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Approaches to in vivo CAR-T therapy technology and considerations of quality control and nonclinical research

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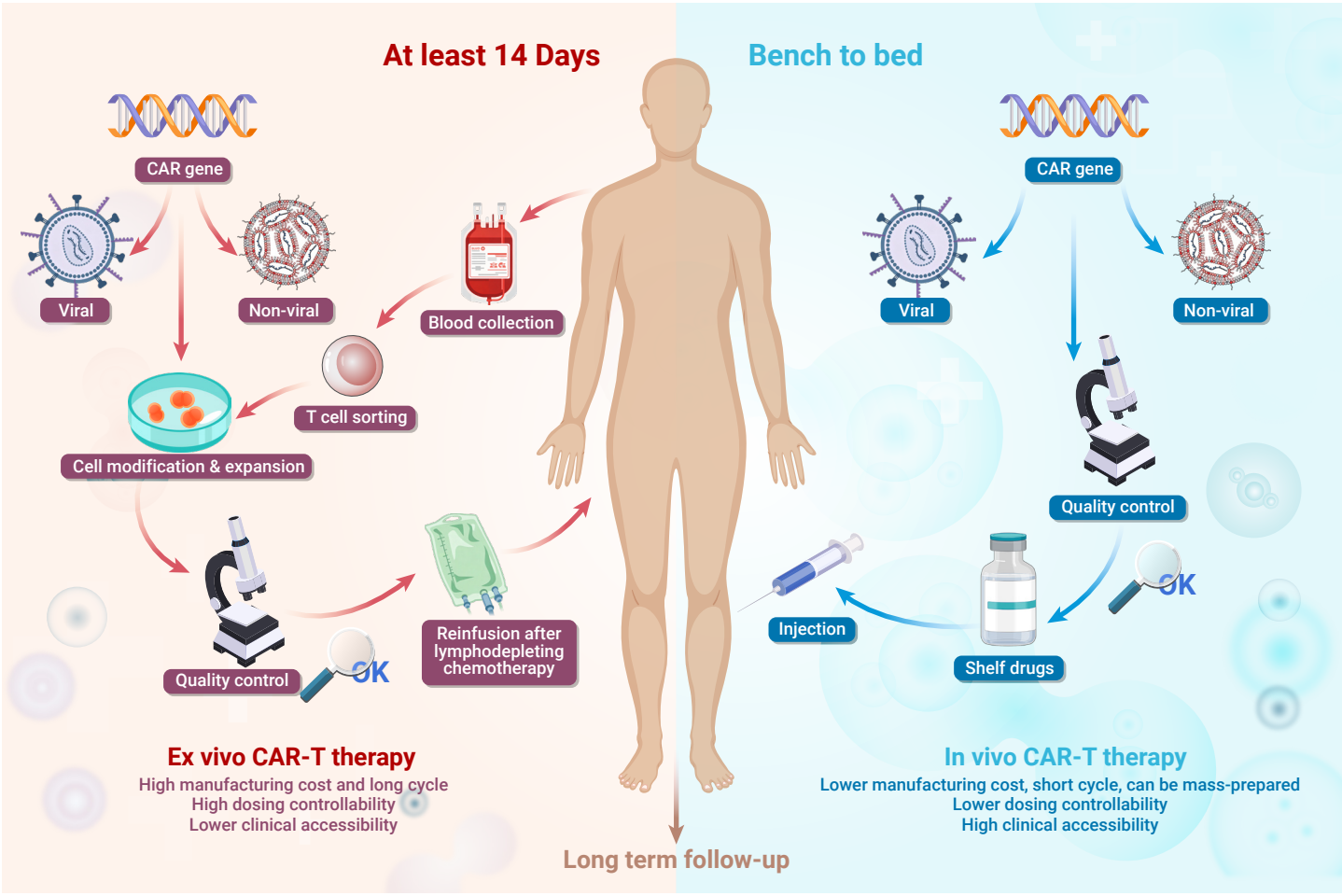
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- This review introduced the mechanisms, clinical advances and optimization strategies of in vivo CAR-T therapy.
- This review focused on considerations for developing quality control strategies for in vivo CAR-T therapy.
- This review focused on discussing key considerations for its nonclinical research.

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Chimeric antigen receptor T-cell (CAR-T) therapy has achieved remarkable success in the treatment of hematological malignancies. However, conventional ex vivo CAR-T therapies are constrained by complex manufacturing processes, long production cycles, and limited clinical applicability. To address these limitations and to simplify the treatment process and enhance accessibility, strategies for in situ delivery of CAR-encoding genes to endogenous T cells have been developed. This review summarizes the design of carriers and payloads for in vivo CAR-T therapy, provides an overview of recent advances in the field, and examines its persistent challenges and potential solutions. The article focuses on discussing key considerations related to quality control and nonclinical research for in vivo CAR-T products based on their characteristics and relevant guidelines for gene therapy and conventional CAR-T cell products, with the aim of providing insights for the translational application of this technology.

INTRODUCTION

Chimeric antigen receptor T-cell (CAR-T) therapy involves the use of genetically engineered T cells to express a chimeric antigen receptor (CAR). This receptor typically consists of an extracellular antigen-binding domain that recognizes specific tumor antigens, coupled with intracellular signaling modules such as the CD3ζ chain and costimulatory domains that activate T-cell effector functions, thereby enabling precise targeting and elimination of malignant cells. CAR-T therapy has achieved remarkable clinical success in hematologic malignancies. Since the approval of the first CAR-T product Kymriah in 2017, a total of 14 CAR-T products have been approved globally by the end of 2025,[1-3] offering hope of long-term remission and cure for patients with relapsed/refractory leukemia, lymphoma, and multiple myeloma.

However, currently available CAR-T products are autologous ex vivo CAR-T therapies, which require the isolation of the patient's own T cells, their genetic modification in vitro, and subsequent reinfusion. Their clinical applications also face a series of limitations. On a production perspective, the complex ex vivo manufacturing process, stringent requirements for specialized equipment, and stringent supply chain demands result in high treatment costs and limited accessibility.[4,5] Additionally, significant product variability complicates quality control. From a clinical perspective, the lengthy preparation period may cause patients to miss the optimal treatment window,[6] and the necessary lymphodepletion pretreatment prior to therapy increases the risk of infection.[7,8] Furthermore, the immunosuppressive microenvironment and the insufficient infiltration capacity of CAR-T cells into solid tissues limit their potential for broader application in diseases such as solid tumors and other solid tissue disorders. Although allogeneic ex vivo CAR-T therapies can shorten the preparation time and reduce product variability through "off-the-shelf" supply, they face various unique immune rejection challenges because graft-versus-host reactions may trigger CAR-T-related toxicities, and host-versus-graft reactions can lead to rapid clearance of CAR-T cells.[9,10] Although the knockout of specific genes can reduce immune rejection,[11] the ex vivo manufacturing process for allogeneic CAR-T remains relatively complex and does not fully overcome limitations such as reliance on lymphodepletion and insufficient infiltration into solid tumors.

In this context, in vivo CAR-T technology, which allows for in situ programming of endogenous T cells directly within the patient's body, has emerged as a promising direction in the development of next-generation CAR-T therapies. In vivo CAR-T addresses inherent drawbacks of ex vivo CAR-T, such as complex processes, long preparation cycles, and the need for chemotherapeutic lymphodepletion. Furthermore, its production process is more closely aligned with standardized biologics, which helps facilitate process control and quality testing and thereby pave a new path for the widespread adoption and enhanced efficiency of cell therapies[12,13] (see the graphical abstract). However, as a novel gene therapy product, its quality control and nonclinical research present considerable uncertainties, lacking translational experience and reference guidelines. Unlike ex vivo CAR-T therapies, where a defined dose of engineered cells is administered, the effective dose of CAR-T cells in the in vivo approach depends on the efficiency of in situ T-cell engineering, making dosing less predictable. Furthermore, in vivo CAR-T faces additional upstream risks associated with vector delivery, such as off-target transduction and random integration. Existing reviews on in vivo CAR-T primarily focus on its technological principles and developmental progress. In contrast, this review aims to provide an integrated perspective bridging technological research and clinical translation. Building upon an introduction to its technical principles, research progress, and optimization strategies, this review focuses on discussing considerations for identifying critical quality attributes, developing quality control strategies for the product, and key considerations for its nonclinical research. By bridging the gap between early-stage R&D and translational application, this review seeks to provide forward-looking insights for the development of in vivo CAR-T products.

THE HISTORY AND TECHNICAL ASPECTS OF IN VIVO CAR-T

The clinical translation of in vivo CAR-T has benefited from breakthroughs in two major technologies: advances in CAR molecular design, which established the foundational theory of CAR-T therapy, and advances in gene delivery vectors, which addressed the core challenge of targeting and genetically modifying specific cell populations in situ (Figure 1). This section reviews the historical development of in vivo CAR-T therapy and describes the technical principles of its main delivery platforms.

CAR construct design

The core mechanism of CAR-T therapy involves genetically engineering T cells to express a synthetically designed chimeric antigen receptor (CAR). The architecture of the CAR construct is fundamental to its therapeutic efficacy. A CAR is a modular synthetic receptor that consists of four functional domains:[4] (1) an extracellular antigen-binding domain; (2) a hinge region that provides flexibility; (3) a transmembrane domain for membrane anchoring; and (4) an intracellular signaling domain that drives T-cell activation upon antigen recognition. T cells expressing this synthetic receptor can specifically recognize and eliminate target cells expressing the corresponding antigen in an major histocompatibility complex (MHC)-independent manner.

The development of CAR technology has progressed through several generations (Figure 2). The widely validated second-generation CAR archi-

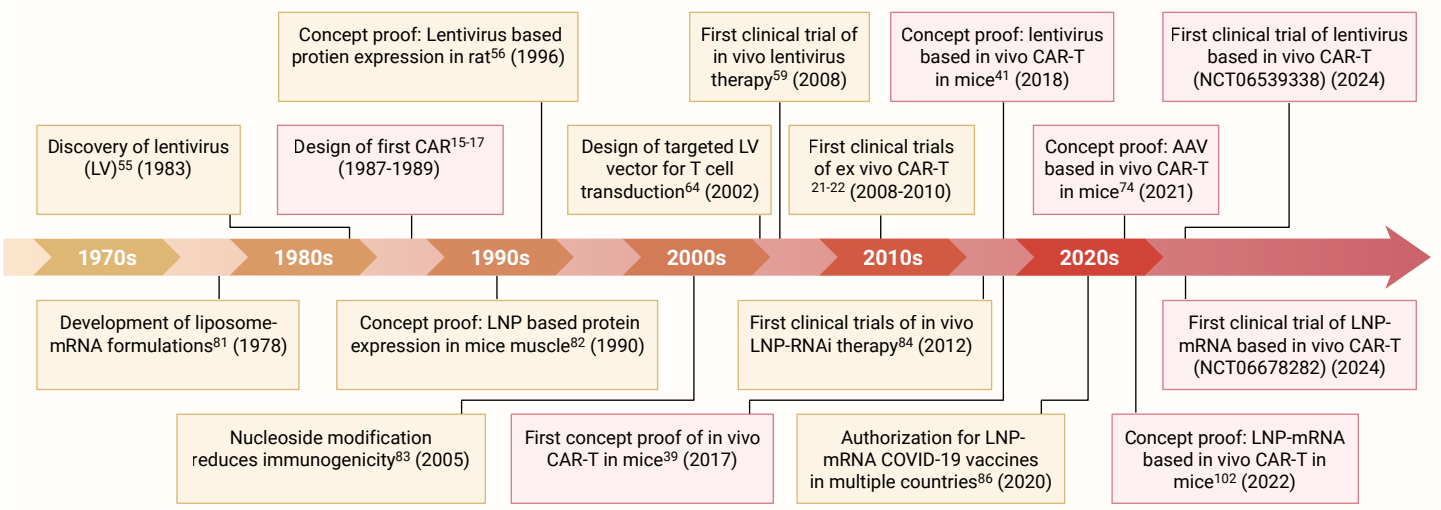

Figure 1. In vivo CAR-T timeline

tecture serves as the foundation for most current in vivo CAR-T candidates. However, its direct application from the ex vivo paradigm requires careful reconsideration of key design elements. First, the selection of the costimulatory domain requires careful re-evaluation. As in vivo administration lacks the capacity for extensive T cell pre-activation compared to ex vivo manufacturing, the distinct metabolic programs and functional states driven by the two mainstream costimulatory domains, CD28 and 4-1BB (e.g., CD28 favoring an effector phenotype, while 4-1BB promoting persistence[33,34]), may yield markedly different therapeutic outcomes in vivo. Second, designs for safety and controllability are of greater urgency. Preclinical studies indicate substantial inter-individual variability in the efficiency and kinetics of CAR-T cell generation following vector administration, [35,36] and direct intervention post-dosing is challenging. Therefore, incorporating safety modules such as inducible suicide switches, a hallmark of fourth-generation CARs, represents a crucial strategy for managing potential toxicities. Finally, flexibility is a key direction for the future. Fifth-generation "split-CARs" designed for target switchability offer a solution to potential antigen escape or the need for sequential therapy in vivo. Nevertheless, their complex multi-component coordination poses higher demands on vector design and in vivo functional validation.

