to overcome these issues.

Keywords: Cancer; *In vivo* CAR-T; Gene editing; Gene delivery

Introduction

Despite considerable research efforts, cancer persists as a significant global health challenge which impacts millions worldwide with a substantial burden on healthcare systems.[1] While conventional cancer treatments like surgery, radiation and chemotherapy have made advancements, their effectiveness remains limited, particularly in cases of advanced or resistant cancers.[2] Recently, several immunotherapy strategies have emerged as a novel strategy for cancer treatment. These include check point inhibitors, chimeric antigen receptor (CAR) T cell therapies, monoclonal antibodies, cancer vaccines and cytokines.[3] Among these strategies, the CAR T therapy demonstrates promising therapeutic outcomes by modifying T cells to induce anti-tumour immune responses, either through direct triggering of cancer cell death or by altering the tumour microenvironment.[4] This pioneering method involves inducing CAR expression in a patient's T cells either *ex vivo* or *in vivo*, allowing them to accurately identify and eliminate cancer cells.[5] Although advancements in *ex vivo* T cell strategy have demonstrated success in addressing different types of blood cancers,[6] with potential applications towards eliminating solid tumours,[6–8] it also faces significant challenges in manufacturing processes and costs, limiting its accessibility to a broad range of patients. To address these issues, the *in vivo* CAR T cell method has been explored and developed in recent years. In this review, we firstly introduce the basics of CAR T therapy, highlighting the current challenges of the *ex vivo* CAR T method. We then discuss the advancements *in vivo* CAR T strategies, including preclinical studies on the development of T cell-targeted delivery systems, progress in industrial development and the technical challenges in developing *in vivo* CAR T therapy. We also emphasise the regulatory considerations and the clinical approval process, providing a clinical perspective on *in vivo* CAR T therapy.

Technological features of CAR T therapies

CAR structure

CARs are modular synthetic cellular receptors with four main regions: an extracellular domain, a hinge region, a transmembrane domain, and intracellular domains; each of which has distinct functions[8] (Fig. 1). First, the extracellular domain, is essential for the targeted recognition and binding to cancer cells. This domain, often derived from a monoclonal antibody, forms a single-chain variable fragment (scFv), which enables CAR T cells to specifically recognise cancer cell surface antigens and trigger T cell activation.[9] Besides scFvs, the extracellular domain can include natural ligands or mutated ligands which are modified to enhance binding specificity and affinity for cancer cell antigens, reducing off-target effects and increasing therapeutic efficacy.[10] It

*Corresponding author. School of Biomedical Engineering, University of Technology Sydney, Ultimo, NSW 2007, Australia.
E-mail address: wei.deng@uts.edu.au (W. Deng).

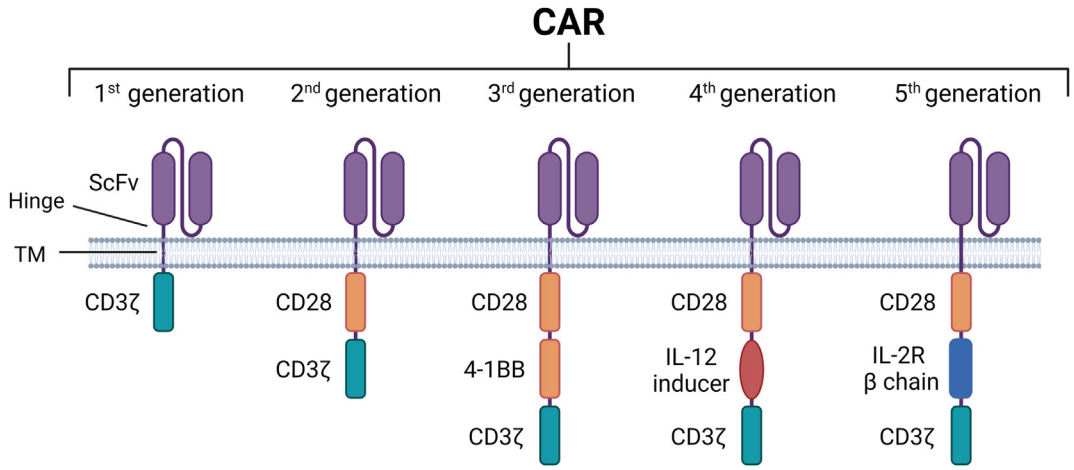

***Fig. 1:* Overview of CAR structure.** All five generations of CAR constructs share common structures with 4 domains: an extracellular domain targeting tumour-specific antigens (ScFV), a hinge region, transmembrane domain (TM), and finally an intracellular domain. As demonstrated, the structure of CAR intracellular domain indicates CAR generation as well as its functional activity. For instance, the CD3ζ domain initiates essential signal transduction pathways necessary for T-cell activation, proliferation, cytokine production, and cytotoxicity. Meanwhile, the CD28 and 4-1BB domains function as co-stimulatory signals, augmenting T-cell activation, persistence, and functionality. The IL-12 inducer domain is employed to prompt cytokine release within the tumour microenvironment, and the IL-2R beta chain mimics IL-2 signalling, enhancing CAR-T cell survival, proliferation, and persistence.

can also include short peptides designed to target specific epitopes on cancer cells, improving stability and binding characteristics as an alternative to traditional scFvs.[11]

The hinge region, the second structure of CAR, is a common connector between the extracellular domain and the transmembrane domain of CAR.[9,12] The hinge region is a common connector between the extracellular domain and the transmembrane domain of CAR.[12] The transmembrane domain anchors the CAR construct through the T cell membrane.[13] Finally, the intracellular domain contains different signalling transduction components similar to those in T cell receptors such as CD3ζ, CD28, IL-2 and 4-1BB.[14,15] This is the domain responsible for triggering the intracellular signal transduction for T cell activation and proliferation, leading to the generation of a new pool of CAR T cells that specifically target and kill cancer cells.[12] Mechanistically, when CAR T cells detect cancer cells, the interaction between the two cell types activates the CAR intracellular domain, which results in the release of cytotoxic molecules by CAR T cells and the initiation of cell death mechanisms in the cancer cells.[16]

To date, CARs have evolved through five generations with different anti-tumour capabilities based on the structure of their intracellular domains (Fig. 1). The first-generation CARs mainly contain the CD3ζ intracellular domain which is responsible for T cell activation with low cytotoxicity and anti-tumour efficacy.[16,17] The second-generation CARs contain CD3ζ and co-stimulatory domain such as CD28, which enhances T cell proliferation.[18,19] The third and fourth CAR generations expanded upon the second CAR generation by incorporating additional signalling domains such as 4-1BB or interleukin 12 (IL-12) cytokines.[20] These constructs have been employed to generate a new cell type known as T cell redirected for antigen-unrestricted cytokine-initiated killing (TRUCKs), which exerts both cytotoxic and cytokine-releasing effects on the targeted cancer cells.[21] The fourth CAR generations also feature dual targeting and logic-gated designs to improve specificity and reduce off-target effects, holding promise for more effective and safer CAR T cell therapy.[22,23] Finally, the fifth-generation CARs possess a structure resembling the second generation but incorporate an additional truncated cytoplasmic IL-2 receptor β-chain domain.[21] This domain allows drug-dependent switches to control CAR T cell functions, thus potentially optimising therapeutic outcomes.[24] However, challenges needs to be overcome for the fifth-generation CARs are off-target and low tumour specificity.[22]

Gene editing tools for CAR T cell generation

Various gene editing systems are employed to induce stable CAR expression in transduced primary T cells. They include transposons, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. Transposon systems, such as Sleeping Beauty and PiggyBac, show potential for introducing CAR constructs into T cells.[25,26] The Sleeping Beauty DNA plasmid system exhibits stable transgene expression with minimal disruption to crucial genes and low genotoxicity.[27] Meanwhile, the PiggyBac

plasmid system can generate CD19 CAR T cells, exhibiting potent anti-tumour activity against acute lymphoblastic leukaemia cells within the central nervous system.[28] However, despite their benefits such as cost-effectiveness and reduced toxicity, challenges with transposon systems remain, including stability issues and the risk of off-target effects.[29,30]

In addition to transposons, ZFN has been employed to examine CAR T therapeutic effects in different animal cancer models as well as in clinical studies.[31] For instance, ZFN has been employed to create HIV-resistant $CD4^+$ T cells via simultaneous deletion of both CCR5 and CXCR4 co-receptors.[32,33] Despite its potential for effective and specific gene editing, optimising the targeting protein molecules in ZFNs can be time-consuming and technically challenging.[34] TALEN, another gene editing tool similar to ZFN, are also being used for CAR production.[35] In clinical trials, TALEN has been employed to introduces CAR constructs specific for certain types of blood cancer, such as acute myeloid leukaemia, advanced lymphoid malignancy, refractory B-ALL, multiple myeloma and B cell acute lymphoblastic leukaemia.[36] However, TALEN has major limitations, including limited space for gene editing tools and the need for extensive nuclease modification for enhanced gene editing outcomes.[35,36] Last but not least, the newly emerged CRISPR/Cas9 offers precise genetic modifications of T cells while improving their effectiveness against cancer cells.[37] The use of CRISPR in generating CAR T cell therapy are currently at early stages of clinical investigation.[38] It is worth noting that, among these four editing tools, the transposon, ZFN and CRISPR systems are suitable for CAR T generation in both *in vivo* and *ex vivo* settings, thus holding promise for novel CAR T-based cancer therapies.[39–41]

Limitations of current *ex vivo* CAR T therapies

Presently, most CAR T cells have been generated outside the body where genetic modification of T cells is performed. This strategy, also known as the *ex vivo* CAR T method, involves harvesting patient- or donor-derived T cells from a blood sample. Next, the isolated T cells are genetically modified to express CAR construct, thus creating a pool of CAR T cells. After that, these modified CAR T cells are expanded in the laboratory before being delivered into the patient's bloodstream via infusion where the therapeutic cells will attack the target cells which present tumour-specific antigens (Fig. 2). This therapy has revolutionised the clinical applications of novel cancer therapies, with several approved products now available to patients with blood cancer and more trials underway for patients with solid tumour (Table 1).

