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In Vivo CAR-T Therapy: The Transformational Leap Toward Affordable and Safer Cancer Immunotherapy

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Abstract. In vivo CAR-T therapy is revolutionizing oncology since it entails modifying a patient's T cells in situ, as opposed to the costly and lengthy ex vivo manufacturing process usually associated with traditional CAR-T therapy. This revolutionary approach utilizes lipid nanoparticles (LNP) or engineered viral vectors (e.g., AAV/LV) to deliver CAR genetic payloads directly to endogenous T cells, greatly reducing production costs while bypassing complex logistics such as leukapheresis and cell shipment. More importantly, it can dramatically lower life-threatening toxicities; early trial data show cytokine release syndrome (CRS) compared with traditional CAR-T, with rapid deployment. By redirecting tissue-resident memory T cells to deliver payloads that counter-tolerize this target (e.g., IL-12, anti-PD-1), it overcame the immunosuppressive barrier created by solid tumor microenvironments, achieving approximately 80% complete remission in relapsed B-ALL. With lyophilized "off-the-shelf" LNPs providing worldwide access (even in resource-restricted settings), this paradigm shift could have turned CAR-T from a boutique therapy into a scalable, less expensive "living drug" that promises to democratize cancer care worldwide.

Keywords: Immunotherapy; Tumor, Autoimmune disease; CAR-T; invivo CAR-T.

1. Introduction

Perhaps cancer is considered the biggest enemy of modern medicine. One group of diseases grows without control and defies all normal regulatory mechanisms [1]. Abnormal cells grow excessively and invade adjacent tissues before eventually taking parts in different organs through the blood vessels or lymphatic system. Genetic alterations manifest changes at the genetic level and epigenetic level, disrupting and breaking the internal cellular homeostasis [2].

These are some of the characteristics that make cancer heterogeneity-the immune escape strategies-and dynamic tumor microenvironment hard to treat [3]. Cancer treatment has dramatically changed within the last decades-from dated patterns to multimodal managing on the basis of disease type, stage, or molecular features. Surgical resection alone is the first-line therapy against localized solid tumors, often for the complete removal with negative margins, and improved techniques in minimally invasive and robotic surgery have increased the precision of such approaches while minimizing morbidity in patients [4].

Radiotherapy, using photons, protons, or heavy ions, specifically targets DNA damage to cancer cells when tumors present a need for non-invasive intervention. Innovations such as stereotactic body radiotherapy (SBRT) and image-guided radiotherapy (IGRT) increased accuracy while minimizing collateral damage. Meanwhile, chemotherapy, widely noted adverse effects generically linked to systemic toxicity, has been refined using nanoparticle carriers (for example, liposomal doxorubicin) or suitably high dosing approaches metronomic regarding chemotherapy's therapeutic index. The most significant advance has been represented by the invention of targeted therapies such as small molecule inhibitors (for example, tyrosine kinase inhibitors) or monoclonal antibodies (for example, trastuzumab for HER2+ cancers) and these have to be guided by companion diagnostics for proper and precise targeting of molecular alterations like inherited defects (mutation of EGFR, for example) or amplifications (like HER2) [5, 6].

Arguably the most radical change has been conferred by immunotherapy: arms the immune system to fight back against cancer. Immune checkpoint inhibitors (e.g., anti-PD-1/PD-L1, CTLA-4 antibodies) help bring exhausted T-cells back to life, while CAR-T cell therapy—a cutting-edge

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adoptive cell therapy—modifies a patient's own T-cells to perceive and eradicate cancer. CAR-T therapy has enjoyed a successful run in blood malignancies since its initial approval by the FDA back in 2017 for acute lymphoblastic leukemia in children. However, antigen escape, T-cell exhaustion, and the immunorespressive tumor microenvironment (TME) have all been factors limiting the efficacy of this treatment modality. Tumors often acquired a metabolic handicap while using preferential competition to rob the immune cells of enough glucose and starving CAR-T cells by consuming glucose at a higher rate than their counterparts. For metabolic contrariety, there are metabolism reengineering strategies, including an increase in the expression of the glucose transporter GLUT1 for the purposes of better CAR-T cell vigor and survival in hostile TMEs. Such strategies would address antigen recognition as well as a long-term therapeutic effect and possibly extend the CAR-T therapy approach into solid tumors [7, 8].