In summary, the rational development of in vivo CAR-T therapies necessitates a context-specific re-evaluation and optimization of each CAR module, grounded in the unique biology of in vivocell engineering and the intended clinical application.

T-cell targeting strategies

For a long time, progress in in vivo CAR-T therapy was impeded by fundamental limitations in early delivery platforms, particularly with respect to targeting specificity, efficiency and safety. Recent advances of targeted vectors, engineered by conjugating T-cell surface marker-specific antibodies or ligands onto the carrier surface, have overcome these limitations, enabling the transition of this approach from concept to application. This section will first explore how to achieve specific in vivodelivery of vectors by selecting appropriate T-cell surface targets and molecular targeting tools.

For T-cell targeting, the predominant antigens include CD3, CD4, CD8, and CD7. Targeting CD3, a core component of the TCR complex, can to some extent directly activate resting T cells [37-39] without the need for IL-7 pre-activation; it facilitates the recruitment of a pan-T-cell pool, including both CD4+ and CD8+ subsets, enabling the CD8+ CAR-T subpopulation to achieve more sustained and potent activation.[40,41] This makes CD3 targeting a widely adopted strategy for in vivo CAR gene delivery.[38-40,42-44] A limitation of this strategy, however, is the inability to precisely control the final ratio of product subsets and the risk of transducing immunosuppressive regulatory T cells. The CD8-targeting strategy can rapidly generate a cytotoxic effector CAR-T cell subset[36,40,45-49] to initiate tumor killing. However, the lack of activated CD4+ T cell help may result in insufficient persistence. Moreover, as CD8 is also expressed on some NK and NKT cells, this may lead to unintended transduction of these cell types.[49] Targeting CD4 primarily activates helper CAR-T cells, which exhibit lower expression levels of exhaustion markers, suggesting they may be more resilient under sustained antigen stimulation.[35] CD7 is expressed on the surface of both T cells and NK cells; therefore, targeting CD7 can generate a mixed effector population of CAR-T and CAR-NK cells, enabling synergistic anti-tumor effects. However, vectors targeting CD7 typically have insufficient transduction efficiency for resting T cells.[37,50]

After identifying the T-cell target, selecting the appropriate molecular targeting ligand is crucial. The mainstream molecular tools for achieving lentiviral vector T-cell targeting include single-chain variable fragments (scFv), nanobodies (variable domain of heavy-chain antibodies, VHHs), and designed ankyrin repeat proteins (DARPins). Single-chain variable fragments (scFvs), which have an extensive clinical track record in conventional CAR-T therapy,[51] remain a reliable option.[37,38,40,52] Nanobodies (VHHs) offer advantages for recognizing complex epitopes due to their small size.[37,40,53] Designed ankyrin repeat proteins (DARPins), characterized by compact size, high modularity, and compatibility with cell-free selection systems, are also actively investigated for in vivo CAR-T development.[35-37,40,48,52] In addition to membrane-anchored formats, targeting can be achieved through non-anchored strategies; for instance, a 2021 study employed an anti-SINV/anti-CD3 bispecific antibody to retarget SINV-LV vectors toward CD3+ T cells.[42]

Current in vivo CAR-T delivery platforms

After defining the targeting strategy, the targeting molecules need to be conjugated to suitable delivery vehicles. Currently, in vivo CAR-T delivery platforms can be categorized into viral and non-viral vectors, and comparisons of their key characteristics are summarized in Table 1.

Viral vector delivery platform. Viral vectors can deliver functional nucleic acids into the human body and are widely used in treating cancer, genetic defect diseases and in vaccines, with LV-based in vivo CAR-T therapies having advanced to clinical trials.

(1) Lentiviral vector delivery platform. Lentivirus (LV) is an enveloped RNA virus. Lentiviral vectors facilitate the stable integration of transgenes into the host genome to achieve sustained, long-term expression[55,56] and have achieved remarkable success in the field of ex vivo CAR-T therapy.[58]

As an enveloped RNA virus, the envelope glycoprotein of lentivirus is responsible for recognizing host cell receptors and mediating membrane fusion, making it a focal point for vector engineering. By replacing or modifying the envelope glycoprotein using pseudotyping technology, its natural tropism can be altered to target specific cell types.[59-62] The most common strategy is to use the vesicular stomatitis virus G glycoprotein (VSV-G) to mediate broad-spectrum transduction by widely expressing low-density lipoprotein receptor (LDLR).[59] However, conventional lentiviral vectors cannot

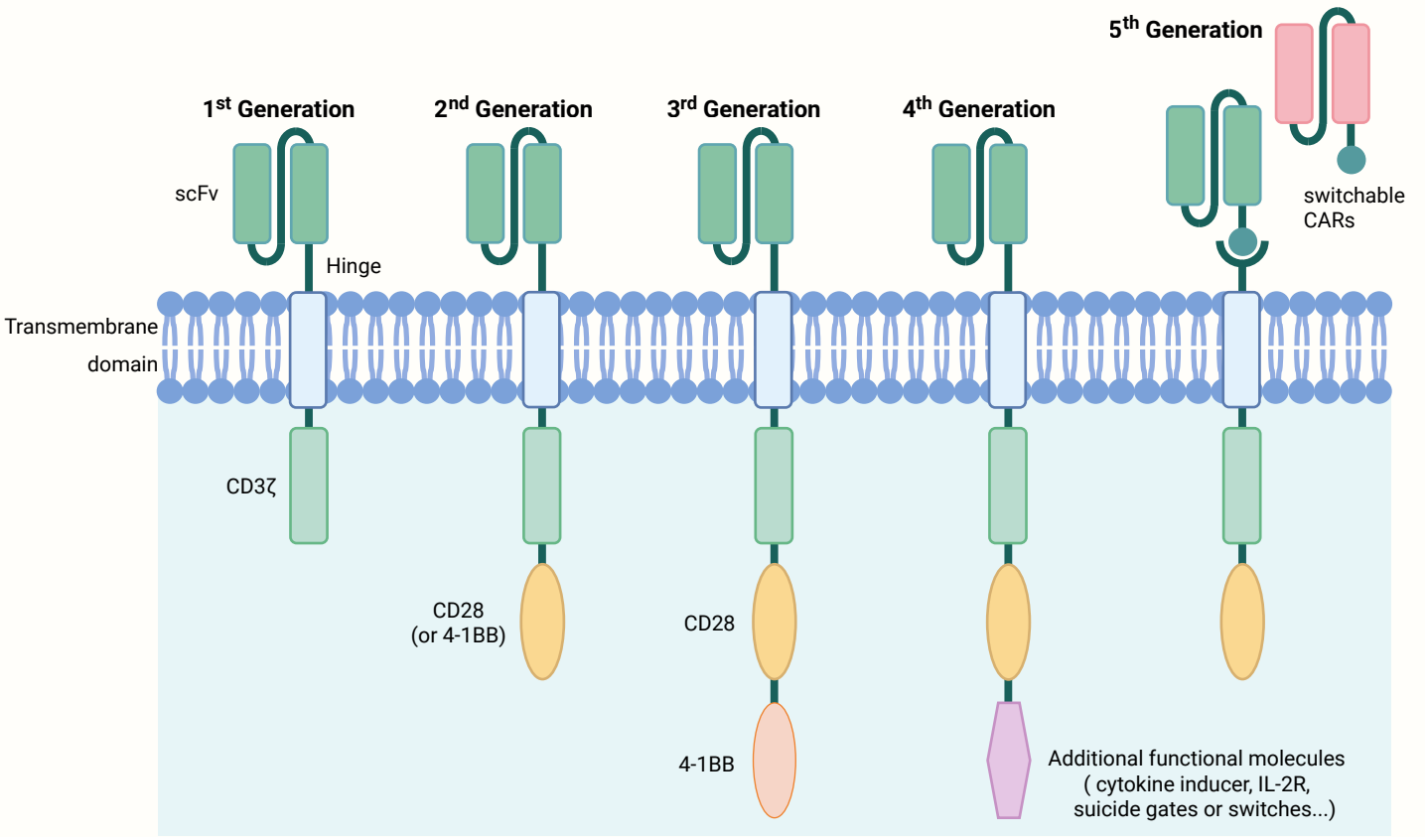

Figure 2. Development of chimeric antigen receptor (CAR) structure First-generation CAR fused the antigen-binding domain solely to the CD3ζ signaling domain[14-16] and demonstrated limited activation capacity. Second-generation CAR incorporated a single costimulatory domain in tandem with CD3ζ to provide a complete activation signal,[17-19] thereby allowing for successful clinical translation.[20-22] Third-generation CAR attempt to amplify signaling by incorporating multiple costimulatory domains.[23] Fourth-generation CAR integrated additional efficacy-enhancing modules[24-26] or introduced suicide switches or logic-gated controls.[11,27-30] Fifth-generation CAR featured switchable split-CAR systems for target flexibility.[31,32]

be directly used for in vivo CAR-T therapy. Taking VSV-G-pseudotyped vectors as an example, their transduction efficiency depends on the LDLR expression level in target cells, making it difficult to efficiently transduce resting T cells in vivo.[63] Furthermore, resting T cells reside in the G0 phase, characterized by low metabolic rates and high expression of restriction factors, which creates an unfavorable environment for lentiviral entry and integration. Additionally, the ubiquitous expression of LDLR across diverse cell types heightens the risk of off-target transduction, while the high seroprevalence of pre-existing antibodies in the human population can mediate vector neutralization,[64] collectively constraining both the efficacy and safety profile of such vectors.

Therefore, a central engineering approach for in vivo CAR-T lentiviral vectors is to decouple and independently optimize the viral "membrane fusion" and "T-cell targeting" functions. For the fusion module, strategies include using VSV-G mutants that lack LDLR-binding capacity[37,65] or employing alternative fusogenic proteins with lower seroprevalence to circumvent issues related to pre-existing antibodies and reduce nonspecific binding.[35,38,40,42] In parallel, incorporating targeting moieties (e.g., scFvs, nanobodies, or DARPins) directed against T-cell surface markers onto the viral envelope can allow for precise cell-specific transduction.[66,67] In 2018, a seminal proof-of-concept study demonstrated that CD8-targeted engineered LVs could generate functional anti-CD19 CAR-T cells in immunodeficient mouse models.[36]

Additional engineering innovations have been developed to enhance the functionality and safety of LV vectors. The VivoVec platform from Umoja presents an anti-CD3 scFv-costimulatory molecule fusion protein on the LV surface, directly providing a complete activation signal to enhance transduction efficiency, as well as the in vivo persistence and anti-tumor function of CAR-T cells.[12] The TetraVecta platform employs TRiP technology to suppress CAR translation and capsid incorporation during vector production, thereby reducing the risk of off-target transduction.[40] Another approach, exemplified by EsoBiotec's ESO-T01, incorporates CD47 into the vector envelope to inhibit phagocytic clearance and reduce immunogenicity.[68]

While most proof-of-concept studies have been conducted in humanized mouse models, their simplified immune systems are insufficient for accurately predicting the complexity of the human immune response. Non-human primate (NHP) models, which possess immune systems more akin to those of humans, therefore provide critical platforms for preclinical validation. Promisingly, several leading targeted LV platforms, such as Umoja's VivoVec™, Kelonia Therapeutics' iGPS, and Interius's CD7-LV, have demonstrated the ability to generate functional CAR-T cells, resulting in substantial and persistent target-cell clearance in NHPs without prior lymphodepletion.[12,13,37]

(2) Adeno-associated virus vector delivery platform. Adeno-associated virus (AAV) is a small, non-enveloped, linear single-stranded DNA virus that lacks autonomous replication capacity.[69] Its genome primarily exists as an episome,[70] and AAV vectors have extensive clinical experience in the field of genetic disease therapy, with several approved products, granting them greater feasibility in terms of product manufacturing and regulatory pathways.[71,72]

All current explorations of AAV-based in vivo CAR-T are at the preclinical stage.[73-76] The primary barriers to its clinical translation are: the high levels of pre-existing antibodies against natural AAV serotypes in the population, which limit repetitive dosage; the inherent liver tropism of natural AAV serotypes upon intravenous administration, posing dose-related toxicity risks; the existence of the transgene as a non-integrating episome—without repeat dosing, rapid T-cell expansion leads to rapid decay of CAR expression levels; and the conservative packaging capacity of AAV vectors, not exceeding 4.7 kb, which poses a greater challenge for CAR transgene cassette design.