There are two distinct therapeutic strategies for *ex vivo* CAR T therapy: autologous and allogeneic. The autologous approach uses a patient's own T cells to recognise tumour-specific antigens on cancer cell surfaces, allowing for a targeted attack on the tumours.[17,20] Some of the common tumour-specific antigens are CD19, B cell maturation antigen (BCMA), CD22, CD20 and EGFR which present in B cell malignancies, acute myelogenous leukaemia (AML), lung and breast cancer.[73] The allogeneic strategy employs T cells from healthy donors, avoiding the need for patient-derived cells by using modified donor T cells.[74] In certain types of blood cancers, there is a risk of CAR T cell fratricide and depletion due to the presence of common antigens between the leukemic and CAR T cells such as CD7 or CD5.[75,76] To cope with this challenge, CD7-specific CAR T cells can be engineered into universal CAR T cells by deleting both CD7 and α chain of T cell receptors using CRISPR system.[77] This method exhibited a fratricide-resistant therapeutic potentials for relapsed and refractory T cell acute lymphoblastic leukaemia (T-ALL).[77] A similar approach has been applied in a phase I study in which CRISPR base editing system was employed to modify T cells derived from healthy donors for expressing a CAR with specificity for CD7 (CAR7).[78] These modifications were intended to delete CD7 and CD52 receptors, along with the β chain of T cell receptors, preventing graft-versus-host disease and T-cell fratricide and enhancing resistance to alemtuzumab-induced depletion.[78] Additionally further modifications such as permanent deletion of the intrinsic α and β chain of T cell receptors by using ZFN technology can be considered.[79]

Although the *ex vivo* CAR T cell approach has shown promising potential as an anti-cancer therapy, many challenges limit its therapeutic efficacy and safety. First, this therapeutic method carries potential side effects, including cytokine release syndrome and neurotoxicity from the immune response.[50] It requires more thorough clinical oversight and adverse event management.[80,81] Second, tumour cells may develop mechanisms to evade CAR T cell recognition and destruction over time, leading to disease relapse.[8] Third, if the *ex vivo* CAR T therapy involves using donor cells (allogeneic cells), the risk of allogeneic graft rejection becomes a concern.[82] This risk arises when the recipient's immune system to recognise the donor cells as foreign and mount an immune response against them, leading to graft rejection.[83] Last but not least, despite promising outcomes in specific blood cancers, the efficacy of the *ex vivo* CAR T-cell therapy in solid tumours remains limited, thus posing a challenge to its extension across diverse cancer types.[8]

In addition to the main obstacles in therapeutic development, the *ex vivo* CAR T therapies also face significant challenges in industrial development and manufacturing processes. First, the procedure involving isolation, modification, and expansion of T-cells outside the body before re-administration is time-consuming and intricate, spanning several weeks.[84] This prolonged process may not suit patients with rapidly advancing

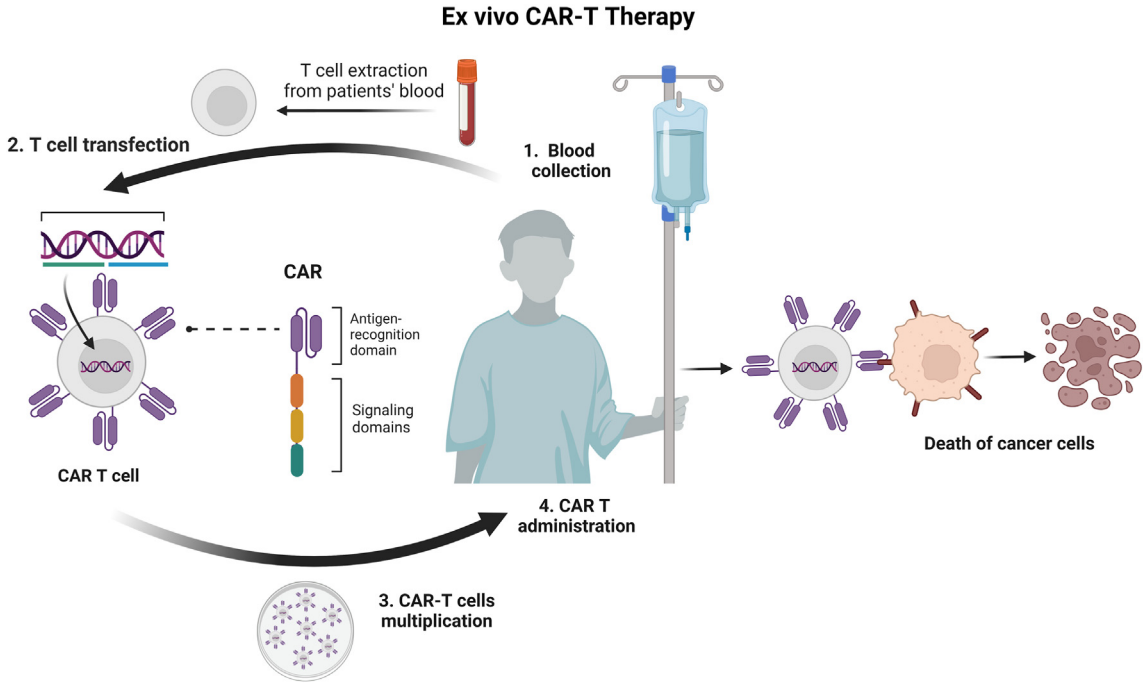

***Fig. 2:* Process of *ex vivo* CAR T therapies.** *Ex vivo* CAR T-cell therapy involves isolating a patient's or healthy donor's T-cells via leukapheresis, genetically modifying them with a CAR construct designed for specific cancer antigens. These modified cells are then cultured and expanded. The expanded and modified T-cells are reintroduced into the patient via infusion. Activated CAR T-cells recognise and destroy cancer cells expressing the targeted antigen, potentially providing long-term immunity against cancer recurrence.

cancers who need urgent treatment.[8] Moreover, this manufacturing process also poses logistical and safety challenges where transport of patient's cells to and from specialised manufacturing facilities is required. It is also technically demanding and costly, thus potentially restricting broad accessibility to this therapy.[85]

In vivo CAR T strategies for cancer treatment

Technical challenges associated with the *ex vivo* CAR T production require the invention of alternative treatment methods that can avoid the complex manufacturing process and reduce costs. Recent advancements have led to the *in vivo* CAR T therapeutic strategy, where T cells are directly engineered into CAR T cells within the patient by using delivery vehicles incorporating gene editing tools (Fig. 3).[86] Compared to the *ex vivo* CAR T therapy, the *in vivo* CAR T method has the potential to reduce manufacturing costs, achieve faster turnaround times and offer greater convenience for patients.[87] In this section, we introduce delivery strategies used for the *in vivo* CAR T method and discuss recent advancements in this field.

Delivery systems for *in vivo* CAR T generation

CAR delivery *in vivo* requires meeting certain criteria including precise T cell targeting, high gene editing efficiency and low toxicity.[88] Currently viral vectors and nanocarriers are widely explored and utilised for *in vivo* delivery of CAR constructs (Fig. 4).[17]

The viral vectors used for CAR T cell therapy are generally produced by transiently transfecting source plasmids into HEK-293T or viral production cell lines to clone the CAR gene into viral vectors. For *in vivo* CAR T cell generation, these viral vectors can be further engineered to specifically target T cells by incorporating T cell-targeting ligands. These ligands can either be genetically fused to the viral envelope protein or attached to the viral vector through chemical conjugation. These provided references offer more detailed methods for viral vectors used *in vivo* CAR T cell generation.[89,90]

The major types of viruses used *in vivo* CAR T cell generation include lentivirus, retrovirus and adeno-associated virus (AAV). Pfeiffer et al. was the first to demonstrate CAR T cells could be generated directly *in vivo* by injection of lentiviral vectors (LV) targeting CD8+ T cells and delivering the CD19-CAR.[91] Following the injection of LV into mice transplanted with human blood cells, CAR T cells were successfully detected in the blood and lymphoid organs. These CAR T cells expanded upon recognizing antigens and effectively eliminated CD19-positive cells. Recently Michels et al. developed anti-CD3 scFv modified LV for *in vivo*

Target	Diseases	Delivery vehicle	Cargo	CAR construct	Approved/Trial phase	Ref
CD19	Relapsed and refractory B cell lymphoma	Lentivirus	Single-stranded RNA genome	AntiCD19-CD8-α-4-1BB- CD3ζ	Tisagenlecleucel	42
CD19	B cell lymphoma	Lentivirus	Single-stranded RNA genome	AntiCD19-CD28-CD3ζ	Axicabtagene ciloleucel	43
CD19	B cell lymphoma	Lentivirus	Single-stranded RNA genome	AntiCD19- IgG4-CD28-4-1BB-CD3ζ	Lisocabtagene maraleucel	44
CD19	Relapsed and refractory B cell lymphoma	Lentivirus	Single-stranded RNA genome	AntiCD19-CD28- CD3ζ	Brexucabtagene autoleucel	45
BMCA	Multiple myeloma	Lentivirus	Single-stranded RNA genome	AntiBMCA-CD8α-4-1BB- CD3ζ	Idecabtagene vicleucel	45
BMCA	Multiple myeloma	Lentivirus	Single-stranded RNA genome	Dual AntiBMCA- CD8α-4-1BB-CD3ζ	Ciltacabtagene autoleucel	46
AJMUC-1	Breast Cancer	Lentivirus	Single-stranded RNA genome (CRISPR/Cas9 editing tool)	AntiMUC1-CD28-CD3ζ	Phase 2	47
MUC1	TnMUC positive solid tumours (triple negative breast cancer, epithelial ovarian cancer, pancreatic cancer and non-small cell lung cancer)	Lentivirus	Single-stranded RNA genome	AntiMUC1-CD8a-CD2- CD3ζ	Phase 1	48
Mesothelin	Triple negative breast cancer	Lentivirus	Single-stranded RNA genome	Antimesothelin-4-1BB- CD3ζ	Phase 1	49,50
EGFR	Glioblastoma	Lentivirus	Single-stranded RNA genome	AntiEGFRvIII-4-1BB- CD3ζ	Phase 1	51
EGFR	Lung, liver and stomach cancer	Lentivirus	Single-stranded RNA genome	AntiPD-1-EGFR-CAR	Phase 2	52,53
CEA	colorectal cancer (CRC), pancreatic cancer (PANC), non-small cell lung cancer (NSCLC)	Lentivirus	Single-stranded RNA genome	AntiCEA-CD8α-CD28-4-1BB-CD CD3ζ	Phase 1/2	54
CLDN18	Advanced gastric/gastroesophageal junction adenocarcinoma Pancreatic cancer	Lentivirus	Single-stranded RNA genome	Humanised anti-CLDN18.2-CD8α-CD28- CD3ζ	Phase 2	55,56
GD2	Neuroblastoma	Retrovirus	Single-stranded RNA genome	AntiGD2- CD28- CD3ζ	Phase 2	57,58
CLDN18	Gastrointestinal cancer	Lentivirus	Single-stranded RNA genome	AntiCLDN18-CD8-CD28- CD3ζ	Phase 1	56
B7H3	Ovarian cancer	Retrovirus	Single-stranded RNA genome	AntiB7H3-CD8α-CD28-CD3ζ	Phase 1, 2	59,60
PSCA	Prostate cancer	Lentivirus	Single-stranded RNA genome	aPSCA-4-1BB/TCRzeta-CD19t	Phase 1	61-63
GPC-3	Hepatocellular carcinoma	Lentivirus	Single-stranded RNA genome	AntiGPC3-CD8-CD28- CD3ζ	Phase 1	64-68
CD70	Relapsed or refractory renal cell carcinoma	Lentivirus	Single-stranded RNA genome	AntiCD70-TRC-Costimulatory domain- CD3ζ	Phase 1	69-71
NKG2D	Unresectable metastatic colorectal cancer	Retrovirus	Single-stranded RNA genome	NKG2DR-CD3ζ	Phase 1	72
CLDN18	Gastrointestinal cancer	Lentivirus	Single-stranded RNA genome	AntiCLDN18-CD8-CD28- CD3ζ	Phase 1	56

[a]We highlighted these ongoing trials because they primarily target common tumour markers in various solid tumours and have reached either phase 1 (for some solid tumours) or phase 2 stage (for other solid tumours).