The next frontier is in vivo CAR-T cell therapy: direct change using a viral vector or lipid nanoparticles in a patient's body. It contrasts with conventional CAR-T, which requires complex ex vivo manufacturing. In vivo CAR-T in this case could dramatically lower costs, delays, and manufacturing hurdles while lessening the T-cell exhaustion linked to lab-based culturing by eliminating leukapheresis and external cell expansion. Together, all these advances highlight that oncology is making rapid strides toward accurate, easily accessible, and curative treatments for both hematologic and solid malignancies [9, 10].

2. The CAR Structure

Chimeric antigen receptors are synthetic fusion proteins that redirect T-cell specificity. The conventional structure of a CAR is composed of three functional domains: 1) an extracellular antigen-binding domain that is usually derived from a single-chain variable fragment (scFv) of monoclonal antibodies and is responsible for target recognition; 2) a transmembrane domain such as CD28 or CD8α that secures the receptor in the cell membrane; and 3) an intracellular signaling module typically containing a CD3ζ activation domain linked to one or more costimulatory domains like CD28, 4-1BB, or OX40 such that T-cell activation and persistence are promoted. The modular design of CARs facilitates the MHC-independent targeting of tumors, which forms the molecular basis for CAR-T therapy. [11]

3. Transformation of the CAR into CAR-T

This process of transforming the chimeric antigen receptor into functional CAR-T cells is highly sophisticated, involving genetic engineering and advanced cell culture procedures, starting from the conceptual design of the synthetic CAR construct made of an extracellular antigen-binding domain (often a scFv targeting tumor-specific markers such as CD19 or BCMA) followed by a flexible hinge region, a transmembrane domain to anchor in the cell membrane, and the intracellular signaling domains including T-cell activation by the CD3ζ chain and either or both costimulatory molecules such as CD28 or 4-1BB. This genetic blueprint is delivered into patient-derived T cells through various viral and non-viral vectors, with lentiviral vectors being the most clinically feasible, based on being able to stably integrate the CAR gene into the host genome while efficiently infecting both proliferating and resting cells, as seen in FDA-approved therapies Novartis's Kymriah and Gilead's Tecartus. Other viral strategies include gamma-retroviral vectors used in Kite Pharma's Yescarta that afford robust long-term expression but need a cell to divide for integration, adenoviral vectors characterized by a large cargo capacity and a high transduction rate but limited by transient expression and immunogenic responses, and studies exploring using adeno-associated viruses for in vivo CAR-T generation though limited by small cargo packaging. The non-viral route is now gaining ground, the mainstay being mRNA electroporated for transient CAR expression- that does not carry the risk of genomic integration, allows rapid production, CRISPR-Cas9 gene editing employed by Allogene Therapeutics to engineer universal CAR-T cells, allowing for precise genomic editing, and transposon systems such as Sleeping Beauty, which could prove cheaper when using for stable genomic insertion.

The genetically modified T cells undergo extensive ex vivo expansion in bioreactors with cytokine support (typically IL-2 and IL-15) for 2-3 weeks to achieve therapeutic quantities before being infused back into lymphodepleted patients, where they further expand and execute their tumor-targeting functions via perforin/granzyme release and Fas/FasL-mediated apoptosis. A huge number of quality controls are instituted from the start of the flow from leukapheresis and T-cell collection to the end-product release to ensure that the pertinent clinical standards are upheld, especially risk considerations such as cytokine release syndrome and neurotoxicity that correlate with the presently approved therapies directed against CD19, while the applied research is primarily aimed at the optimization of the vector selection with hybrid viral-CRISPR systems, improvement of their persistence via strengthened co-stimulatory domains, and enhancement of application to solid tumors through new targeting strategies and modulation of the tumor microenvironment [12, 13].