The natural serotypes of AAV lack sufficient tissue and cell-type specificity, necessitating improved T-cell targeting through capsid directed evolution or the insertion of targeting moieties.[77] The directed evolution strategy improves

Table 1. Comparison of main vector platforms for in vivo CAR-T therapy

Vector		Payload	Integration risk, expression duration	Capacity	T-cell targeting specificity	Repetitive dosage	Major translational limitations
Viral vector	Lentivirus	dsDNA	Integrative, sustained expression	~8kb	High	Not applicable	Immunotoxicity at high doses, risk of insertional tumorigenesis, uncontrolled long-term CAR-T expansion
	Adeno-associated virus	ssDNA/ scDNA	Low-integrative, sustained expression	~4.7kb	Medium	Usually not applicable	CAR-T cells fail to expand effectively, repetitive dosage is not applicable due to anti-drug antibodies
Non-viral vector	Lipid nanoparticle	mRNA/ circRNA	Non-integrative, transient expression	Adjustable	High	Applicable	Transient expression, immunogenicity risk upon repetitive dosage
	Cationic polymer	DNA	Payload-dependent	Adjustable	Medium	Applicable	Non-specific transduction, payload-dependent risks (integration-related or repetitive dosage-related)

T-cell tropism by screening recombined capsids. For example, an AAV-DJ-based vector was used in 2021 to generate CAR-T cells in vivoand clear tumors for the first time, completing the proof-of-concept for AAV-mediated in vivo CAR-T;[73] In 2026, an AAV6-M2 variant also successfully generated CD62L+ CAR-T cells and cleared pathogenic B cells in a systemic lupus erythematosus mouse model.[74] However, capsids obtained through directed evolution often retain targeting ability towards other cell types.[73,75] In contrast, strategies involving the insertion of targeting moieties can achieve more precise targeting.[78,79]

Into HSPG-binding-deficient AAV2 or AAV6 capsids produced vectors that transduced >90% of CD8+ T cells with high specificity following a single injection while eliminating native liver tropism in mice.[45,46] Similarly, AAVivo's AVO-100, which decorates the AAV capsid with targeting peptides, effectively prolonged survival in a Raji lymphoma mouse model.[76] Biomimetic membrane coating of AAV is a helpful strategy to overcome the immunogenicity challenges associated with repeat AAV dosing.[80,81] For example, one study encapsulated the AAV6 capsid in red blood cell-derived membrane vesicles and conjugated anti-CD3 antibodies onto the vesicle surface to generate a T-cell-targeting artificial enveloped AAV (αCD3-AEV6); repeated administration in a diffuse large B-cell lymphoma mouse model resulted in significant tumor suppression.[81]

Non-viral vector delivery platform. Lipid nanoparticles (LNPs) and cationic polymer represent two primary non-viral delivery platforms for in vivo CAR-T therapy, which generally exhibit lower immunogenicity than viral vectors. Their payloads are typically mRNA, which enables transient CAR expression and thereby prevents the potential long-term toxicity risks associated with persistent CAR-T activity. LNP-mRNA based in vivo CAR-T products having already entered clinical trials, while cationic polymers and other non-viral vectors remain at the proof-of-concept stage.

(1) Lipid nanoparticle vector delivery platform. The feasibility of the LNP-mRNA platform has been extensively validated in applications such as mRNA vaccines.[87] A standard LNP formulation typically comprises four key components: an ionizable lipid, a phospholipid, cholesterol, and a PEGylated lipid. Its primary function is to efficiently encapsulate and deliver nucleic acid therapeutics, releasing the payload into the cytoplasm via endosomal escape to achieve transient transgene expression.[87,88] However, unmodified LNPs accumulate in the liver upon intravenous administration, limiting therapeutic efficacy and posing dose-related toxicity risks.[89,90]

The lipid composition of LNPs is a key determinant of their performance, directly influencing delivery efficiency and organ tropism. In terms of enhancing transfection efficiency, substituting conventional cholesterol with 7α-hydroxycholesterol as a helper lipid can enhance LNP stability and promote endosomal escape, resulting in approximately twofold improvement in the in vitrotransfection efficiency of human primary T cells;[91] Structural modification of the ionizable lipid, on the other hand, influences membrane fusion properties and cellular selectivity, thereby promoting T-cell transfection.[92-95] Regarding the modulation of organ tropism, lipid engineering can alter the natural liver-accumulation tendency of LNPs. Emerging non-cationic thiourea-based LNPs, which bind mRNA via hydrogen bonding, show reduced cation-associated toxicity and a stronger propensity for spleen targeting.[96] Furthermore, Selective ORgan Targeting (SORT) technology, by introducing permanently anionic lipids, can promote the delivery of LNPs to the spleen.[97-99] However, relying solely on organ-level tropism cannot meet the high-precision requirements for T-cell gene engineering and may trigger unwanted immune responses due to off-target delivery to antigen-presenting cells.

Targeted lipid nanoparticles (tLNPs), constructed by displaying T-cell-specific ligands on the LNP surface,[100] enable precise delivery to specific immune cells.[43,44,50,101-104] In 2022, CD5-targeted tLNPs generated antifibrotic CAR-T cells in a mouse model of heart failure, validating the feasibility of this platform for non-oncological indications.[103] Subsequently, CD3-targeted tLNPs effectively generated CAR-T cells and cleared tumors in both leukemia and solid tumor mouse models.[43,44] However, conventionally formulated tLNPs may still exhibit nonspecific hepatic accumulation and ligand-independent transfection.[103,105] Therefore, enhancing the lymphoid organ targeting of tLNPs by combining lipid engineering or biomimetic modifications is necessary. For example, one study displayed erythrocyte membrane proteins on the LNP surface, leveraging the natural spleen-homing property of red blood cells to enhance splenic tropism and achieve highly specific transfection of splenic CD11b+ myeloid cells.[106]

The selection of payload format requires a trade-off between safety and durability based on therapeutic goals. The LNP-mRNA platform circumvents the risk of genomic integration through transient expression, offering relatively controllable CAR expression[103,104] and avoiding the exhaustion of CAR-T cells caused by persistent antigen stimulation.[107] This makes it suitable for autoimmune diseases requiring controlled intervention. The duration of its expression can be extended through repeat dosing,[101] the use of circular RNA, or nucleic acid chemical modifications.[43,108,109] For malignancies requiring durable efficacy, LNPs can also co-deliver DNA and transposase mRNA to achieve long-term, genomically integrated expression,[50,110] though this necessitates weighing the corresponding integration risks.

Furthermore, the large capacity of LNPs supports co-encapsulation strategies, allowing for the simultaneous delivery of CAR genes and immunomodulatory units to synchronously regulate cell function and safety during in vivo-programming. For example, co-delivery of a CAR gene and an IL-6-targeting interfering RNA gene can mitigate cytokine release syndrome.[43] Co-delivery of a CAR and a competitive receptor for the immunosuppressive molecule CD24 can reverse the immunosuppressive microenvironment.[111] These strategies highlight the flexibility of the LNP platform in integrating multiple therapeutic functions, providing possibilities for its application in more complex disease scenarios. Future work can further incorporate well-established immunomodulatory concepts from conventional CAR-T therapy to expand its application scope.[112]

Beyond murine models, several LNP platforms have also demonstrated excellent performance in non-human primate (NHP) models: Capstan Therapeutics' CellSeeker™ platform achieved efficient CD8+ T cell transfection and profound B cell depletion in cynomolgus macaques;[49] Mina Therapeutics' MT-303 enabled targeted expression of a GPC3-specific CAR in monocytes, providing preliminary validation of its potential for solid tumor therapy.[113]

(2) Cationic polymer nanoparticle vector delivery platform. Cationic polymer nanoparticles efficiently load nucleic acids via electrostatic interactions and can be chemically modified for cell-specific targeting.[114] Traditional cationic polymers readily undergo nonspecific interactions with blood components and are rapidly cleared by the liver, resulting in poor targeting specificity and transduction efficiency. Therefore, strategies such as conjugating T-cell-targeting moieties are required to improve their targeting.

In 2017, a study using CD3ε-targeting poly(beta-amino ester) (PBAE) polymers to deliver the Sleeping Beauty transposon system together with a murine anti-CD19 CAR plasmid significantly prolonged survival in an immunocompetent leukemia mouse model.[39] This system represents the first complete proof-of-concept demonstration of in vivo CAR-T. Subsequently, in 2020, the same group extended this strategy to solid tumor models. Using CD8-targeting particles, they generated anti-Receptor tyrosine kinase-like orphan receptor 1 (ROR1) CAR-T cells in vivo in a prostate cancer mouse model and extended median survival by 40 days.[47] The work highlights the platform's translational potential for overcoming challenges in the complex tumor microenvironment.

The application of cationic polymer vectors has also been extended to other immune effector cells. In an innovative study on brainstem glioma, an RP-182 peptide-modified synthetic DNA nanocarrier loaded with an ErbB2-targeting CAR gene was developed. The construct could specifically target and reprogram tumor-associated macrophages (TAMs) to generate CAR-macrophages in vivo, prolonging survival in NOG mice bearing glioma, with 75% of treated mice surviving beyond 100 days.[115]

(3) Other non-viral vectors. Beyond mainstream non-viral vectors such as LNPs and cationic polymers, a series of novel carrier platforms based on peptides, biomimetic vesicles, and biopolymer hydrogel technologies are also in the preclinical proof-of-concept stage in the field of in vivo CAR-T, offering diversified solutions to overcome existing delivery bottlenecks.

Peptide-based nanoparticles (PEP-NPs) represent a novel class of nanocarriers that exploit the self-assembly properties of amphiphilic short peptides for efficient nucleic acid loading and extrahepatic targeted delivery. A 2025 study showed that a single intravenous injection of eGFP mRNA-loaded PEP-NPs achieved transfection efficiencies of 15-22% in T cells in C57BL/6 mice and, at the cellular level, demonstrated the ability to generate functional anti-CD19 CAR-T cells.[116] Although comprehensive in vivo efficacy validation remains pending, this study provided preliminary evidence supporting the platform's application potential.

Virus-mimetic fusogenic nanovesicles (FuNVs) are biomimetic vesicular carriers that emulate viral membrane-fusion mechanisms to directly deliver CAR proteins pre-loaded onto the vesicle membrane to T cells. A 2024 study engineered FuNVs by fusing measles virus or mammalian orthoreovirus fusogenic proteins with an anti-CD3 scFv and generated vesicles that displayed this fusion protein together with an anti-CD19 CAR on their membrane. In a B-cell lymphoma mouse model, this approach successfully converted approximately 2% of peripheral T cells into anti-CD19 CAR-T cells in vivo and achieved tumor inhibition rates exceeding 50% with repeated dosing, without inducing cytokine release syndrome, which demonstrates strong translational promise.[117]

Extracellular vesicles (EVs), which are naturally derived from the plasma membrane of living cells and characterized by high biocompatibility, represent an emerging platform for nucleic acid delivery.[118] A 2023 study used exosomes modified with CD3/CD28 scFvs to deliver CAR mRNA, successfully generating CAR-T cells in vitro.[119] As engineerable delivery vehicles, EVs can directly deliver CAR genes and immunomodulatory factors while simultaneously displaying functional molecules on their surface to further modulate immune responses. For instance, surface presentation of MHC-antigen peptide complexes together with co-stimulatory ligands can directly activate antigen-specific T cells.[120] If further engineered to display tumor-targeting antibodies, EVs may enhance T-cell recognition and tumor binding in a manner analogous to bispecific antibodies.[121] Although in vivo validation studies are yet to be published, successful in vitro delivery by EVs offers a novel conceptual framework for the development of carriers in in vivo CAR-T generation.