Table 1: **FDA approved *ex vivo* CAR T products for blood cancer and other representative trials for solid tumour treatment.[a]**

generating anti-CD19 CAR in CD3+ T cells, exhibiting selective expansion in rapamycin and achieving B-cell malignancies elimination in xenograft mouse model.[92] Agarwalla et al. developed an implantable and multi-functional scaffold to simultaneously load patient-derived T cells and CD19-encoding retroviral particles for *in vivo* CAR T cell manufacturing, condensing the entire process to a single day.[93] This scaffold facilitates *in vivo* activation and expansion of T cells through αCD3-and αCD28-mediated cell activation and interleukin-mediated proliferation upon subcutaneous implantation. It sustainably releases fully functional CAR T cells over 5 days and achieves equivalent efficacy in controlling tumour growth as the *ex vivo* CAR T method, while offering better cell expansion and presence in post-tumour clearance.

AAV has also been applied for *in vivo* generation of CAR T cells for human T-cell leukaemia regression in a mouse model.[94] In this work, authors utilised AAV-DJ, a chimera of type 2, type 8 and type 9 AAVs, to deliver a plasmid encoding CD4 CAR gene to T cells in a humanised leukaemic mouse model. However, AAV lacks a membrane envelope for directed delivery to the target sites, thus posing critical technical challenges. Therefore, *in vivo* gene delivery by AAV vectors requires precise selection of AAV serotypes for tissue-specific delivery or modification of AAV capsid proteins with T cell targeting constructs.[88] For instance, the designed ankyrin repeat proteins (DARPins) can be inserted into AAV2 capsid protein to specifically target murine CD8 T cells, resulting in a 20-fold increase in mouse splenocyte CAR gene delivery.[95] This group further engineered bispecific DAPRins-AAV2 to dual-target CD4 and CD32a, enhancing T cell targeting and CAR gene generation *in vivo*.[96] Despite the promising outcomes shown in the preclinical stage, viral vectors raise safety

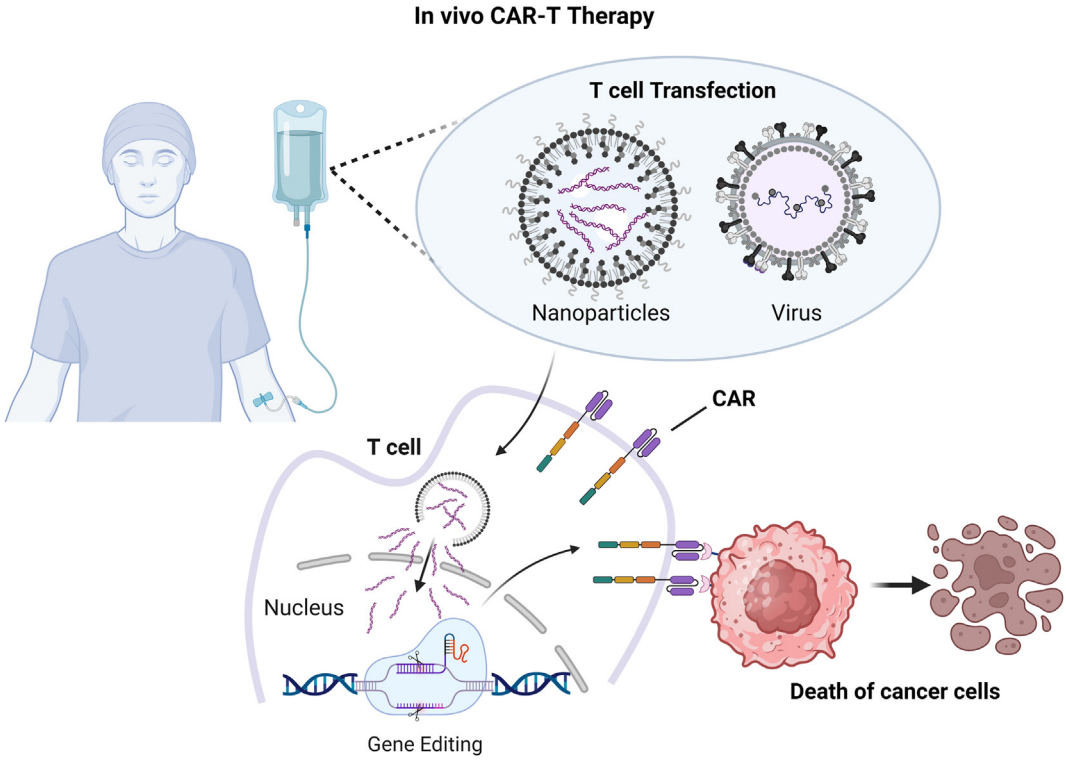

***Fig. 3:* Schematic explanation of the *in vivo* CAR T therapy for cancer treatment.** The *in vivo* CAR T therapy simplifies *ex vivo* CAR T method by systemic administration of the CAR gene editing construct enveloped in viral vectors or nanoparticles. These carriers specifically target T cells to unload gene editing cargo, thus inducing the expression of the CAR construct on the T cell surface. The resulting CAR T cells can then specifically detect cancer cells, thus activating themselves and expanding to effectively eliminate cancer cells in the bloodstream or malignant tumours.

concerns due to their potential to trigger immune responses, causing tissue inflammation.[97]

Nanocarriers have also shown promise for delivering genetic material such as mRNA, plasmid and protein to introduce CAR construct into T cells *in vivo*.[98] The general synthesis methods for these nanocarriers are summarised in several reviews.[99–101] For example, microfluidics has been used to prepare lipid nanoparticles for gene delivery including CAR T constructs.[102] This technique allows precise control over preparation parameters (such as flow rates, components concentrations and mixing ratios), facilitating scalable production for industrial development.[103] Compared with viral vectors, the nanocarriers provide minimal off-target toxicity and immunogenicity[98,104] and they can be manufactured in a large scale.[105] Furthermore, nanocarriers can be custom designed by conjugating with different targeting biomaterials to achieve targeting capability.[104] In addition, they can be chemically conjugated and lyophilised for extended stability.[99]

Currently polymers, lipids and exosomes are the most used nanocarriers for *in vivo* CAR T generation.[99] Cationic polymer nanoparticles can form complexes with negative nucleic acids via electrostatic condensation. The positive surface charge of the complexes also enhances cellular uptake through electrostatic binding to the negatively charged cell membrane.[106] These complexes are more stable due to their ability to avoid enzymatic degradation and escape endosomes thanks to their proton sponge effect.[107] Smith et al. developed polymer nanocarriers for the *in vivo* CAR T therapy, achieving leukaemia regression through targeted T cell programming with minimal side effects.[108] This system, later adapted for CAR-mRNA and T cell receptor (TCR)-mRNA delivery, effectively reprogrammed T cells *in situ* in mouse models of leukaemia, prostate cancer and hepatitis B induced-hepatocellular carcinoma.[109] However, the high toxicity of cationic polymers requires further engineering, including lipid co-complexation, to mitigate toxicity and enhance functionality.[110–113] Lipid nanoparticles and exosomes have also shown promise in facilitating T cell reprogramming *in vivo*.[73,103] Fan et al. developed a CAR T inspiration platform via tumour-antigen stimulated dendritic cell-derived exosomes (tDC-Exo) modified with anti-CD3 and anti-epidermal growth factor receptors (EGFR) antibodies, allowing

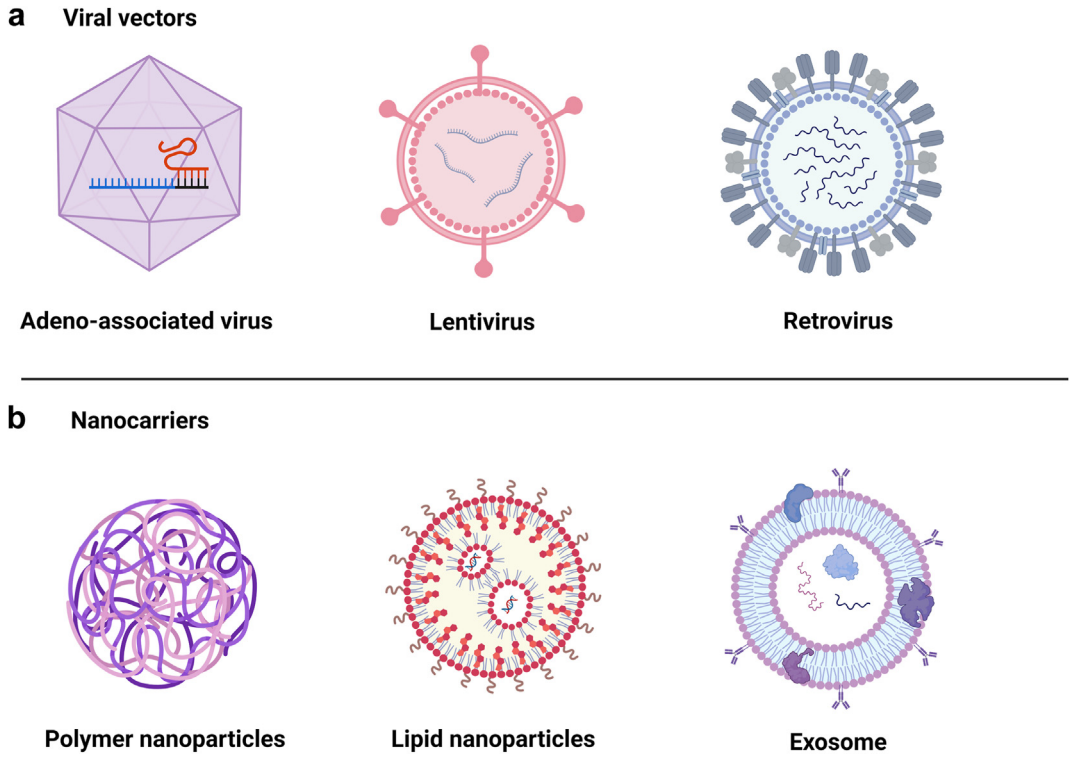

Fig. 4: **Delivery vehicles for *in vivo* CAR T generation.** a. Viral vectors: Adeno-associated virus, lentivirus and retrovirus; b. Nanocarriers: Polymer nanocarriers, lipid nanoparticles and exosome.

simultaneously targeting endogenous T cells and cancer cells.[73] However, the small size of exosomes makes it difficult to effectively package large gene editing tools such as CRISPR/Cas9 RNP. To tackle this challenge, scientists have explored various strategies, including exosome engineering[114,115] and hybrid delivery systems.[116]

Progress of T cell-targeted delivery of CAR construct

As stated above, precise targeting of T cells is a critical factor affecting both the efficacy and safety of the *in vivo* CAR T cell therapy. In this section, we mainly explore recent strategies for developing T cell-targeted CAR delivery by using viral vectors and nanocarriers, with more studies on the *in vivo* T cell therapy listed in Table 2.

Researchers have developed viral vectors to improve the targeting capabilities and maximise *in vivo* T cell editing. For instance, one study reported that LV modified with the T-cell receptor-CD3 complex (CD3-LVs) can specifically identify and transfect circulating T cells to produce CAR gene.[118] This study has demonstrated that CD3-LVs successfully delivered CD19-specific CAR transgenes into T lymphocytes *in vivo* in humanised NSG mice, leading to the elimination of human CD19+ cells from the bloodstream.[118] In another investigation, bispecific LV platform was engineered through the modification of azide group-modified LV with anti-CD3 antibody.[120] These LVs were intravenously administered into humanised NOD-scid-IL2Rγnull (huNSG) mice engrafted with Nalm6-luc cells. The results demonstrated targeted delivery of CD19-CAR genes to T cells both *in vitro* and *in vivo*, with ongoing exploration of their efficiency in tumour elimination.

Research has also shown significant interest in delivering CAR constructs by using targeted nanocarriers. In a recent work, Zhou et al. developed CD3-targeted lipid nanocarriers loaded with plasmids containing interleukin 6 short hairpin RNA (IL-6 shRNA) and CD19-CAR combination genes (AntiCD3-LNP/CAR19 + shIL6).[103] Following tail vein injection into leukaemic nude mice, the nanocarriers were observed to selectively target circulating T cells. This resulted in increased CAR expression and extended anti-tumour effects of CAR T cells in leukaemic mice for up to 41 days post-administration, comparable to *ex vivo* T cell therapy. Additionally, the co-administration of IL6-shRNA for IL-6 knockdown reduced the risk of cytokine release syndrome. In another study, researchers utilised CD3-targeted biodegradable poly (β-amino ester)-based nanoparticles to deliver leukaemia-specific CAR genes to T cells in a leukaemic mouse model.[108] After 4 h of tail vein injection, these T cell-targeted polymer nanoparticles have been found to successfully transfect CAR genes to circulating T cells. This resulted

Disease model	Delivery vector	Cargo	T cell target	CAR construct	Outcomes	Ref
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD8	AntiCD8-LV^{CD19CAR}	Successful production of *in vivo* CAR T cells Clearance of CD19^{+} B malignant cells in 7/10 animals Cytokine release syndrome detected	91
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD8	AntiCD8-CD19-CAR	CD8-LV successfully deliver CD19-CAR Tumour reduction 2 weeks post treatment CAR-high expression in spleen and blood	86
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD4	AntiCD4-CD19-hCD28-hCD3z-CAR	CD4-LV leads 40–60% CAR-positive cells among CD4+ T cells CAR + T cells shows Th1/Th2 phenotype and eliminate CD19+ B cells Tumour cell lysis faster after CD4-LV treatment compared to CD8-LV or CD4/CD8-LV.	117
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD3	AntiCD3-CD19-CD28-CD3z-myc-CAR	CD3+ T cells are efficiently and exclusively transduced. CAR-T generation can be observed Human CD19+ B cells eliminated 20 days post-injection	118
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD3	AntiCD3-CD19-CD8-CD28-CD3z-CAR (SINV-CAR)	Increased CAR T cell count after 24 days of treatment Reduced tumour growth in spleen Prolonged survival time	119
B cell leukaemia	Lentiviral	Single-stranded RNA genome	CD3	AntiCD3-CD19-FMC63-CD3z-4-1BB-CAR (VivoVec)	Dose-dependent CAR T cell transduction Tumour elimination in mice treated with VivoVec Intranodal injection in canines successfully transduced immune cells, exhibiting favourable biodistribution characteristics	92
T cell leukaemia	AAV	Single-stranded DNA genome	CD4	AntiCD4-CD28-4-1BB-CD3z-CAR	Successful *in vivo* CAR T cell generation Tumour regression from day 10 post-treatment Complete tumour regression in 4/6 mice	94
T cell leukaemia	Poly (β-amino ester) polymer nanoparticle	Plasmid DNA	CD3	AntiCD3-CD194-1BB-CD3z-CAR	DNA-carrying nanoparticles effectively bring CAR-gene into T cell nuclei Tumour regression from day 12 post-treatment	108
Acute lymphobastic leukaemia	DSPE-PEG-CD3 antibody-targeted lipid nanoparticles	IL6 shRNA, CAR gene (Double-stranded DNA)	CD3	AntiCD3-LNP/CAR19+shIL6	*In vivo* transfection efficiency of around 8% in AntiCD3-LNP/CAR19+shIL6-treated animals CAR T cell production peaking at day 21 with sustained expression towards day 90 after injection.	103

Table 2: **Preclinical research on *in vivo* CAR T for cancer treatment.**

in tumour regression and enhanced survival by 5 days during a 120-day follow-up period. In a follow-up study, similar polymer nanoparticles were used to transport CAR mRNA to the targeted T cells in animal models of lymphoma, prostate cancer, and HBV-induced hepatocellular carcinoma.[109] This study has shown that these CD3-and CD8-targeted nanoparticles could elevate CAR T cell count, leading to tumour elimination and increased survival rates. More importantly, this therapeutic strategy demonstrated efficiency in immunocompetent leukaemic mice as well as immunodeficient mice carrying solid tumours. However, the lack of evaluation regarding CAR transgene delivery in both studies poses a challenge in quantifying the extent of CAR production via these nanocarrier platforms. Additionally, the poor biodegradability and instability nature of cationic polymers within host could reduce CAR production capacity and compromise the therapeutic effects of the *in vivo* CAR T therapies.[121]

Industrial development of *in vivo* CAR T therapy

The prospects of the viral-based *in vivo* CAR T therapies within the industrial landscape are evolving, thanks to the progress in surface-engineered lentiviral vectors that enable the targeted delivery of CARs to specific T cell subsets. For example, EXUMA Biotech claims to have developed a lentiviral vector encoding a CD19 CAR to target and activate CD3^{+} T cells in the humanised NSG-SGM3 mouse model.[122] Their preclinical data show that *in vivo* editing the CD3^{+} T cells can produce functional CAR T cells and eliminate pre-existing B cells at the same time.[122] In addition to addressing haematological disorders, this company has developed CCT303-406 as a new therapeutic strategy against HER2-positive relapsed or refractory stage IV metastatic solid tumours. This study is currently under a phase I clinical investigation.[119] In a parallel venture to EXUMA Biotech, Umoja Biopharma introduces VivoVec™, an innovative surface-engineered lentiviral vectors designed for *in vivo* CAR-T cell generation.[123] This system consists of lentiviral particles modified with a multi-domain fusion protein on their surface and carries a CD19-targeted CAR transgene. After a single dose of VivoVec™ particles to immune-competent non-human primates, this platform could induce potent and specific generation of functional CAR T cells, peaking between day 7 and day 42, with another peak observed at day 51. More importantly, this treatment leads to persistent B cell aplasia up to 76 days and is well-tolerated, showing no signs of toxicity in the treated primates. This proof-of-concept

study suggests the potential for an off-the-shelf therapy to address the limitations of current *ex vivo* CAR T therapies.

Along with viral vectors, industrial advancements in the nanoparticle-based *in vivo* CAR T therapies also present significant progress. For instance, Ensoma has developed virus-like-particle Engenious™ platform to deliver gene materials up to 35 kilobases of packaged DNA, which is more than seven times the limit of AAV vector.[124] Capstan Therapeutics has successfully generated *in vivo* CD5-specific CAR T cells in a mouse model of heart disease by using mRNA-lipid nanoparticle platform.[125] This company employed CD5-targeting ionisable lipid nanoparticles (CD5/LNP) to encapsulate CAR mRNA molecules targeting fibroblast activation protein during *in vivo* CAR T generation. At 48 h following LNP injection, a new subset of CAR T cells emerged accounting for 20% of the total T cell population.[126] This new CAR T cell population was comprised of 87% $CD4^+$ T cells and 9–10% $CD8^+$ T cells, a ratio indicated a high efficacy of CAR-based treatment.

Challenges of *in vivo* CAR T therapy development

Despite the promising data, the *in vivo* CAR T therapy encounters its own technical challenges. One major issue is efficiently delivering CAR transgenes to the targeted T cells within the body.[127] Viral vectors boast high gene transduction efficiency due to their inherent viral properties that facilitate cellular uptake and nucleus penetration.[128] However, directly infusing patients with viral vectors carrying CAR constructs is associated with limited targeting capability.[129] Moreover, this method also poses the risk of off-target effects, where other cell types may be inadvertently transfected instead of the targeted T cells.[8] To address this issue, researchers have focused on the engineering of viral vectors specifically for targeted delivery to T cells. For example, Jesse Green et al. developed T cell-targeted viral vector by engineering fusogen with a novel single-chain variable fragment targeting human $CD8^+$ T cells.[130] These engineered vectors exhibited the capability to selectively target and transduce non-activated $CD8^+$ T cells that can eliminate CD19-expressing tumours both *in vitro* and *in vivo* settings. Given the risks and high production costs associated with viral vector-based therapies, nanocarrier platforms appear to be more suitable for targeting T cells *in vivo*. Their surface can be conjugated with ligands to target the receptors highly expressed by T cells.[131] To date, the frequently selected receptors include general T cell markers such as CD3,[108] CD4,[132] CD7[133] and CD8,[134] which could be taken into consideration when designing T cell targeting strategies. Moreover, additional research is necessary to enhance the stability and biocompatibility of these nanoparticles within biological environments. This is crucial to prevent degradation and potential adverse reactions in the body before they can effectively reach and targeted T cells.