Hydrogel-based three-dimensional carrier systems can mimic the extracellular matrix microenvironment, enabling the in situ recruitment and engineering of T cells within the tumor microenvironment for efficient and durable in vivo CAR-T manufacturing, making them suitable for solid tumor therapy.[122,123] Studies show that multifunctional alginate-based scaffolds (MASTER) loaded with T-cell activators and encapsulated retroviral vectors carrying an anti-CD19 CAR gene, when implanted subcutaneously into NSG mice bearing a Daudi lymphoma model, increased the 100-day tumor-free survival rate from 16.6% for ex vivo CAR-T to 50%.[122] Another study administered an intratumoral injection of a hydrogel assembled from α-cyclodextrin and a cationic polymer PPP conjugated to anti-CD3 F(ab') fragments, which generated functional CAR-T cells in humanized mice, significantly enhancing cytotoxic T-cell infiltration and reversing the immunosuppressive tumor microenvironment.[123]

Although further validation is required, these distinctive novel carrier platforms open up more possibilities for the application of in vivo CAR-T.

ADVANCES IN IN VIVO CAR-T CLINICAL RESEARCH AND DEVELOPMENT

The in vivo CAR-T research and development pipeline has experienced rapid expansion since 2022. According to statistics from the Synapse Drug Database (synapse.zhihuiya.com), more than 90 publicly disclosed in vivo CAR-T candidate assets were reported worldwide as of early January 2026, spanning multiple therapeutic areas, including hematologic malignancies, solid tumors, and autoimmune diseases.

With respect to target distribution, CD19 and CD19-containing dual-target combinations dominate the disclosed in vivo CAR-T pipeline, followed by other B-cell antigens such as BCMA, CD20, and CD22. This distribution closely mirrors the clinical success of traditional ex vivo CAR-T in hematologic malignancies.[124] Concurrently, the development of drugs targeting entities such as GPC3 and TROP2 suggests increasing confidence in the applicability of in vivo CAR-T strategies for solid tumors.

In vivo CAR-T therapies are currently being explored across a broad spectrum of indications, including hematologic malignancies, autoimmune diseases, and solid tumors. Notably, a significant portion of the disclosed preclinical pipeline is focused on autoimmune diseases, and this underscores the perceived potential of this technology to address areas of high unmet medical need.

By the end of January 2026, a total of 16 in vivo CAR-T drug candidates worldwide had investigator-initiated trials (IITs) underway (excluding terminated or suspended trials); 9 in vivo CAR-T products have received IND approval. These studies involve 16 lentiviral vector platforms and 9 LNP-mRNA platforms. The explored indications include hematological malignancies, solid tumors, and autoimmune diseases, as detailed in Tables 2, Table 3 and Figure 3, which would be described separately below.

In vivo CAR-T clinical advances for hematologic malignancies

The application of in vivo CAR-T technology to hematologic malignancies builds upon the established success of conventional ex vivo CAR-T therapies. To date, 20 investigational in vivo CAR-T products targeting hematologic malignancies have entered clinical trials. BCMA and CD19 represent the most advanced targets in this area, demonstrating favorable safety and efficacy profiles in preclinical studies, with preliminary data from early clinical trials now emerging.[13,44,125]

In B-cell malignancies, therapies targeting CD19 or BCMA are of particular interest. JY-231, developed by Shenzhen GYGene Bio (a CD3-scFv-targeted lentiviral vector encoding an anti-CD19 CAR), was evaluated in a 64-year-old patient with relapsed/refractory diffuse large B-cell lymphoma (DLBCL). Following a single infusion, in vivo-generated CAR-T cells peaked on day 17, and the patient achieved a complete response at month 3. Treatment-related adverse events primarily included myelosuppression (hematologic toxicity) and grade 1 cytokine release syndrome (CRS), with no reported neurotoxicity or serious infections. This case provided preliminary proof-of-concept for in vivo CAR-T therapy in B-cell malignancies.[126] However, this case report originates from a conference abstract and is a single-case design; its conclusions should be interpreted with caution.

ESO-T01 (a CD47-modified, TCR-targeted lentivirus encoding an anti-BCMA CAR), co-developed by Wuhan Union Hospital and EsoBiotec, was evaluated in four patients with relapsed/refractory multiple myeloma

Table 2. In vivo CAR-T in IIT clinical trials

Drug	Vector	CAR target	Indications	Trial ID	Trial location	Investigator
LV009	Lentivirus	CD19	B-NHL, B-ALL	ChiCTR2500110470	China	PersonGen BioTherapeutics
SNC116	Lentivirus	CD19	B-NHL	ChiCTR2500110417	China	Simnova Bio
JY-231	Lentivirus	CD19	B-NHL, B-ALL	ChiCTR2500106833, NCT06940960, NCT06890065, NCT06689917, NCT06678282	China	Shenzhen Genocury Biotech
			B-ALL	NCT06902025, NCT06514768	China	
			B-NHL	NCT06879470	China	
			B-cell lymphoma	ChiCTR2400091980, NCT06045585	China	
			B-cell malignancies	NCT07065279	China	
			Autoimmune diseases	NCT07059169, NCT06887985, NCT06243159	China	
			AID of nervous system	NCT06797024	China	
			SLE	NCT06675422	China	
IMV101	Lentivirus	CD19	B-NHL	NCT07376642	China	Suzhou Immunofoco Biotechnology
OriV508	Lentivirus	CD19×BCMA	Multiple myeloma, B-NHL	NCT07101705	China	OriCell Therapeutics
LVIVO-TaVec100	Lentivirus	CD19×CD20	B-cell malignancies	NCT07002112	China	Nanjing Legend Biotech
ESO-T01	Lentivirus	BCMA	Multiple myeloma	NCT06791681	China	EsoBiotec
IASO206	Lentivirus	BCMA	Multiple myeloma	NCT07322159	China	Nanjing IASO Biotechnology
IASO208	Lentivirus	CD20	B-cell malignancies	NCT07309900	China	Nanjing IASO Biotechnology
STR-P004	LNP-mRNA	CD19	B-ALL	NCT07245251/ ChiCTR2500111445	China	Starna Therapeutics
			B-NHL	NCT07003178	China	
			Autoimmune diseases	NCT07143617	China	
GT801	LNP-mRNA	CD19	B-leukemia-lymphoma	NCT07205315	China	GRIT BioTherapeutics
			B-ALL	ChiCTR2500111445	China	
			B-NHL	NCT07003178	China	
JCXH-213	LNP-mRNA	CD19	B-NHL	ChiCTR2400092722, NCT06618313	China	Immorna (Hangzhou) Biotechnology
HN2301	LNP-mRNA	CD19	Multiple myeloma	NCT06965309	China	Shenzhen MagicRNA Biotechnology
			SLE	NCT06801119	China	
RXIM-002	LNP-mRNA	CD19	Autoimmune diseases	NCT07322718	China	RiboX Therapeutics
InViVoCAR1920	LNP-mRNA	CD19×CD20	B-cell lymphoma/ B-cell leukemia	NCT07321301	China	Chinese PLA General Hospital

Abbreviations: B-NHL, B-cell non-hodgkin lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; SLE, systemic lupus erythematosus; AID, Autoimmune diseases. Data sources: Clinicaltrials.gov and Chinese Clinical Trial Registry.

(RRMM). Administered as a single low-dose (2.0×10^8 TU) intravenous infusion without lymphodepleting chemotherapy, CAR-T cells peaked in peripheral blood between days 10 and 17. All patients achieved minimal residual disease (MRD) negativity by day 28, and two patients showed complete resolution of extramedullary disease, meeting criteria for stringent complete response. The safety profile included grade 3 CRS (three patients), grade 1 immune effector cell-associated neurotoxicity syndrome (ICANS) (one patient), and reversible grade 3-4 hematologic toxicities, with no severe non-hematologic events reported.[66] Kelonia Therapeutics' KLN-1010 (a CD3-targeted lentiviral vector encoding an anti-BCMA CAR) was evaluated in a first-in-human Phase 1 study involving three RRMM patients. Without prior lymphodepletion, all patients achieved MRD negativity (10^{-5} to 10^{-6} sensitivity) and at least a partial response within one month. The patient with the longest follow-up remained MRD-negative at three months and maintained a very good partial response. Treatment was associated with infusion reactions and grade 2 CRS; hematologic toxicity was mild and transient, and no neurotoxicity or infections were reported.[127] These early findings collectively underscore the therapeutic potential of in vivo CAR-T in refractory multiple myeloma. These findings are based on small sample sizes, short follow-up durations, and data primarily from conference abstracts or IIT disclosures; they should be interpreted with caution until validated in larger-scale, peer-reviewed clinical studies.

In addition to CD19 and BCMA, CD20-targeting strategies have shown promising efficacy in murine and non-human primate (NHP) preclinical models, indicative of their strong potential for future clinical translation.[44]

In vivo CAR-T clinical advances for solid tumors

The application of conventional CAR-T therapy to solid tumors has long been hindered by inadequate T-cell homing, limited tumor infiltration, and an immunosuppressive microenvironment that impairs effector cell function.[128] In vivo CAR-T strategies offer several distinct theoretical advantages: the small size of delivery vectors may facilitate tissue penetration, and sustained CAR expression could help maintain prolonged immune pressure in the tumor microenvironment.

Table 3. In vivo CAR-T in IND clinical trials

Drug	Vector	CAR target	Indications	Approval date	Approval country	Investigator
INT2104	Lentivirus	CD20	B-cell malignancies	2024.7.9; 2025	Australia, Germany	Interius BioTherapeutics
UB-VV400/410	Lentivirus	CD22	B-NHL, Autoimmune diseases	2024.12 2025.8	USA	Umoja Biopharma/IASO Biotechnology
UB-VV111	Lentivirus	CD19	Hematological malignancies	2024.7.31	USA	Umoja Biopharma
SYS6055	Lentivirus	CD19	B-cell lymphoma	2026.1.29	China	CSPC Pharma
KLN-1010	Lentivirus	BCMA	Multiple myeloma	2025.8.19; 2026.1.7	Australia, USA	Kelonia Therapeutics
CPTX-2309	LNP	CD19	Autoimmune diseases	2025.6	USA	Capstan Therapeutics/AbbVie Inc.
ORN-252	LNP+circRNA	CD19	Autoimmune diseases	2026.2	USA	Eli Lilly
MT-302	LNP	Trop-2	Solid tumor	/	USA	Myeloid Therapeutics
MT-303	LNP	Gpc3	Liver tumor	/	USA	Myeloid Therapeutics

Data sources: Clinicaltrials.gov and Chinese Clinical Trial Registry.

Preclinical studies have provided preliminary validation of this concept. For instance, in a mouse melanoma model, anti-CD3-targeted lipid nanoparticles (tLNPs) delivering TRP1-specific CAR mRNA successfully generated functional CAR-T cells capable of infiltrating tumors and selectively eliminating TRP1-positive cancer cells.[55]

Clinical translation of this approach is underway. CREATE Medicines' MT-302, a myeloid-cell-targeted LNP encoding an anti-TROP2 CAR, was evaluated in a Phase I trial involving 27 patients with advanced solid tumors (11 subtypes). Without lymphodepletion, repeated infusions resulted in infiltration of CAR-positive myeloid cells into tumor tissue, accompanied by T-cell recruitment, enhanced antigen presentation, and pro-inflammatory remodeling of the tumor microenvironment. The best overall response observed was a partial response lasting 16 months in a patient with HR-positive breast cancer. The therapy has an acceptable safety profile, with adverse events primarily consisting of low-grade CRS, fever, and hematologic toxicity; a single case of grade 4 ICANS occurred only in the highest dose cohort.[129] Biomarker analyses confirmed rapid CAR expression in circulating myeloid cells and co-localization of CAR-positive myeloid cells with TROP2-positive tumor cells in biopsy specimens.[130]

Despite these advances, substantial challenges remain for in vivo CAR-T in solid tumors, including tumor microenvironment-mediated suppression, potential off-target transduction of immunosuppressive T-cell subsets, and limited efficacy or persistence of CAR-T cells generated from functionally exhausted patient T cells.[131,132] Future optimization strategies may include incorporating vectors with co-stimulatory or agonistic antibodies,[12,48] co-delivering immunomodulatory cytokines,[133] employing local biomaterial scaffolds to remodel the tumor microenvironment,[122,123] developing dual-targeting systems to enhance specificity, or redirecting this approach toward reprogramming more readily infiltrating myeloid cells.[105,129,134]

In vivo CAR-T clinical advances for autoimmune diseases

The application of in vivo CAR-T technology to autoimmune diseases is receiving increasing attention. Compared to traditional antibody therapies, CAR-T may enable deeper and more sustained B-cell depletion.[135] The in vivo approach eliminates the need for ex vivo manufacturing and lymphodepleting chemotherapy, reduces treatment complexity and associated risk while allowing repeat dosing for adjustable treatment intensity, which may offer unique advantages for long-term disease management.