Another major issue associated with nanocarriers is their limited transfection efficiency, which becomes more apparent when they are used for *in vivo* transfection of genes including CAR constructs. Even with lipid nanoparticles which have been used in clinical settings, they exhibit a very limited ability to escape from the endo/lysosomal compartments before entering the cytoplasm, which can result in enzymatic degradation of genetic cargo (such as mRNA) within the lysosomes.[135] It is important to note that an additional barrier exists for plasmid DNA cargo, which must be transported into the nucleus for transcription and subsequent translation into proteins in the cytosol.[136] To tackle this problem, various strategies have been developed including use of pH-responsive nanocarriers and incorporation of components that trigger escape mechanisms, such as pore-forming proteins or peptides.[137,138]

Achieving sustained functionality and persistence of *in vivo* CAR T cells presents another critical issue in optimising therapeutic outcomes for patients with cancer.[139] Researchers are exploring various strategies to overcome this challenge. One approach involves refining CAR design by incorporating co-stimulatory domains that enhance T cell longevity and activity.[140] Modulating intracellular signalling pathways within CAR T cells using co-stimulatory domains can prolong their functionality.[141] Strategies to prevent T cell exhaustion, such as checkpoint inhibition or cytokine support, are also investigated.[142] Moreover, efforts have been made to promote the formation of memory CAR T cells, enabling lasting anti-tumour responses of *in vivo* CAR T cells in both haematological and solid malignancies.[143]

Similar to the *ex vivo* CAR T therapy, the *in vivo* CART cell therapy for solid tumours is much more challenging compared to haematological malignancies. Solid tumours commonly exhibit immunosuppressive tumour microenvironment which can inhibit T cell activities, thus reducing the efficacy of CAR T therapies. One strategy to enhance the efficacy for solid tumours involves improving the homing of CAR T cells to tumour sites by remodelling tumour vasculature with agents such as Bevacizumab,[144] combretastatin A-4 phosphate[145] or blood–brain barrier permeabilizer NEO100.[60] Moreover, the heterogeneity of antigens in solid tumours poses challenges for CAR-T cells, preventing them from effectively detecting cancer cells and significantly limiting their antitumour function.[146] This issue is linked to the inadequate production of CAR-T cells *in vivo* that target various tumour-associated antigens, thus resulting in the limited efficacy of CAR-T therapy for solid tumours and the occurrence of disease relapse.[147] To address this challenge, several methods have been explored such as co-expression of

several CARs on a single T cell and expression of a chimeric receptor including two or more antigen recognition domains.[148]

In addition to the above mentioned challenges, considering the clinical relevance of the animal models utilised in preclinical studies is also crucial for successful therapy development.[149] Presently, the evaluation of antitumour activity of the *in vivo* CAR T cell therapy primarily relies on xenograft model on immunodeficient mice engrafted with human tumour cell lines.[150] However, these approaches hinder in-depth understanding of cancer progression mechanisms as observed in patients, thereby limiting treatment options. To address this limitation, researchers have adopted patient-derived xenograft (PDX) models, where patient-specific cancer cells are engrafted into immunodeficient mice.[151] This approach enables the assessment of immune responses to primary cancer cells, enhancing the relevance to clinical applications.[151] Nonetheless, the major drawback of the PDX model lies in their use of immunodeficient mouse strains like NOG/NSG, which might evoke different immune responses compared to those potentially detected in patients with cancer, thus restricting the efficacy evaluation of therapeutic strategies.[152] An alternative advancement in preclinical study design involves employing humanised mouse models where nude mice are transplanted with human immune cells to develop fully functional human haematopoietic and immune systems.[150] These models facilitate the examination of safety and efficacy of the *in vivo* CAR T therapy in immunocompetent hosts, providing insights on how human CAR T constructs interact with human tumours while circumventing allogeneic and anti-mouse xeno-antigens.[149] For instance, findings from humanised mouse models suggest that the VivoVec™ consistently exhibits low toxicity and robust generation of functional CAR T cells *in vivo* in non-human primates.[123] This implies its potential safety and efficacy for further clinical investigations.

Regulatory consideration and approval of the *in vivo* CAR T therapy

Similar to the *ex vivo* CAR T therapy, transitioning *in vivo* CAR T products from preclinical success to the clinical settings requires overcoming regulatory and translational hurdles, ensuring theirs safety and efficacy in the clinical setting.[153] Regulatory bodies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA) require viral and nanocarrier vectors to adhere to stringent quality, safety, and efficacy standards prior to approval.[154,155] For instance, toxicology evaluations are crucial for ensuring lipid nanocarrier compliance with regulations, as the accumulation of lipid nanocarriers in healthy tissues can lead to cyto- and/or geno-toxicity.[156] This may be due to the cationic lipid components used in lipid formulations.[157] Meanwhile, viral vectors used in the clinic for CAR gene delivery are also required to carefully test their purity, safety, stability, and functionality.[128,156] Certain viral vectors used to deliver CARs have been implicated to occasional instances of cancer in other gene therapy applications. This is because they carry substantial risks of oncogenic insertional mutagenesis or triggering unwanted inflammatory responses in the modified T cells.[139,158]

Another regulatory concern regarding the *in vivo* CAR T therapeutic strategies is the safety profile of gene editing tools, such as the CRISPR/Cas9 system.[159] For example, it may result in unwanted gene editing events with the introduction of variable length insertion/deletion (indel) at off-target sites within the targeted T cells.[160] These off-target effects can promote functional gene disruption and epigenetic modifications, thus potentially leading to low therapeutic efficacy or even hazardous consequences such as excessive cytokine releases or adverse immune responses within the hosts.[161] To minimise unintended off-target effects of CRISPR/Cas9 editing tool during T cell modification both *in vivo* and *ex vivo*, high-fidelity Cas9 variants could be considered.[160]

In addition to the regulatory considerations, the approval process for the *in vivo* CAR T therapy also pose the challenges during setup of clinical trials.[162] First, establishing suitable patient eligibility criteria for clinical trials is a crucial step. This involves accurately identifying individuals who are most likely to benefit from the treatment with minimal potential risks.[163] Additionally, regulatory agencies demand compelling evidence of efficacy of the *in vivo* CAR T therapy in treating the targeted cancer.[162] This often requires well-designed clinical trials with appropriate dose level, analysis of safety outcomes such as neurotoxicity, cytokine release syndrome events and dose-limiting toxicity alongside with assessment on clinical benefits.[162] Moreover, long-term follow-up data are indispensable for evaluating the safety and efficacy of the therapy over time.[164] This includes monitoring patients for delayed adverse events and assessing treatment response durability.[162] Lastly, determining the appropriate regulatory pathway for *in vivo* CAR T therapy can be complex and time-consuming. Depending on the therapy's classification, it may require approval through various regulatory channels, such as the FDA's Biologics License Application (BLA) or the EMA's Marketing Authorization Application (MAA).[165]

Outstanding questions

In preclinical studies, *in vivo* CAR gene delivery using nanocarriers or viral vectors has produced therapeutic CAR T cells comparable to those generated by the *ex vivo* method. These advancements have shown promise in enhancing cancer treatment strategies. Moving forward,

Search strategy and selection criteria

We conducted a comprehensive review by searching PubMed and Google Scholar and other relevant articles to identify the data for this study. The search terms used included "CAR T therapy," "*In vivo* CAR T", "CAR T delivery systems," "Industrial development of CAR T therapy" and "Ethics consideration of CAR T and gene therapy". Our search was limited to articles in English published within the last thirty years.

current research efforts are attempting to address outstanding questions and limitations, including how to precisely target T cells *in vivo* while improving the transfection efficiency of nanocarriers. Researchers are also exploring methods to enhance the long-term effectiveness of CAR T cells and refine the components of genetic cargo to minimise unintended off-target effects. Furthermore, nonhuman primate models will be crucial for evaluating the safety and pharmacokinetic profiles of the *in vivo* CAR T cell products. Additionally, it is also essential to address manufacturing practices, quality assurance and regulatory considerations to advance this treatment into clinical applications.

Conclusions

While existing *ex vivo* CAR T products are effective, their complexity and high cost limit accessibility and benefit to all patients. As a feasible off-the-shelf alternative, the *in vivo* CAR therapy may address the significant concerns associated with *ex vivo* CAR T therapy. It has the potential to enhance safety and efficacy, reduce treatment waiting times, eliminate the need for lymphodepleting chemotherapy and offer greater convenience to patients. Although some questions and challenges remain, ongoing research efforts and industry investments are essential for realising the full potential of the *in vivo* CAR T cell therapy in revolutionising cancer medicine and benefiting numerous patients.

Contributors

T. Bui and H. Mei conducted the literature search, drafted the manuscript and designed figures. R. Sang drafted the section on delivery systems. D. Ortega contributed to editing the manuscript. W. Deng contributed to conceptualisation of the study and manuscript review and editing. All authors read and approved the final version of the manuscript.

Declaration of interests

The authors report no conflicts of interest in this work.

Acknowledgements

This work was financially supported by the funding (GNT1181889) from the Australian National Health and Medical Research Council and fellowship award (2019/CDF1013) from Cancer Institute NSW, Australia. The funders had no role in paper design, data collection, data analysis, interpretation, writing of the paper.

References

1 Kocarnik JM, Compton K, Dean FE, et al. Cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life years for 29 cancer groups from 2010 to 2019: a systematic analysis for the global burden of disease study 2019. *JAMA Oncol*. 2022;8(3):420–444.
2 Debela DT, Muzazu SG, Heraro KD, et al. New approaches and procedures for cancer treatment: current perspectives. *SAGE Open Med*. 2021;9:20503121211034366.
3 Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med*. 2016;14:73.
4 Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol*. 2020;20(11):651–668.
5 Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA*. 1993;90(2):720–724.
6 Weber EW, Maus MV, Mackall CL. The emerging landscape of immune cell therapies. *Cell*. 2020;181(1):46–62.
7 Sharma P, Allison JP. The future of immune checkpoint therapy. *Science*. 2015;348(6230):56–61.
8 Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J*. 2021;11(4):69.
9 Chailyan A, Marcatili P, Tramontano A. The association of heavy and light chain variable domains in antibodies: implications for antigen specificity. *FEBS J*. 2011;278(16):2858–2866.
10 Ramírez-Chacón A, Betriu-Méndez S, Bartoló-Ibars A, González A, Martí M, Juan M. Ligand-based CAR-T cell: different strategies to drive T cells in future new treatments. *Front Immunol*. 2022;13:932559.
11 Zoine JT, Immadisetty K, Ibanez-Vega J, et al. Peptide-scFv antigen recognition domains effectively confer CAR T cell multiantigen specificity. *Cell Rep Med*. 2024;5(2):101422.
12 Jensen MC, Riddell SR. Designing chimeric antigen receptors to effectively and safely target tumors. *Curr Opin Immunol*. 2015;33:9–15.
13 Guedan S, Posey AD Jr, Shaw C, et al. Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation. *JCI Insight*. 2018;3(1):e96976.
14 Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA*. 1989;86(24):10024–10028.
15 Maher J, Brentjens RJ, Gunset G, Rivière I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta/CD28 receptor. *Nat Biotechnol*. 2002;20(1):70–75.
16 Tokarew N, Ogonek J, Endres S, von Bergwelt-Baildon M, Kobold S. Teaching an old dog new tricks: next-generation CAR T cells. *Br J Cancer*. 2019;120(1):26–37.
17 Zhang C, Liu J, Zhong JF, Zhang X. Engineering CAR-T cells. *Biomark Res*. 2017;5:22.
18 Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol*. 2003;3(12):939–951.
19 Hombach A, Wieczarkowiecz A, Marquardt T, et al. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. *J Immunol*. 2001;167(11):6123–6131.
20 Smith AJ, Oertle J, Warren D, Prato D. Chimeric antigen receptor (CAR) T cell therapy for malignant cancers: summary and perspective. *J Cell Immunother*. 2016;2(2):59–68.
21 Dummy. From CARs to TRUCKs and Beyond: Safely en Route to Adoptive T-cell Therapy for Cancer. *EBioMedicine*. 2016;14:1–2.
22 Dagar G, Gupta A, Masoodi T, et al. Harnessing the potential of CAR-T cell therapy: progress, challenges, and future directions in hematological and solid tumor treatments. *J Transl Med*. 2023;21(1):449.
23 Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. *Expert Opin Biol Ther*. 2015;15(8):1145–1154.

24 Kagoya Y, Tanaka S, Guo T, et al. A novel chimeric antigen receptor containing a JAK–STAT signaling domain mediates superior antitumor effects. *Nat Med*. 2018;24(3):352–359.
25 Huang X, Wilber AC, Bao L, et al. Stable gene transfer and expression in human primary T cells by the sleeping beauty transposon system. *Blood*. 2006;107(2):483–491.
26 Bishop DC, Xu N, Tse B, et al. PiggyBac-engineered T cells expressing CD19-specific CARs that lack IgG1 Fc spacers have potent activity against B-ALL xenografts. *Mol Ther*. 2018;26(8):1883–1895.
27 Maiti SN, Huls H, Singh H, et al. Sleeping beauty system to redirect T-cell specificity for human applications. *J Immunother*. 2013;36(2):112–123.
28 Tanaka K, Kato I, Tanaka M, et al. Direct delivery of piggyBac CD19 CAR T cells has potent anti-tumor activity against ALL cells in CNS in a xenograft mouse model. *Mol Ther Oncolytics*. 2020;18:37–46.
29 Hudecek M, Ivics Z. Non-viral therapeutic cell engineering with the Sleeping Beauty transposon system. *Curr Opin Genet Dev*. 2018;52:100–108.
30 Monjezi R, Miskey C, Gogishvili T, et al. Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. *Leukemia*. 2017;31(1):186–194.
31 Urnov FD, Miller JC, Lee YL, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*. 2005;435(7042):646–651.
32 Didigu CA, Wilen CB, Wang J, et al. Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection. *Blood*. 2014;123(1):61–69.
33 Liu Z, Chen S, Jin X, et al. Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4(+) T cells from HIV-1 infection. *Cell Biosci*. 2017;7:47.
34 Paschon DE, Lussier S, Wangzor T, et al. Diversifying the structure of zinc finger nucleases for high-precision genome editing. *Nat Commun*. 2019;10(1):1133.
35 Holkers M, Maggio I, Liu J, et al. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res*. 2013;41(5):e63.
36 Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: current and future perspectives. *Biosci Rep*. 2020;40(4).
37 Razeghian E, Nasution MKM, Rahman HS, et al. A deep insight into CRISPR/Cas9 application in CAR-T cell-based tumor immunotherapies. *Stem Cell Res Ther*. 2021;12(1):428.
38 Ottaviano G, Georgiadis C, Gkazi SA, et al. Phase 1 clinical trial of CRISPR-engineered CAR19 universal T cells for treatment of children with refractory B cell leukemia. *Sci Transl Med*. 2022;14(668):eabq3010.
39 Harmatz P, Prada CE, Burton BK, et al. First-in-human in vivo genome editing via AAV-zinc-finger nucleases for mucopolysaccharidosis I/II and hemophilia B. *Mol Ther*. 2022;30(12):3587–3600.
40 Prommersberger S, Reiser M, Beckmann J, et al. CARAMBA: a first-in-human clinical trial with SLAMF7 CAR-T cells prepared by virus-free Sleeping Beauty gene transfer to treat multiple myeloma. *Gene Ther*. 2021;28(9):560–571.
41 Hamilton JR, Chen E, Perez BS, et al. In vivo human T cell engineering with enveloped delivery vehicles. *Nat Biotechnol*. 2024. https://doi.org/10.1038/s41587-023-02085-z.
42 Vairy S, Garcia JL, Teira P, Bittencourt H. CTL019 (tisagenlecleucel): CAR-T therapy for relapsed and refractory B-cell acute lymphoblastic leukemia. *Drug Des Devel Ther*. 2018;12:3885–3898.
43 Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017;377(26):2531–2544.
44 Abramson JS, Solomon SR, Arnason J, et al. Lisocabtagene maraleucel as second-line therapy for large B-cell lymphoma: primary analysis of the phase 3 TRANSFORM study. *Blood*. 2023;141(14):1675–1684.
45 Wang Y, Jain P, Locke FL, et al. Brexucabtagene autoleucel for relapsed or refractory mantle cell lymphoma in standard-of-care practice: results from the US lymphoma CAR T consortium. *J Clin Oncol*. 2023;41(14):2594–2606.
46 Madduri D, Berdeja JG, Usmani SZ, et al. CARTITUDE-1: phase 1b/2 study of ciltacabtagene autoleucel, a B-cell maturation antigen-directed chimeric antigen receptor T cell therapy, in relapsed/refractory multiple myeloma. *Blood*. 2020;136(Supplement 1):22–25.
47 ClinicalTrials.gov [Internet]. *Identifier NCT05812326, PD-1 knockout anti-MUC1 CAR-T cells in the treatment of advanced breast cancer.* Bethesda (MD): National Library of Medicine (US); 2022. Available from: https://clinicaltrials.gov/study/NCT05812326.
48 ClinicalTrials.gov [Internet]. *Identifier NCT04025216, A study of CART-TnMUC1 in patients with TnMUC1-positive advanced cancers.* Bethesda (MD): National Library of Medicine (US); 2019. Available from: https://clinicaltrials.gov/study/NCT04025216.
49 Uslu U, Da T, Assenmacher CA, et al. Chimeric antigen receptor T cells as adjuvant therapy for unresectable adenocarcinoma. *Sci Adv*. 2023;9(2):eade2526.
50 ClinicalTrials.gov [Internet]. *Identifier NCT05623488, CAR T cells in mesothelin-expressing breast cancer.* Bethesda (MD): National Library of Medicine (US); 2022. Available from: https://clinicaltrials.gov/study/NCT05623488.
51 Bagley SJ, Binder ZA, Lamrani L, et al. Repeated peripheral infusions of anti-EGFRvIII CAR T cells in combination with pembrolizumab show no efficacy in glioblastoma: a phase 1 trial. *Nat Cancer*. 2024;5(3):517–531.
52 ClinicalTrials.gov [Internet]. *Identifier NCT02873390, PD-1 antibody expressing CAR-T cells for EGFR family member positive advanced solid tumor.* Bethesda (MD): National Library of Medicine (US); 2016. Available from: https://clinicaltrials.gov/study/NCT02873390.
53 ClinicalTrials.gov [Internet]. *Identifier NCT02862028, PD-1 antibody expressing CAR-T cells for EGFR family member positive advanced solid tumour (lung, liver and stomach).* Bethesda (MD): National Library of Medicine (US); 2016. Available from: https://clinicaltrials.gov/study/NCT02862028.
54 Punekar SR, Welling T, Randolph Hecht J, et al. 634 EVEREST-1: a seamless phase 1/2 study of CEA logic-gated Tmod CAR T-cell therapy (A2B530) in patients with solid tumors associated with CEA expression also exhibiting HLA loss of heterozygosity (LOH). *J ImmunoTherapy Cancer*. 2023;11(Suppl 1):A724–A725.
55 Qi C, Liu C, Gong J, et al. Safety, tolerability, and preliminary efficacy results in patients with advanced gastric/gastroesophageal junction adenocarcinoma from a phase Ib/II study of CLDN18.2 CAR T-cell therapy (CT041). *J Clin Oncol*. 2022;40(16_suppl):4017.
56 Qi C, Gong J, Li J, et al. Claudin18.2-specific CAR T cells in gastrointestinal cancers: phase 1 trial interim results. *Nat Med*. 2022;28(6):1189–1198.
57 Del Bufalo F, De Angelis B, Caruana I, et al. GD2-CART01 for relapsed or refractory high-risk neuroblastoma. *N Engl J Med*. 2023;388(14):1284–1295.
58 ClinicalTrials.gov [Internet]. *Identifier NCT03373097, anti-GD2 CAR T cells in pediatric patients affected by high risk and/or Relapsed/Refractory neuroblastoma or other GD2-positive solid tumours.* Bethesda (MD): National Library of Medicine (US); 2017. Available from: https://clinicaltrials.gov/study/NCT03373097.
59 Liu Y, Dang Y, Zhang C, et al. IL-21-armored B7H3 CAR-iNKT cells exert potent antitumor effects. *iScience*. 2024;27(1):108597.
60 Wang W, Marin-Ramos NI, He H, et al. NEO100 enables brain delivery of blood–brain barrier impermeable therapeutics. *Neuro Oncol*. 2021;23(1):63–75.
61 Liu Z, Zhou Z, Dang Q, et al. Immunosuppression in tumor immune microenvironment and its optimization from CAR-T cell therapy. *Theranostics*. 2022;12(14):6273–6290.
62 Dorff TB, Blanchard S, Martirosyan H, et al. Final results from phase I study of PSCA-targeted chimeric antigen receptor (CAR) T cells in patients with metastatic castration resistant prostate cancer (mCRPC). *J Clin Oncol*. 2023;41(16_suppl):5019.
63 Priceman SJ, Gerdts EA, Tilakawardane D, et al. Co-stimulatory signaling determines tumor antigen sensitivity and persistence of CAR T cells targeting PSCA+ metastatic prostate cancer. *OncoImmunology*. 2018;7(2):e1380764.
64 Shi Y, Shi D, Chi J, et al. Combined local therapy and CAR-GPC3 T-cell therapy in advanced hepatocellular carcinoma: a proof-of-concept treatment strategy. *Cancer Commun*. 2023;43(9):1064–1068.
65 Sun H, Xing C, Jiang S, et al. Long term complete response of advanced hepatocellular carcinoma to glypican-3 specific chimeric antigen receptor T-Cells plus sorafenib, a case report. *Front Immunol*. 2022;13:963031.
66 Wu X, Luo H, Shi B, et al. Combined antitumor effects of sorafenib and GPC3-CAR T cells in mouse models of hepatocellular carcinoma. *Mol Ther*. 2019;27(8):1483–1494.
67 Shi D, Shi Y, Kaseb AO, et al. Chimeric antigen receptor-glypican-3 T-cell therapy for advanced hepatocellular carcinoma: results of phase I trials. *Clin Cancer Res*. 2020;26(15):3979–3989.
68 Gao H, Li K, Tu H, et al. Development of T cells redirected to glypican-3 for the treatment of hepatocellular carcinoma. *Clin Cancer Res*. 2014;20(24):6418–6428.