B-cell-depleting strategies have been demonstrated to be effective in several autoimmune conditions.[136] Preclinical studies across multiple platforms have demonstrated robust B-cell clearance mediated by anti-CD19 in in vivo CAR-T, providing a rationale for clinical investigation in autoimmune indications. In small animal models, platforms developed by Aera Therapeutics (tLNP) demonstrated dose-dependent and substantial B-cell depletion in humanized mice;[137] And the SAIL (tLNP-eRNA) platform achieved profound depletion of multiple B-cell subsets in Peripheral blood mononuclear cell (PBMC)-humanized mice at a low dose of 0.01 mg/kg.[138] In large animal models, platforms developed by Capstan Therapeutics (CD8-tLNP)[103] and Sanofi (CD8-tLNP)[139] achieved outstanding B-cell clearance in cynomolgus macaques with manageable toxicity profiles, indicative of their translational potential.

In systemic lupus erythematosus (SLE), a disease characterized by dysregulated B-cell activity,[140] MagicRNA's HN2301 (an anti-CD19 CAR-T therapy) showed preliminary clinical activity in patients with refractory SLE. CAR-T cells were detectable in peripheral blood within 6 hours post-infusion. In a medium-dose cohort (4 mg), repeated infusions resulted in CAR-T cells constituting over 60% of peripheral T cells, accompanied by complete B-cell aplasia lasting 7-10 days. Clinically, patients exhibited reductions in anti-double-stranded DNA antibody titers, normalization of complement levels within 2-3 months, and marked improvement in disease activity scores (SLEDAI-2000). The therapy had an acceptable safety profile, with low-grade CRS as the primary adverse event and no reported treatment-related infections or neurotoxicity.[141] In the future, efficacy needs to be further confirmed in studies with larger cohorts and longer follow-up durations.

Compared to integrative platforms, the tLNP-mRNA platform circumvents the risk of genomic integration, and the CAR-T cells it generates exhibit limited expansion while effectively depleting B cells and promoting immune reconstitution. This profile significantly improves the risk-benefit ratio for treating autoimmune diseases, offering clear translational advantages and holding promise as a novel therapeutic strategy.

In vivo CAR-T clinical advances for other diseases

While not yet in clinical trials, in vivo CAR-T has shown exploratory promise in areas like antifibrotic therapy and targeting inflammatory aging. Fibrosis, driven by excessive extracellular matrix from activated fibroblasts, lacks therapies that directly eliminate these pathogenic cells. A 2022 proof-of-concept study used CD5-targeted tLNPs to deliver anti-FAP CAR mRNA in a cardiac injury model, generating CAR-T cells that cleared activated fibroblasts, reduced collagen, and improved function.[103] The transient CAR expression may avoid interfering with normal tissue repair, offering a tunable strategy for other fibrotic diseases. An alternative approach targets inflammatory aging—a chronic, pro-fibrotic inflammatory state. In a liver fibrosis model, T-cell-tropic LNPs delivering anti-uPAR CAR circRNA cleared senescent cells, reduced injury markers, and attenuated scarring,[95] supporting senescence clearance as a repair strategy. Studies in allergic[143] and infectious diseases[144] further highlight the platform's potential for conditions poorly addressed by conventional therapies.

IN VIVO CAR-T RESEARCH AND DEVELOPMENT CHALLENGES AND DESIGN OPTIMIZATION

Safety challenges

Off-target risk. Off-target vector delivery represents a primary safety challenge in the clinical translation of in vivo CAR-T technologies, distinct from ex vivotherapies. The associated risks and consequences vary depending on the type of off-target cell and the vector involved. Unlike traditional ex vivo CAR-T, which ensures cellular purity through in vitro selection and expansion, in vivo strategies rely on vector systems to directly transduce or transfect endogenous T cells in situ, thereby introducing the possibility of unintended transduction of non-T cells.

If vectors are delivered to non-immune cells (e.g., fibroblasts, epithelial cells) that subsequently express the CAR, the CAR protein may bind tumor antigens via an "epitope masking" mechanism, physically blocking recognition by therapeutic CAR-T cells and potentially causing treatment resistance.[145] Careful selection of the CAR-recognition epitope to minimize steric hindrance can mitigate this effect.[133] Unintended transduction of immunosuppressive cell subsets[146] may lead to CAR expression within these populations, potentially reinforcing the immunosuppressive microenvironment and exacerbating resistance. Furthermore, for integrative vectors (such as lentivirus), inadvertent integration into stem or germ cell genomes poses a potential risk of insertional mutagenesis, which could lead to secondary malignancies or genotoxicity. During vector production (particularly for viral vectors), CAR expression must be carefully monitored to prevent incorporation of the CAR protein into capsids, which could otherwise facilitate unintended transduction of tumor cells.[40]

Strategies to minimize off-target delivery are as follows: local administration to solid tumors;[123] Dual-targeting vector design to enhance T-cell specificity;[147] Engineering microenvironment-responsive "smart" vectors activated selectively within tumor tissue;[148] Incorporation of tissue-specific promoters to restrict CAR expression to immune cells;[133] And implementation of systems such as the TRAP protein to suppress CAR expression and prevent capsid incorporation during vector production.[40]

On target, off tumor risk. In addition to vector off-target effects, CAR-T cells themselves may cause "on-target, off-tumor" toxicity to normal tissues due to low-level expression of the target antigen in healthy tissues or antigen cross-reactivity resulting from suboptimal CAR design.[149,150] The design of bispecific or multispecific CARs, which require the simultaneous recognition of two or more tumor-associated antigens to trigger full activation, is an effective strategy to enhance targeting specificity while also improving the ability to counteract antigen escape.[151-153]

Genomic random integration risk. In vivo CAR-T therapies based on integrating vectors such as lentiviruses carry the inherent risk of random insertion of the transgene into the host genome, which warrants particular attention. Although ex vivo CAR-T can mitigate this risk through T-cell selection to ensure the vector transduces only the T-cell subset, and has demonstrated an acceptable clinical safety profile overall (despite rare reports of clonal expansion and secondary tumorigenesis[154]), in vivovector delivery introduces the additional concern of off-target integration into long-lived cells such as stem cells. This makes the assessment of integration-site preferences critically important.

The core strategy to reduce this risk is to shift from random to site-specific integration. This approach can be achieved by co-delivering the CAR gene template with a transiently expressed gene-editing system (e.g., CRISPR-Cas9 or a transposase) to direct precise CAR insertion into genomic safe-harbor loci (e.g., the AAVS1 locus), thereby maximizing the avoidance of insertional mutagenesis.[155-159]

Efficacy Challenges

Low in vivo transduction/transfection efficiency. Based on preclinical data, the transduction or transfection efficiency for generating CAR-T cells in vivo is generally low. Across various platforms (lentivirus, LNP, AAV, etc.) and in multiple animal models, the proportion of CAR+ T cells among sampled cells mostly falls within the single-digit to low double-digit percentage range.[44,46,47,52,56,58,59,64,98,102] Delivery efficiency is constrained by multiple factors: physical barriers that limit tissue penetration;[160] Pre-existing antibodies (especially against viral vectors) that accelerate vector clearance;[161] The inherent resistance of resting T cells to transduction;[42,58] And suboptimal cellular uptake and endosomal escape.

Improving efficiency requires coordinated optimization across three dimensions: vector design, payload engineering, and delivery strategies. In terms of vector design, taking LNP-based vectors as an example, optimizing lipid composition to enhance endosomal escape can directly improve delivery efficiency.[162] For viral vectors, technologies such as capsid engineering and biomimetic envelope modification can alter the capsid surface structure to evade vector clearance caused by pre-existing antibodies.[44, 47, 79, 80] Modifying or structurally optimizing the vector/payload to extend its half-life (e.g., through circular RNA design) can prolong its window of engagement with

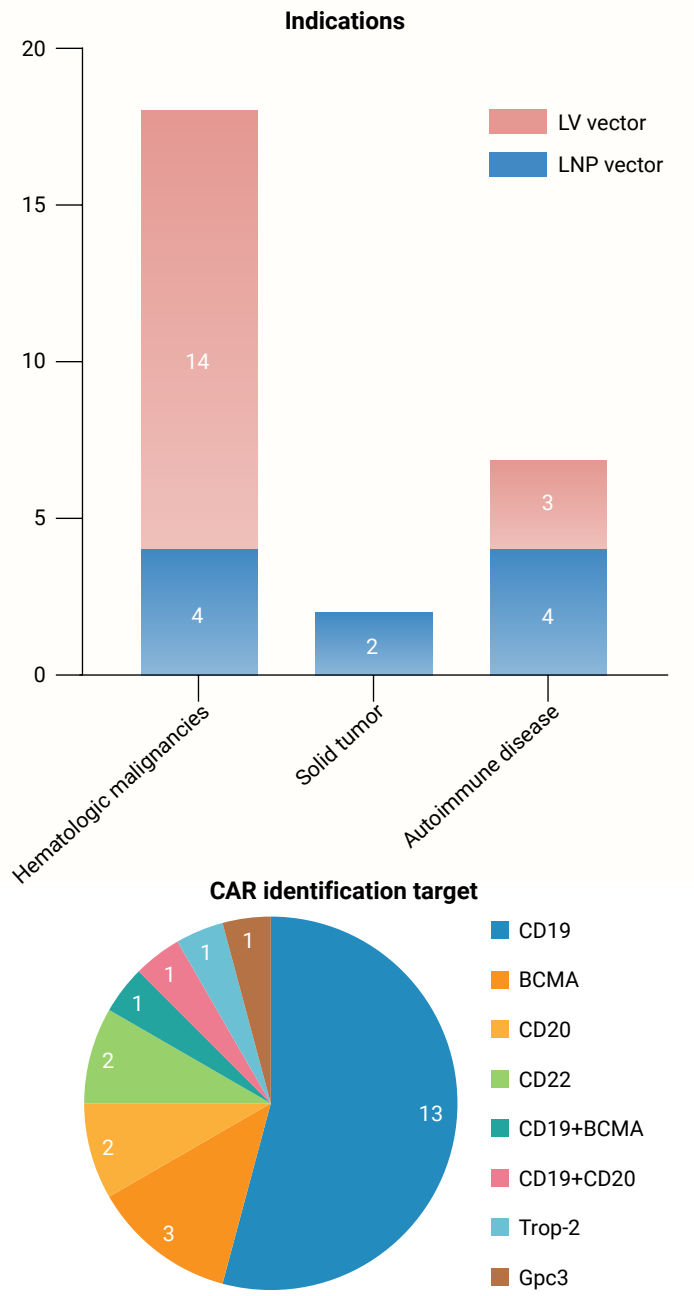

Figure 3. Clinical trial indications and corresponding CAR targets of in vivo CAR-T therapy By the end of January 2026, 9 in vivo CAR-T products have received IND approvals, and 16 have been registered for investigator-initiated clinical trials (excluding terminated or suspended trials). (Left) Clinical indication distribution of in vivo CAR-T therapies by vector platform (multiple trials for the same drug within the same indication category are counted once). (Right) CAR target distribution of in vivo CAR-T candidates in clinical trials.

target cells, indirectly boosting transduction efficiency.[105,107] From an administration perspective, local delivery (e.g., intratumoral injection) can bypass systemic barriers, and repeat dosing can cumulatively improve overall transduction/transfection outcomes.