69 Liu S, Deng B, Yin Z, et al. Corticosteroids do not influence the efficacy and kinetics of CAR-T cells for B-cell acute lymphoblastic leukemia. *Blood Cancer J*. 2020;10(2):15.
70 Strati P, Ahmed S, Furqan F, et al. Prognostic impact of corticosteroids on efficacy of chimeric antigen receptor T-cell therapy in large B-cell lymphoma. *Blood*. 2021;137(23):3272–3276.
71 Pal SK, Tran B, Haanen J, et al. CD70-Targeted allogeneic CAR T-cell therapy for advanced clear cell renal cell carcinoma. *Cancer Discov*. 2024;14:1176–1189.
72 Sallman DA, Kerre T, Havelange V, et al. CYAD-01, an autologous NKG2D-based CAR T-cell therapy, in relapsed or refractory acute myeloid leukaemia and myelodysplastic syndromes or multiple myeloma (THINK): haematological cohorts of the dose escalation segment of a phase 1 trial. *Lancet Haematol*. 2023;10(3):e191–e202.
73 Fan M, Liu H, Yan H, et al. A CAR T-inspiring platform based on antibody-engineered exosomes from antigen-feeding dendritic cells for precise solid tumor therapy. *Biomaterials*. 2022;282:121424.
74 Jo S, Das S, Williams A, et al. Endowing universal CAR T-cell with immune-evasive properties using TALEN-gene editing. *Nat Commun*. 2022;13(1):3453.
75 Gomes-Silva D, Srinivasan M, Sharma S, et al. CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies. *Blood*. 2017;130(3):285–296.
76 Mamonkin M, Rouce RH, Tashiro H, Brenner MK. A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies. *Blood*. 2015;126(8):983–992.
77 Cooper ML, Choi J, Staser K, et al. An "off-the-shelf" fratricide-resistant CAR-T for the treatment of T cell hematologic malignancies. *Leukemia*. 2018;32(9):1970–1983.
78 Chiesa R, Georgiadis C, Syed F, et al. Base-Edited CAR7 T cells for relapsed T-cell acute lymphoblastic leukemia. *N Engl J Med*. 2023;389(10):899–910.
79 Torikai H, Reik A, Liu PQ, et al. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood*. 2012;119(24):5697–5705.
80 Fitzgerald JC, Weiss SL, Maude SL, et al. Cytokine release syndrome after chimeric antigen receptor T cell therapy for acute lymphoblastic leukemia. *Crit Care Med*. 2017;45(2):e124–e131.
81 Belin C, Devic P, Ayrignac X, et al. Description of neurotoxicity in a series of patients treated with CAR T-cell therapy. *Sci Rep*. 2020;10(1):18997.
82 Martinez-Cibrian N, Espanol-Rego M, Pascal M, Delgado J, Ortiz-Maldonado V. Practical aspects of chimeric antigen receptor T-cell administration: from commercial to point-of-care manufacturing. *Front Immunol*. 2022;13:1005457.
83 Biernacki MA, Sheth VS, Bleakley M. T cell optimization for graft-versus-leukemia responses. *JCI Insight*. 2020;5(9):e134939.
84 Levine BL, Miskin J, Wonnacott K, Keir C. Global manufacturing of CAR T cell therapy. *Mol Ther Methods Clin Dev*. 2017;4:92–101.
85 Wang X, Rivière I. Clinical manufacturing of CAR T cells: foundation of a promising therapy. *Mol Ther Oncolytics*. 2016;3:16015.
86 Agarwal S, Weidner T, Thalheimer FB, Buchholz CJ. In vivo generated human CAR T cells eradicate tumor cells. *OncoImmunology*. 2019;8(12):e1671761.
87 Xin T, Cheng L, Zhou C, Zhao Y, Hu Z, Wu X. In-vivo induced CAR-T cell for the potential breakthrough to overcome the barriers of current CAR-T cell therapy. *Front Oncol*. 2022;12:809754.
88 Michels A, Ho N, Buchholz CJ. Precision medicine: in vivo CAR therapy as a showcase for receptor-targeted vector platforms. *Mol Ther*. 2022;30(7):2401–2415.
89 Zhao G, Zhang Y, Xu CF, Wang J. In vivo production of CAR-T cells using virus-mimetic fusogenic nanovesicles. *Sci Bull*. 2024;69(3):354–366.
90 Hou R, Zhang X, Wang X, et al. In vivo manufacture and manipulation of CAR-T cells for better druggability. *Cancer Metastasis Rev*. 2024. https://doi.org/10.1007/s10555-024-10185-8.
91 Pfeiffer A, Thalheimer FB, Hartmann S, et al. In vivo generation of human CD19-CAR T cells results in B-cell depletion and signs of cytokine release syndrome. *EMBO Mol Med*. 2018;10(11):e9158.
92 Michels KR, Sheih A, Hernandez SA, et al. Preclinical proof of concept for VivoVec, a lentiviral-based platform for in vivo CAR T-cell engineering. *J Immunother Cancer*. 2023;11(3):e006292.
93 Agarwalla P, Ogunnaike EA, Ahn S, et al. Bioinstructive implantable scaffolds for rapid in vivo manufacture and release of CAR-T cells. *Nat Biotechnol*. 2022;40(8):1250–1258.
94 Nawaz W, Huang B, Xu S, et al. AAV-mediated in vivo CAR gene therapy for targeting human T-cell leukemia. *Blood Cancer J*. 2021;11(6):119.
95 Michels A, Frank AM, Gunther DM, et al. Lentiviral and adeno-associated vectors efficiently transduce mouse T lymphocytes when targeted to murine CD8. *Mol Ther Methods Clin Dev*. 2021;23:334–347.
96 Theuerkauf SA, Herrera-Carrillo E, John F, et al. AAV vectors displaying bispecific DARPins enable dual-control targeted gene delivery. *Biomaterials*. 2023;303:122399.
97 Shirley JL, de Jong YP, Terhorst C, Herzog RW. Immune responses to viral gene therapy vectors. *Mol Ther*. 2020;28(3):709–722.
98 Yang Y. Cancer immunotherapy: harnessing the immune system to battle cancer. *J Clin Invest*. 2015;125(9):3335–3337.
99 Pinto IS, Cordeiro RA, Faneca H. Polymer-and lipid-based gene delivery technology for CAR T cell therapy. *J Contr Release*. 2023;353:196–215.
100 Gharatape A, Sadeghi-Abandansari H, Seifalian A, Faridi-Majidi R, Basiri M. Nanocarrier-based gene delivery for immune cell engineering. *J Mater Chem B*. 2024;12(14):3356–3375.
101 Lin Y, Chen Y, Luo Z, Wu YL. Recent advances in biomaterial designs for assisting CAR-T cell therapy towards potential solid tumor treatment. *Nanoscale*. 2024;16(7):3226–3242.
102 Kitte R, Rabel M, Geczy R, et al. Lipid nanoparticles outperform electroporation in mRNA-based CAR T cell engineering. *Mol Ther Methods Clin Dev*. 2023;31:101139.
103 Zhou J-e, Sun L, Jia Y, et al. Lipid nanoparticles produce chimeric antigen receptor T cells with interleukin-6 knockdown in vivo. *J Contr Release*. 2022;350:298–307.
104 Shin S, Lee P, Han J, et al. Nanoparticle-based chimeric antigen receptor therapy for cancer immunotherapy. *Tissue Eng Regen Med*. 2023;20(3):371–387.
105 Nawaz W, Xu S, Li Y, Huang B, Wu X, Wu Z. Nanotechnology and immunoengineering: how nanotechnology can boost CAR-T therapy. *Acta Biomater*. 2020;109:21–36.
106 Ita K. Polyplexes for gene and nucleic acid delivery: progress and bottlenecks. *J Eur J Pharm Sci*. 2020;150:105358.
107 Qin Y-T, Li Y-P, He X-W, Wang X, Li W-Y, Zhang Y-K. Biomaterials promote in vivo generation and immunotherapy of CAR-T cells. *J Front Immunol*. 2023;14:1165576.
108 Smith TT, Stephan SB, Moffett HF, et al. In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers. *J Nat Nanotechnol*. 2017;12(8):813–820.
109 Parayath N, Stephan S, Koehne A, Nelson P, Stephan M. In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo. *Nat Commun*. 2020;11(1):6080.
110 Kaczmarek JC, Patel AK, Kauffman KJ, et al. Polymer–lipid nanoparticles for systemic delivery of mRNA to the lungs. *Angew Chem*. 2016;128(44):14012–14016.
111 Miller JB, Zhang S, Kos P, et al. Non-viral CRISPR/Cas gene editing in vitro and in vivo enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA. *Angew Chem*. 2017;129(4):1079–1083.
112 Sun W, Ji W, Hall JM, et al. Self-assembled DNA nanoclews for the efficient delivery of CRISPR–Cas9 for genome editing. *Angew Chem*. 2015;127(41):12197–12201.
113 Zhang Z, Wan T, Chen Y, et al. Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing. *Macromol Rapid Commun*. 2019;40(5):1800068.
114 Yao X, Lyu P, Yoo K, et al. Engineered extracellular vesicles as versatile ribonucleoprotein delivery vehicles for efficient and safe CRISPR genome editing. *J Extracell Vesicles*. 2021;10(5):e12076.
115 Li Z, Zhou X, Wei M, et al. In vitro and in vivo RNA inhibition by CD9-HuR functionalized exosomes encapsulated with miRNA or CRISPR/dCas9. *Nano Lett*. 2018;19(1):19–28.
116 Lin Y, Wu J, Gu W, et al. Exosome–liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. *Adv Sci*. 2018;5(4):1700611.
117 Agarwal S, Hanauer JDS, Frank AM, Riechert V, Thalheimer FB, Buchholz CJ. In vivo generation of CAR T cells selectively in human CD4(+) lymphocytes. *Mol Ther*. 2020;28(8):1783–1794.
118 Frank AM, Braun AH, Scheib L, et al. Combining T-cell-specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. *Blood Adv*. 2020;4(22):5702–5715.
119 Huckaby JT, Landoni E, Jacobs TM, Savoldo B, Dotti G, Lai SK. Bispecific binder redirected lentiviral vector enables in vivo engineering of CAR-T cells. *J Immunother Cancer*. 2021;9(9):e002737.