T-cell heterogeneity, exhaustion, and insufficient activation. The high heterogeneity and functionally exhausted state of a patient's endogenous T-cell pool are fundamental challenges limiting the efficacy of in vivo CAR-T. The functional properties of CAR-T cells are dependent on the originating T-cell subset. Due to the still limited targeting precision of current delivery vectors, the transduced T-cell population contains different functional subtypes. Naïve and central memory T cells generate CAR-T cells with superior expansion and persistence potential, whereas γδ T and NKT cells may confer enhanced solid-tumor infiltration and direct cytotoxicity.[131] Ex vivo-

manufacturing allows for selective enrichment and expansion of desired subsets, whereas in vivo CAR-T relies on the patient's heterogeneous and variable endogenous T-cell pool, making it difficult to precisely control the final subset composition of the product.

T-cell exhaustion is another major obstacle. In cancer patients, endogenous T cells are often chronically exposed to tumor antigens, resulting in an exhausted phenotype characterized by high expression of inhibitory receptors and reduced effector function.[163] Generating CAR-T cells from these exhausted precursors may severely compromise anti-tumor efficacy.[131,132] Furthermore, the vector delivery and transduction process itself may impose additional cellular stress, potentially further exacerbating the exhausted phenotype.[164]

Key strategies to address these challenges include: designing vectors that preferentially target specific surface markers associated with T-cell subsets possessing greater proliferative and persistence capacity; combining therapy with immune checkpoint inhibitors to reverse T-cell exhaustion in vivo;[165] And extending CAR technology to other immune cells (e.g., CAR-macrophages or CAR-NK cells), which may demonstrate superior tumor infiltration and microenvironment remodeling capabilities.[105,129,134]

To enhance the activity of generated CAR-T cells, vector designs may incorporate co-display of T-cell co-stimulatory ligands;[12] Co-delivery of the CAR gene with immunomodulatory agents (e.g., cytokines or checkpoint inhibitors);[24,166] Or localized delivery systems (e.g., hydrogels) to create an activation-favorable microenvironment.[122,123]

Other challenges

The dose-response relationship for in vivo CAR-T therapies is more complex and less predictable than for ex vivo approaches. The administered product is a vector rather than cells. Its pharmacokinetics and transduction efficiency are influenced by individual genetic background (e.g., expression levels of target molecules such as CD3 or CD8), immune status (including T-cell fitness and pre-existing antibody levels), and disease stage.[131,132] Unlike ex vivo manufacturing, direct quality control of the resulting cellular product, such as precise quantification of CAR expression, viability, and phenotype by flow cytometry, and accurate control of the infused cell dose are not feasible. To mitigate safety risks associated with dosing uncertainty, in addition to accumulating preclinical and clinical data to refine predictive models, greater controllability over CAR-T cell generation and clearance is required. Two strategic approaches include: (1) employing transient expression systems (e.g., LNP-mRNA platforms) to minimize long-term toxicity; and (2) incorporating safety switches[27,167] and regulable CAR expression systems[28,168,169] that enable rapid elimination of CAR-T cells in the event of severe adverse reactions.

Further reducing manufacturing costs and improving therapeutic accessibility are critical for democratizing in vivo CAR-T therapy. Universal CAR technologies, such as switchable, split-CAR systems, enable target flexibility. For instance, a CAR engineered to target a biotin-binding immune receptor can engage any biotin-labeled antigen;[31] Administration of different biotin-conjugated targeting antibodies allows dynamic switching of tumor targets, enabling a single platform to address multiple indications while significantly reducing both R&D costs and development timelines. From a manufacturing perspective, advances in large-scale vector production have markedly reduced production costs, while improvements in formulation technologies (e.g., LNP lyophilization) have greatly decreased logistical complexity and barriers to use, thereby facilitating broader accessibility of in vivo CAR-T therapies.[170]

Combination therapy strategies, which enhance efficacy by combining CAR-T with cytokine therapy, antibody therapy, tumor vaccines, chemoradiotherapy, and other treatments, are well-established in conventional ex vivo CAR-T therapy. For in vivo CAR-T, a core challenge is the low efficiency of vector transduction in resting T cells. Therefore, pre-treatment with cytokines such as IL-7 to pre-activate T cells in vivoand push them into the cell cycle may serve as a crucial adjuvant strategy for improving transduction efficiency. Beyond such external combination approaches, a more sophisticated strategy involves designing drug-responsive elements directly into CAR-T cells. For example, Umoja's UB-VV100 incorporates an FKBP-IL2R fusion protein within the CAR's intracellular domain. In the presence of the small molecule drug rapamycin, this system not only competitively protects the mTOR pathway from inhibition but also simultaneously activates the IL-2 signaling cascade, thereby enhancing T cell activation.[133]

QUALITY CONSIDERATIONS OF IN VIVO CAR-T THERAPY

The final product of in vivo CAR-T is the CAR gene-loaded vector. Its efficacy and safety depend primarily on the vector's in vivo distribution, targeting specificity, and transduction efficiency. Unlike ex vivo CAR-T, where quality control focuses on the cellular product, in vivo CAR-T quality control must focus on the nucleic acid and its delivery vector, identifying critical attributes based on the delivery platform's design. This entails controls for starting materials, manufacturing processes, and final product release, each with defined specifications. Process validation and stability studies are also crucial. As in vivo CAR-T is a gene therapy product, overall quality management should follow guidelines like ICH Q9/Q10. Control strategies for materials and processes can reference gene therapy guidelines,[171-173] while potency assays can refer to CAR-T drug guidelines.[174-176] The two main clinical-stage platforms, lentiviral vectors and LNPs, differ in their manufacturing processes (Figure 4) and underlying mechanisms for mediating transgene expression. These differences define their distinct critical quality attributes and control challenges.

Quality considerations of lentiviral vector-based in vivo CAR-T products

The core advantage of lentiviral vectors is achieving long-term CAR gene expression through genomic integration, but this also introduces risks of insertional mutagenesis and off-target transduction. Therefore, quality control should focus on: the precision and stability of targeting modifications; stringent control of vector purity (e.g., empty capsid ratio, replication-competent lentivirus); and analysis of integration sites to assess mutagenesis risk.

Control of production materials. Starting materials for lentiviral vector production include plasmids, production cells, culture media, additives, cryoprotectants, and excipients. As these materials are not unique to in vivo CAR-T, their control standards can be established following ICH Q5D and other relevant guidelines for viral vectors.[171-173]

Process parameters, in-process control, and process validation. A stable lentivirus production process must be established, with control over key parameters in the manufacturing process to ensure batch-to-batch consistency and product safety. The production process for lentiviral vectors is complex (Figure 4). In general, reasonable ranges for process parameters should be defined in accordance with relevant guidelines.[171-173] Intermediate samples should be taken to monitor their key quality attributes (e.g., cell viability, vector titer, vector particle size, and surface molecular characteristics) in real time for process oversight. Unlike ex vivo CAR-T, which is typically filled based on infection activity, in vivo CAR-T should be consistent with other gene therapy products and filled based on physical titer. This approach aims to ensure efficacy while stringently controlling the safety risks associated with the infusion dose.

Process validation aims to prove the process can consistently produce product meeting quality standards. This includes: process performance qualification via three consecutive commercial-scale batches; validation of key process parameter ranges; cleaning validation to prevent cross-contamination; and analytical method validation to ensure reliable testing.

Drug characteristic analysis. Lentiviral vectors used for in vivo CAR-T are classified as in vivo gene therapy products. Applicable guidelines[171-173] require their comprehensive identification and structural analysis at multiple levels (genomic, protein, viral) to ensure consistency. QC attributes related to activity, purity, impurities, and safety are likely to become the critical release criteria for in vivo CAR-T first-in-human trials

Unlike ex vivo CAR-T, which delivers purified cells, in vivo CAR-T delivers the vector directly to patients, necessitating stringent control of its structural integrity. Targeting modifications (e.g., pseudotyped envelopes, displayed moieties like scFv or DARPin) require verification of sequence, density, and target-binding activity (e.g., to CD3/CD8) using methods like mass spectrometry or flow cytometry. The stability of these modifications is a key quality attribute, directly impacting in vivo targeting specificity.

Direct intravenous administration imposes stricter requirements for purity and impurity control. Assays for residuals like HCP/HCD (via ELISA/qPCR)

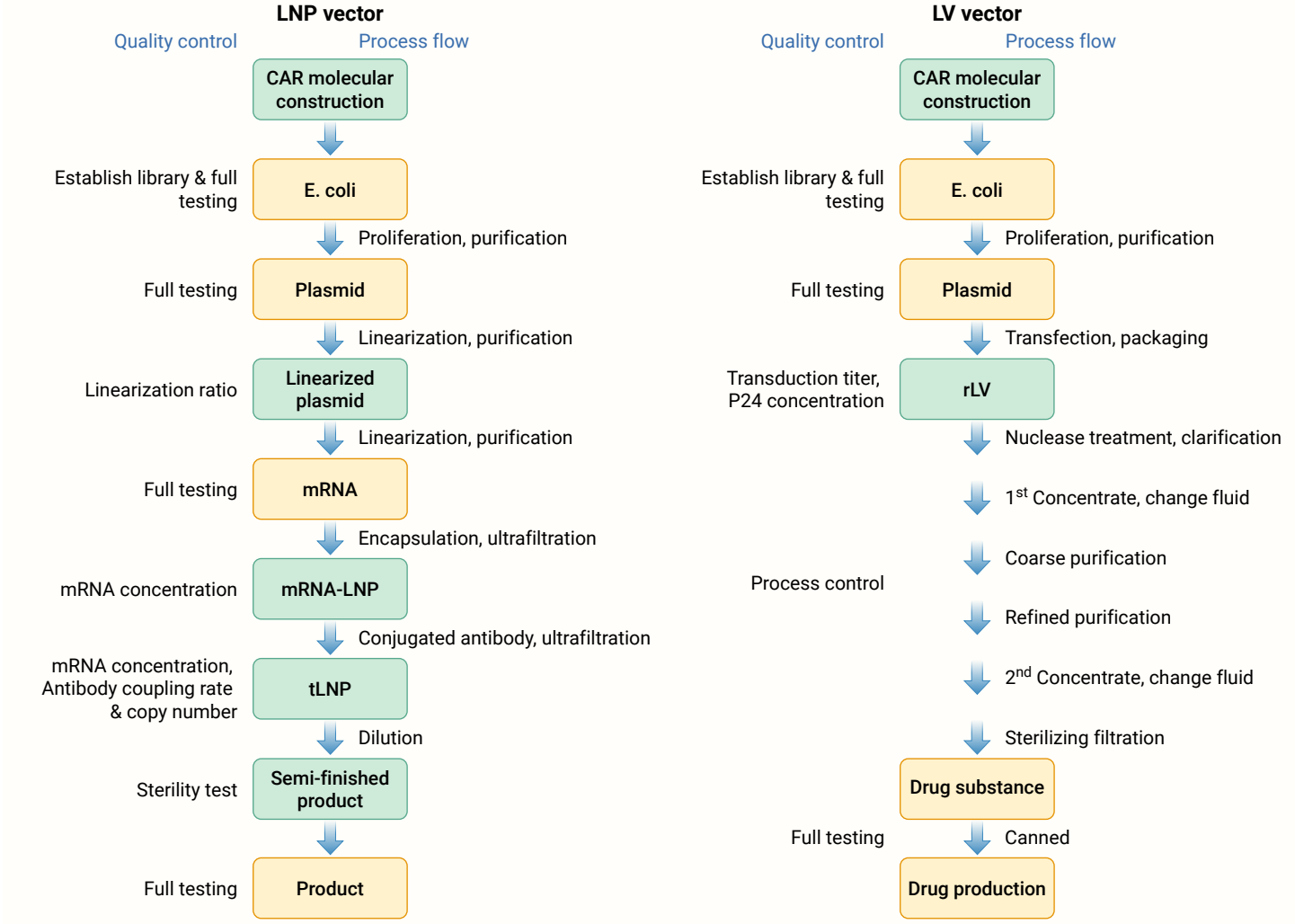

Figure 4. Comparison of manufacturing and quality control processes of LNP-based and lentiviral-based in vivo CAR-T therapy Products (or intermediate products) recommended for full-testing are marked with a yellow background, while those requiring only partial testing are marked with green.

require higher sensitivity. Product-related impurities (e.g., aggregates, free proteins) and risks like capsid-mis-packaged CAR antigens[49] must be strictly controlled.