120 Mei H, Chen Z, Hu Y, Mei H. Bio-orthogonally redirected bispecific lentiviral vectors for in vivo CAR-T cell generation. *Blood*. 2023;142(Supplement 1):2062.
121 Zhang J, Cai X, Dou R, et al. Poly(beta-amino ester)s-based nanovehicles: structural regulation and gene delivery. *Mol Ther Nucleic Acids*. 2023;32:568–581.
122 Challener CA. Technology for in vivo CAR T-cell therapy advances. https://www.biopharminternational.com/view/technology-for-in-vivo-car-t-cell-therapy-advances; 2023.
123 Parker M, Ulrich-Lewis J, Tang W, et al. Vivovec™ surface-engineered lentiviral particles mediate in vivo CAR T generation with potent and highly durable activity in non-human primates and tumor-bearing humanized mice. *Blood*. 2023;142(Supplement 1):765.
124 Li C, Georgakopoulou A, Newby GA, et al. In vivo HSC prime editing rescues sickle cell disease in a mouse model. *Blood*. 2023;141(17):2085–2099.
125 Rurik JG, Tombácz I, Yadegari A, et al. CAR T cells produced in vivo to treat cardiac injury. *Science*. 2022;375(6576):91–96.
126 Sommermeyer D, Hudecek M, Kosasih PL, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. *Leukemia*. 2016;30(2):492–500.
127 Frank AM, Buchholz CJ. Surface-engineered lentiviral vectors for selective gene transfer into subtypes of lymphocytes. *Mol Ther Methods Clin Dev*. 2019;12:19–31.
128 Bulcha JT, Wang Y, Ma H, Tai PWL, Gao G. Viral vector platforms within the gene therapy landscape. *Signal Transduct Target Ther*. 2021;6(1):53.
129 Short L, Holt RA, Cullis PR, Evgin L. Direct in vivo CAR T cell engineering. *Trends Pharmacol Sci*. 2024;45(5):406–418.
130 Green J, Johnson A, Granger B, et al. CD8-Targeted, integrating viral vectors transduce resting T cells and enable extracorporeal delivery (ECD) for rapid CAR T cell therapies. *Blood*. 2022;140(Supplement 1):7768–7769.
131 Cevaal PM, Ali A, Czuba-Wojnilowicz E, et al. In vivo T cell-targeting nanoparticle drug delivery systems: considerations for rational design. *ACS Nano*. 2021;15(3):3736–3753.
132 Ramishetti S, Kedmi R, Goldsmith M, et al. Systemic gene silencing in primary T lymphocytes using targeted lipid nanoparticles. *ACS Nano*. 2015;9(7):6706–6716.
133 Lee J, Yun KS, Choi CS, et al. T cell-specific siRNA delivery using antibody-conjugated chitosan nanoparticles. *Bioconjug Chem*. 2012;23(6):1174–1180.
134 Yang YS, Moynihan KD, Bekdemir A, et al. Targeting small molecule drugs to T cells with antibody-directed cell-penetrating gold nanoparticles. *Biomater Sci*. 2018;7(1):113–124.
135 Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol*. 2015;33(9):941–951.
136 Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-viral gene transfer. *Adv Drug Deliv Rev*. 2005;57(5):755–767.
137 Qiu C, Xia F, Zhang J, et al. Advanced strategies for overcoming endosomal/lysosomal barrier in nanodrug delivery. *Research*. 2023;6:148.
138 Klipp A, Burger M, Leroux JC. Get out or die trying: Peptide- and protein-based endosomal escape of RNA therapeutics. *Adv Drug Deliv Rev*. 2023;200:115047.
139 Cappell KM, Kochenderfer JN. Long-term outcomes following CAR T cell therapy: what we know so far. *Nat Rev Clin Oncol*. 2023;20(6):359–371.
140 Kawalekar OU, O'Connor RS, Fraietta JA, et al. Distinct signaling of coreceptors regulates specific metabolism pathways and impacts memory development in CAR T cells. *Immunity*. 2016;44(2):380–390.
141 Harrison AJ, Du X, von Scheidt B, Kershaw MH, Slaney CY. Enhancing co-stimulation of CAR T cells to improve treatment outcomes in solid cancers. *Immunother Adv*. 2021;1(1):ltab016.
142 Kishton RJ, Sukumar M, Restifo NP. Metabolic regulation of T cell longevity and function in tumor immunotherapy. *Cell Metab*. 2017;26(1):94–109.
143 Jung IY, Noguera-Ortega E, Bartoszek R, et al. Tissue-resident memory CAR T cells with stem-like characteristics display enhanced efficacy against solid and liquid tumors. *Cell Rep Med*. 2023;4(6):101053.
144 Bocca P, Di Carlo E, Caruana I, et al. Bevacizumab-mediated tumor vasculature remodelling improves tumor infiltration and antitumor efficacy of GD2-CAR T cells in a human neuroblastoma preclinical model. *OncoImmunology*. 2017;7(1):e1378843.
145 Deng C, Zhao J, Zhou S, et al. The vascular disrupting agent CA4P improves the antitumor efficacy of CAR-T cells in preclinical models of solid human tumors. *Mol Ther*. 2020;28(1):75–88.
146 Safarzadeh Kozani P, Safarzadeh Kozani P, Ahmadi Najafabadi M, Yousefi F, Mirarefin SMJ, Rahbarizadeh F. Recent advances in solid tumor CAR-T cell therapy: driving tumor cells from hero to zero? *Front Immunol*. 2022;13:795164.
147 Anurathapan U, Chan RC, Hindi HF, et al. Kinetics of tumor destruction by chimeric antigen receptor-modified T cells. *Mol Ther*. 2014;22(3):623–633.
148 Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat Rev Clin Oncol*. 2020;17(3):147–167.
149 Duncan BB, Dunbar CE, Ishii K. Applying a clinical lens to animal models of CAR-T cell therapies. *Mol Ther Methods Clin Dev*. 2022;27:17–31.
150 Jin CH, Xia J, Rafiq S, et al. Modeling anti-CD19 CAR T cell therapy in humanized mice with human immunity and autologous leukemia. *EBioMedicine*. 2019;39:173–181.
151 Cassidy JW, Caldas C, Bruna A. Maintaining tumor heterogeneity in patient-derived tumor xenografts. *Cancer Res*. 2015;75(15):2963–2968.
152 Garrido-Laguna I, Uson M, Rajeshkumar NV, et al. Tumor engraftment in nude mice and enrichment in stroma- related gene pathways predict poor survival and resistance to gemcitabine in patients with pancreatic cancer. *Clin Cancer Res*. 2011;17(17):5793–5800.
153 Singh AP, Chen W, Zheng X, et al. Bench-to-bedside translation of chimeric antigen receptor (CAR) T cells using a multiscale systems pharmacokinetic-pharmacodynamic model: a case study with anti-BCMA CAR-T. *CPT Pharmacometrics Syst Pharmacol*. 2021;10(4):362–376.
154 Mitchell MJ, Billingsley MM, Haley RM, Wechsler ME, Peppas NA, Langer R. Engineering precision nanoparticles for drug delivery. *Nat Rev Drug Discov*. 2021;20(2):101–124.
155 *Multidisciplinary: gene therapy*; 2024. https://www.ema.europa.eu/en/human-regulatory-overview/research-and-development/scientific-guidelines/multidisciplinary-guidelines/multidisciplinary-gene-therapy. Accessed May 6, 2024.
156 Xuan L, Ju Z, Skonieczna M, Zhou PK, Huang R. Nanoparticles-induced potential toxicity on human health: applications, toxicity mechanisms, and evaluation models. *MedComm*. 2023;4(4):e327.
157 Foroozandeh P, Aziz AA. Insight into cellular uptake and intracellular trafficking of nanoparticles. *Nanoscale Res Lett*. 2018;13(1):339.
158 Johnson PA, Miyanohara A, Levine F, Cahill T, Friedmann T. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J Virol*. 1992;66(5):2952–2965.
159 Sun S, Hao H, Yang G, Zhang Y, Fu Y. Immunotherapy with CAR-modified T cells: toxicities and overcoming strategies. *J Immunol Res*. 2018;2018:2386187.
160 Dimitri A, Herbst F, Fraietta JA. Engineering the next-generation of CAR T-cells with CRISPR-Cas9 gene editing. *Mol Cancer*. 2022;21(1):78.
161 Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduct Target Ther*. 2020;5(1):1.
162 Bulsara S, Wu M, Wang T. Phase I CAR-T clinical trials review. *Anticancer Res*. 2022;42(12):5673–5684.
163 *Cancer clinical trial eligibility criteria: patients with organ dysfunction or prior or concurrent malignancies: guidance for industry*; 2020. https://consultations.tga.gov.au/tga/international-scientific-guidelines-r01-2023/user_uploads/cancer-clinical-trial-eligibility-criteria-patients-with-organ-dysfunction-or-prior-concurrent-maligancies.pdf.
164 Osarogiagbon RU, Vega DM, Fashoyin-Aje L, et al. Modernizing clinical trial eligibility criteria: recommendations of the ASCO-friends of cancer research prior therapies work group. *Clin Cancer Res*. 2021;27(9):2408–2415.
165 Iglesias-Lopez C, Obach M, Vallano A, Agusti A. Comparison of regulatory pathways for the approval of advanced therapies in the European Union and the United States. *Cytotherapy*. 2021;23(3):261–274.