The risk of insertional mutagenesis from random integration is a key safety concern for in vivo use.[68] Although long-term follow-up data show stable clonal expansion,[179] sensitive integration-site analysis and long-term monitoring remain essential. Integration risk analysis should assess site preference via sequencing post-in vitro transduction. Sensitive RCL detection assays are mandatory. Off-target transduction risk must be evaluated using relevant non-target primary human cells (e.g., hepatocytes). Processes with CAR mis-packaging risk require corresponding monitoring methods.

Biological activity, critical for efficacy, must be evaluated using predictive in vitro surrogate methods. Common approaches involve transducing human PBMCs/primary T cells to measure CAR+ rates. For tumor therapies, co-culture assays to assess cytokine release and killing are standard; disease-specific assays should be developed otherwise. All new methods require rigorous validation. Furthermore, in vivo transduction efficiency directly determines efficacy. Assessing T-cell-specific transduction efficiency is crucial for clinical dose-finding, demanding reliable and sensitive methods.

Establishment of quality standards. Quality standards are used for product release and stability studies. For lentiviral vector drugs, detailed quality standards covering all raw materials, intermediates, drug substance, and drug product should be established in accordance with relevant guidelines such as ICH Q6B and Q5A. The analytical methods and acceptance criteria for each test item should be clearly defined, and method validation should be performed.[175-177]

The selection of quality control test items for in vivo CAR-T products should be based on thorough product characterization, taking into account the production process, clinical indication, product-specific attributes, and current scientific knowledge. The analytical methods can be optimized as the field advances. The test items should typically include identification, purity and impurities, potency, content, general safety tests, and general tests. Identification includes identification of the envelope and nucleic acid. Compared to CAR-T cell products, purity and impurities require stricter control. This includes purity, molecular size variants, product-related impurities (such as virus aggregates, replication-competent lentivirus, empty capsid rate), and process-related impurities (such as residual host cell DNA and residual plasmid DNA). For potency, the target gene expression level and biological activity can generally be established with reference to gene therapy products; the CAR-positive rate can be established with reference to CAR-T cell products. If the product is used for tumor therapy, with reference to oncolytic virus products, the tumor cell killing rate and selective killing activity can be established after T cell transduction. Content can include the number of viral particles, genome copy number, and infectious titer. General safety items include endotoxin, sterility testing, and mycoplasma. General tests can include osmolality, pH, and appearance.

Stability studies should cover the long-term stability, accelerated stability, and stress conditions (e.g., temperature, light, freeze-thaw cycles) of the vector, with a focus on the maintenance of biological activity and the control of impurities. The study data should be used to define the product's storage conditions, shelf life, and transportation requirements.

Quality considerations of LNP-based in vivo CAR-T products

Unlike lentiviral vectors, LNP-mRNA platforms offer transient expression without integration risk, but their efficacy depends on efficient T-cell targeting and cytosolic mRNA expression kinetics. Thus, quality control focuses on: 1) nanoparticle physicochemical uniformity (size, encapsulation efficiency, polydispersity); 2) accurate chemical composition (lipid ratios, antibody conjugation rate); and 3) mRNA integrity (capping efficiency, poly(A) tail length, purity). The following sections detail key quality considerations for each platform. For tLNPs with conjugated targeting moieties, characterization of these components must be added to standard LNP controls.

Control of production materials. tLNPs involve more complex materials than standard LNPs. Per guidelines,[171-173] rigorous control of the mRNA, lipids, and targeting components is needed to ensure quality and consistency. mRNA quality is paramount. DNA template banks should be established and qualified, and mRNA standards set per existing guidelines. These include sequence identity, concentration, purity, integrity, sterility, endotoxin, capping efficiency, poly(A) tail length, and modified nucleotide incorporation—all critical for translation efficiency, stability, and immunogenicity. Lipid components require defined chemical structures and synthesis routes. Key controls are chemical identity, purity, residual solvents, and functional properties (e.g., apparent pKa for ionizable lipids). Targeting antibodies, produced from qualified cell banks, must meet monoclonal antibody standards for purity, aggregates, titer, binding, and Fc function. If engineered (e.g., fragmented, conjugated), intermediates also require strict standards.

Process parameters, in-process control, and process validation. LNP production should follow guideline-recommended process controls.[171-173] For tLNPs, the antibody conjugation rate and free antibody residual are additional key in-process measures.

As with lentiviral vectors, tLNP process validation requires three consecutive commercial-scale batches to demonstrate a consistent process meeting CQAs. Cleaning validation, analytical method validation, and specific validation of the conjugation process are also needed.

Drug characterization analysis. The in vivo function of LNP-mRNA vectors depends highly on the accuracy of their chemical composition and the uniformity of their nanostructure. Characterization focuses on the following aspects.

Chemical composition accuracy: For the ionizable lipids, helper lipids, cholesterol, PEGylated lipids, and targeting antibodies described in Section 2.3.2.1, methods such as HPLC-MS and nuclear magnetic resonance should be used to confirm the purity, ratio, and antibody conjugation rate of each component. Parameters such as the pKa of ionizable lipids and the chain length of PEGylated lipids directly affect endosomal escape efficiency and in vivo half-life and should be included as key indicators.

mRNA quality and integrity: The capping efficiency (preferably >90%), poly(A) tail length distribution, and modified nucleotide incorporation rate of the mRNA directly impact translational efficiency and immunogenicity. Strict quality control via methods such as capillary electrophoresis and LC-MS is required. For novel payloads like circular RNA (circRNA) (see Section 2.2.2.1), dedicated methods for assessing circularization efficiency and purity must be established.

Nanostructural uniformity: Methods such as dynamic light scattering, transmission electron microscopy, and microfluidic chips should be used to measure particle size (typically 50–150 nm), polydispersity index (PDI < 0.2), and encapsulation efficiency (>90%) to ensure batch-to-batch consistency. These parameters are directly related to in vivo spleen targeting and T cell transfection efficiency.

Biological activity is referenced to the in vivo activity evaluation of lentiviral vector CAR-T as described in 5.1.3. Compared to vaccine-like products, therapeutic LNP products impose higher requirements on purity and impurities to enhance efficacy and reduce immunogenicity. Therefore, consistent with lentiviral vectors, QC attributes related to activity, purity, and impurities are likely to become the true critical release attributes for the first-in-human clinical trial products of this type of vector. The sensitivity of the detection methods, the limit of detection, and the substitutability of in vitro activity testing will face challenges.

Establishment of quality standards. LNP-mRNA products also require the establishment of detailed quality standards covering all raw materials, intermediates, drug substance, and drug product, including analytical methods, acceptance criteria, and method validation.[171-173] Special release standards must be established for antibody conjugation-related parameters (conjugation rate, free antibody, binding activity, etc.).

Test items can be set with reference to approved COVID-19 LNP-mRNA vaccines, with adjustments for different drug purposes and mechanisms. Test items should include identification, purity and impurities, activity, content, general safety items, and general tests. Identification should include identification of LNP components, surface antibodies, and nucleic acids. Purity and impurities include mRNA integrity and antibody conjugation rate, as well as product-related impurities such as mRNA degradation products, lipid degradation products, mRNA-lipid adducts, and free antibodies, along with process-related impurities such as residual solvents. Potency testing should include evaluation of target gene expression level and biological activity, as well as antibody binding activity. With reference to CAR-T cell products, the CAR-positive rate may be included. For products used in tumor therapy, with reference to oncolytic virus products, assays such as tumor cell killing rate and selective killing activity can be established after T cell transduction. Content determination should include mRNA concentration, individual lipid component content, and excipient content. General safety testing should include bacterial endotoxin and sterility assessment. General tests may include encapsulation efficiency, particle size and distribution, and pH.

Stability studies can be conducted in accordance with the relevant requirements of ICH Q5C. Test items generally include long-term stability (-20°C or lower), accelerated stability, stress testing, transportation stability, and in-use stability. In addition to conventional physicochemical indicators (e.g., particle size, PDI, encapsulation efficiency), key indicators that should be monitored include changes in mRNA integrity, CAR expression potency (in vitro activity), antibody conjugation stability, and binding activity. These data provide the scientific basis for defining appropriate product production, packaging, storage, and transportation conditions.

CONSIDERATIONS FOR NON-CLINICAL RESEARCH OF IN VIVO CAR-T THERAPY

Non-clinical studies for in vivo CAR-T therapies are designed to characterize biological function and mechanism of action, define pharmacologically active dose ranges, and identify potential toxicological risks. These studies provide critical support for first-in-human trial design, including selection of the subject population, dosing regimen, route of administration, efficacy evaluation endpoints, and risk monitoring strategies.

From a regulatory perspective, in vivo CAR-T therapies exhibit a dual functional identity. In terms of pharmaceutical form, the product is classified as a gene therapy product because it delivers the CAR transgene via a vector. However, its mechanism of action, exerted through the genetically modified cells generated in the body, aligns it closely with cell therapy products. Consequently, non-clinical studies should integrate regulatory guidance applicable to both gene therapy products and cell products[178-183] while specifically addressing the unique challenges associated with in situ CAR-T cell generation.

The in vivo generation of CAR-T cells introduces two primary challenges: (1) defining the appropriate patient population, route of administration, dose level, and dosing regimen for clinical trials through robust preclinical evaluation; and (2) addressing safety concerns inherent to novel vectors, including off-target toxicity, unintended random integration, and tumorigenesis associated with integrating vectors. Furthermore, due to substantial variations in in vivo biological behaviors across different vector platforms and therapeutic indications, non-clinical study design must be conducted on a case-by-case basis. Based on the distinct technical characteristics of different vector platforms described in Section 2.2, non-clinical study protocols should be designed differentially: differences between lentiviral vectors and LNP-mRNA vectors in integration risk, targeting specificity, immunogenicity, and expression durability dictate varied emphases in animal species selection, key safety evaluation endpoints, and pharmacokinetic study objectives.

Selection of formulations and animals

Test article and route of administration. The test article used in non-clinical studies should be representative of the intended clinical candidate with

respect to manufacturing process and critical quality attributes, and must comply with predefined release specifications. Due to target species specificity, the same CAR molecule cannot be used across species. Studies using animal models therefore require animal-derived surrogate CAR molecules, whose production and quality should closely mirror the clinical candidate, with their representativeness justified. The route of administration in animal studies should, where feasible, replicate the intended clinical route. If not feasible, a scientifically sound rationale for the alternative must be provided. For formulations intended for repeated dosing (e.g., LNP-mRNA products[101]), nonclinical studies must ensure consistent quality of the formulation throughout the dosing period.

Animal species. The selected animal species should be physiologically comparable with humans, exhibiting similar vector biodistribution, transduction, CAR expression, and resulting pharmacological activity. Common models for in vivo CAR-T include immune-reconstituted human xenograft mice (e.g., with human PBMCs or CD34+ cells)[36,76] and non-human primates (NHPs).[49,113]

For lentiviral vectors: The species specificity of targeting moieties and CAR antigens often necessitates the use of humanized mouse models to evaluate CAR-T generation and targeting. If cross-reactive surrogates exist, NHPs can further assess long-term integration safety and immunogenicity.[12,13,37] For products without suitable surrogates, humanized mice are used but their incomplete immune system limits full immunogenicity assessment. Additionally, hyperactive immunity in PBMC-reconstituted mice may overestimate safety risks.

For LNP-mRNA vectors: Immunogenicity assessment of lipids and mRNA requires animals with intact immune systems. NHPs, due to similarity in immune cell distribution and lipid metabolism, are preferred for evaluating immunotoxicity and cytokine release syndrome (CRS) after repeated dosing.[48,113]

Both models have pros and cons. Selection should be driven by study objectives. When cross-reactive surrogates are available, NHP models can assess targeting and toxicity profiles, though affinity differences may obscure some risks.

Pharmacodynamics study

Pharmacodynamic evaluation requires an integrated approach combining both in vitro and in vivo experiments.

In vitro pharmacodynamics. In vitro pharmacodynamic studies, typically using human peripheral blood mononuclear cells (PBMCs) or primary T cells, evaluate drug-induced activation of target immune cells, vector transfection/transduction efficiency, persistence of CAR expression in T cells, and the specific cytolytic activity of the generated CAR-T cells against target cells. Because CAR-T cell activation is dependent on target-cell antigen density,[179] it is advisable to test killing efficacy against a panel of cells expressing varying antigen levels. If CAR-T cells are engineered to co-express other functional elements (e.g., cytokine-inducing domains or suicide switches), dedicated functional validation assays should be performed;[43,68,111] If these functional elements require induction by other drugs, corresponding dosing regimen studies for those drugs should be conducted in the non-clinical stage.

Organoids are three-dimensional tissue models derived from adult stem cells, mimicking the structure and function of real organs. Compared to traditional cell lines, they better simulate organ complexity and can be cultured long-term.[184] This model has been used to evaluate the efficacy of conventional CAR-T against solid tumors,[185] and is applicable to in vivo CAR-T pharmacodynamics. While not yet a full replacement for animal models in assessing overall pharmacokinetics, pharmacodynamics, and systemic toxicity, organoid findings provide valuable prospective references for in vivo evaluation.

In vivo pharmacodynamics. In vivo pharmacodynamic studies for in vivo CAR-T products commonly utilize humanized mouse models and NHPs, with emphasis on CAR-T generation efficiency, expansion, persistence, and functional activity. For oncology indications, immunodeficient MHC-dKO-NXG mice engrafted with human PBMCs and tumor cells can be used to establish a tumor model with a reconstituted human immune system to verify in situ CAR-T generation and antitumor effects. For products intended for lymph node administration, intraperitoneal injection in mice may serve as a practical alternative. For autoimmune disease models, NXG mice engrafted with human hematopoietic stem cells (HSCs) may be used to validate CAR-T generation and B-cell depletion activity.

To further validate biological function and mechanism, larger animal models such as dogs or NHPs can be used when suitable cross-reactive surrogate formulations are available. For instance, Kelonia Therapeutics' iGPS lentiviral platform, in a proof-of-concept NHP study with a single dose of a surrogate particle targeting CD20, achieved complete B-cell depletion lasting over two months;[13] Capstan Therapeutics' CellSeeker™ tLNP platform surrogate CPTX2309-S (CD8-tLNP delivering an anti-CD20 CAR) mediated efficient transient CAR transfection of CD8+T cells following a single dose in cynomolgus monkeys;[49] And a CD8-tLNP platform developed by Hunter et al. induced profound transient B-cell depletion followed by naïve B-cell reconstitution after treatment cessation in cynomolgus monkeys.[104] Preclinical efficacy data indicate that lentiviral vector platforms, due to their genomic integration, can often induce responses with a single dose, whereas LNP-mRNA platforms relying on transient expression typically require multiple administrations to maintain therapeutic efficacy. This difference should be reflected in pharmacodynamic studies and provide a basis for designing the clinical dosing regimen.

Pharmacokinetics Study

Pharmacokinetic studies aim to characterize the in vivo dynamics of the vector and the accompanying biological behavior of the generated CAR-T cells. ICH S12 provides globally recognized standards for designing biodistribution studies for gene therapy products.

Distribution. Biodistribution studies for in vivo CAR-T assess the distribution, retention, and clearance of the vector and the genetically modified cells at the administration site, as well as in target organs, cell types and non-target tissues in relevant animal species. These studies for investigational products can be conducted independently or integrated with pharmacological and/or toxicological investigations.[186] These studies are conducted in model animals using qRT-PCR. For products with a risk of genomic integration (e.g., lentiviral vectors), RNA in situ hybridization (ISH) can be used to identify specific cell types expressing the CAR transgene in tissues with high distribution. A study case shows that UB-VV111 has the highest distribution in the liver and spleen in an HSC-reconstituted mouse model. ISH shows that CAR is expressed only in mouse macrophages and human T cells, with no detectable expression in non-immune cells. In beagle dogs, this product also failed to transduce non-lymphoid tissues, suggesting a high degree of in vivo targeting specificity.[187]

For integrating vectors such as lentivirus, analysis should focus on whether vector sequences are present in long-lived cells such as gonadal tissues and bone marrow hematopoietic stem cells, to assess the long-term potential risks of genomic integration. For non-integrating vectors (e.g., LNP-mRNA), it is recommended to evaluate their inherent liver tropism. If novel components with unknown risks are involved, it is advisable to investigate the systemic clearance kinetics of the vector components.

Shedding/excretion. Shedding or excretion refers to the release or secretion of the gene therapy product from the body. Although current in vivo CAR-T platforms use replication-defective vectors, shedding studies may still be required. For example, following intravenously or intraperitoneally administration of UB-VV111 in mice, no vector genomes were detected in excreta or secretions.[187] For novel T-cell-targeting antibody-conjugated LNPs, nonspecific binding can be evaluated through human tissue cross-reactivity assays and membrane protein array screening.[188]

Safety study

Safety assessment should encompass safety pharmacology, general toxicology, and formulation-related safety studies, conducted in compliance with Good Laboratory Practice (GLP) and relevant guidelines from the NMPA, FDA, and ICH. Specialized toxicology studies addressing product-specific risks, such as the genotoxicity and tumorigenic potential of integrating vectors, are also required.

Safety pharmacology. These studies evaluate potential adverse effects on physiological functions (e.g., central nervous, cardiovascular, and respiratory

systems) at or above the therapeutic dose range. For in vivo CAR-T products, this evaluation is often integrated into general toxicology or pharmacokinetic studies since neurotoxicity is frequently induced indirectly by intense immune activation.[189] Assessments should ideally be conducted in animal models capable of mimicking cytokine release syndrome. For example, Capstan Therapeutics evaluated the safety of its alternative tLNP formulation in cynomolgus monkeys and reported no central nervous system-related adverse finding following intravenous injection.[104]

General toxicology. General toxicology studies for in vivo CAR-T products are typically conducted in humanized mice or non-human primates (NHPs) using clinically relevant dosing regimens and in alignment with guidelines such as ICH M3. Standard endpoints include body weight, clinical observations, clinical pathology parameters, peripheral blood immunophenotyping, assessment of CAR-T generation, immunotoxicity markers, and histopathological examination. The recovery period should be defined based on the persistence of pharmacological effects and the reversal of observed toxicity.

Immunogenicity and immunotoxicity are key safety considerations. Both lentiviral and LNP-based vectors are inherently immunogenic. For viral vectors, the influence of pre-existing host antibodies on transduction efficiency and safety should be considered during vector design. For example, VSV-G pseudotyped lentivirus has a high prevalence of pre-existing antibodies prone to neutralization, prompting some platforms to use alternative fusogens (e.g., Nipah or Sindbis envelope proteins) to lower immunogenicity. For LNP platforms, components (ionizable lipids, PEGylated lipids) and the mRNA are immunogenic, requiring toxicology studies to include cytokine profiles and to focus on the dose relationship to CRS incidence. As LNP-mRNA platforms often require repeated dosing, toxicology studies should cover the full dosing cycle and recovery to assess immune tolerance and cumulative toxicity. Given that CAR-T therapy's mechanism involves immune cell activation, cytokine release syndrome (CRS) is a core anticipated toxicity that requires specific monitoring and management.

In preclinical evaluations, candidate products like UB-VV111 and Interius's INT2104 have shown favorable tolerability. In an HSC-reconstituted mouse model, UB-VV111 administered intraperitoneally or intravenously at up to 5.1×10^7 TU was well tolerated, with no test-article-related adverse effects in body weight, clinical chemistry, hematology, cytokine release, or histopathology.[187] Similarly, in a comparable model, INT2104 administered intravenously at up to 2.5×10^7 TU was well tolerated, with no test-article-related body weight loss.[37]

Reproductive toxicity. As in vivo CAR-T therapies expand into broader clinical indications and patient populations compared to conventional CAR-T, potential reproductive and developmental toxicity risks should be assessed based on product characteristics, mechanism of action, intended clinical indications and population, findings from general toxicology studies, and biodistribution data, in accordance with relevant gene therapy guidelines. If general toxicology studies indicate a potential reproductive organ toxicity, fertility and early embryonic development toxicity studies should be conducted. Studies should assess whether the vector persists in gonads and whether there is potential germ cell exposure; if exposure is confirmed, germline transmission studies should be conducted. For integrating vectors, the risk of germline integration must also be evaluated. For non-integrating vectors (e.g., LNP-mRNA), focus should be placed on their tropism for reproductive organs and the potential reproductive/developmental toxicity risks thereby incurred.

Genotoxicity and tumorigenicity studies. In conventional CAR-T therapy, genetic modification is confined to terminally differentiated T cells. Although rare cases of secondary malignancies associated with integrating lentiviral vectors have been reported, the overall safety profile has remained acceptable. In vivo CAR-T strategies, however, introduce the risk of off-target transduction in stem cells, making the integration profile of the vector a primary safety concern.[186]

For lentiviral vector-based products, safety assessment includes evaluation of nonspecific transduction by comparing vector efficiency across immune versus non-immune cell types. Genomic integration risk is typically analyzed using methods such as amplicon sequencing to map integration sites and characterize their distribution patterns. For example, the candidate product UB-VV111 exhibited high specificity for T cells, with no transduction of human primary non-immune cells even at the maximum achievable in vivo multiplicity of infection.[37] Moreover, CAR-T cells generated in vitro using this vector showed a vector copy number (VCN) and integration site profile (e.g., low VCN, low propensity for integration into repetitive sites, with insertions primarily within intronic regions) comparable to conventional lentiviral vectors.[37]

LNP-mRNA platforms, which enable transient and non-integrating gene expression, generally avoid the risk of insertional mutagenesis and therefore may not require integration site analysis. For platforms employing transposon systems or gene-editing technologies to achieve site-specific integration, safety assessment must address distinct concerns, such as genotoxicity arising from transposon footprint sequences or off-target editing events.

In addition to the direct risk of insertional mutagenesis, other mechanisms that may promote tumor growth should be considered, such as cellular injury and aberrant repair processes triggered by off-target immune responses.

Formulation safety. Formulation safety should be evaluated using the intended clinical administration route. For intravenously injected products, local irritation assessments can be incorporated into general toxicology studies, and in vitro hemolysis testing should also be performed.

CONCLUSION AND FUTURE PERSPECTIVES

In vivo CAR-T technology addresses several key limitations of conventional CAR-T approaches by generating therapeutic CAR-T cells directly within the patient. This strategy offers significant advantages, including a streamlined treatment process, improved cost-effectiveness, and the potential for broader accessibility, and therefore represents a promising direction for future therapeutic development with broad prospects. The field has progressed from conceptual exploration to the stage of clinical application, demonstrating encouraging preliminary outcomes in hematologic malignancies, solid tumors, and autoimmune diseases. Nevertheless, significant challenges remain, particularly concerning inadequate vector-targeting specificity, off-target effects, and dose controllability, all of which necessitate ongoing technical refinement and rigorous clinical research.

Looking ahead, the continued evolution of this technology will likely concentrate on several key directions. First, advancing technological maturity through continuous optimization of targeted delivery systems, particularly by improving T-cell transduction efficiency and targeting precision of tLNP platforms, refining viral vectors to enhance specificity while mitigating risks associated with random genomic integration, and enhancing the ability to target and transduce resting T cells across different platforms. Second, addressing industrialization and accessibility by overcoming barriers to cost-effective, scalable production through technological innovation and process optimization to enable large-scale manufacturing and worldwide distribution of in vivo CAR-T products. Third, accelerating clinical translation for non-oncological diseases and extending the application of in vivo CAR-T to fields such as autoimmune disorders and tissue fibrosis. Fourth, establishing standardized manufacturing processes and quality control to support the development of affordable therapeutic platforms, reduce treatment costs, and expand accessibility by establishing comparable standards for different delivery platforms applied to various diseases, and developing more clinically meaningful and robust potency assays. Furthermore, the integration of gene-editing technologies with in vivo CAR-T platforms, together with the application of artificial intelligence and organ-on-a-chip systems in CAR construct design, targeted delivery optimization, and efficacy prediction, may create new avenues for technological advancement in this field.

Currently, although in vivo CAR-T research is advancing rapidly with continuous emergence of new technologies, there is a lack of corresponding regulatory guidelines and regulatory experience. Based on the drug characteristics of in vivo CAR-T, this review discusses its quality control and nonclinical evaluation during clinical translation. It is anticipated that with technological progress and increased understanding, regulatory science in this field will gradually improve, thereby accelerating the market approval process for in vivo CAR-T therapies.

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DECLARATION OF INTERESTS

Sun Z. is employed by CSPC Pharmaceutical Group Co., Ltd. Huo Y. is employed by TriApex Laboratories Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AND CODE AVAILABILITY

Not applicable.