4. CAR Generations

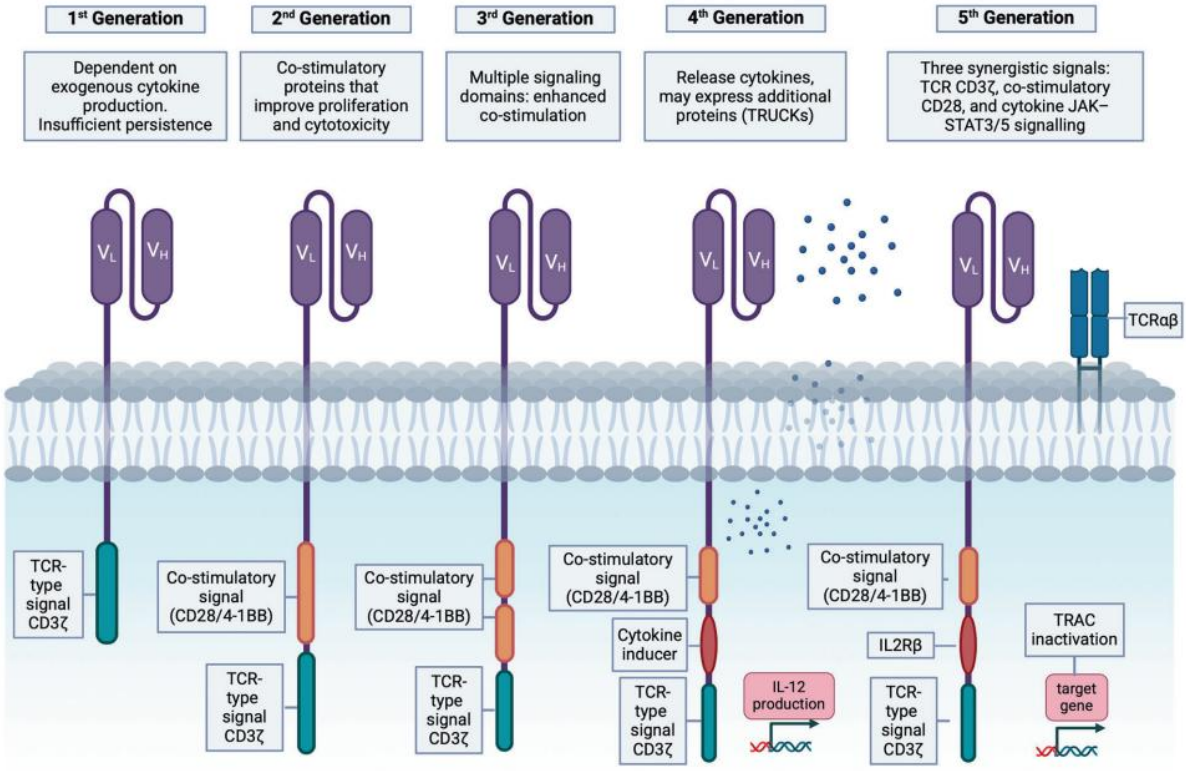

Figure 1. CAR Generations (Inés Zugasti, et al. CAR-T cell therapy for cancer: current challenges and future directions. Signal Transduct Target Ther 10: 210 (2025). [14])

4.1. First-Generation CAR-T Cells

The first of its kind, first-generation CAR-T cells were indeed a very exciting proof of concept in adoptive cell therapy, demonstrating that T cells could be genetically reprogrammed to target tumor antigens solely by the minimalist design of their CD3ζ signaling domain without any unnecessary co-stimulatory inclusion in their design. Notwithstanding, these pioneering constructs, while successful in mediating in vitro antigen-specific tumor cell lysis, had critical limitations related to their potential clinical applications. Because of the lack of co-stimulation, T-cell exhaustion happened quite fast and T cells became undetectable usually within a few weeks after infusion; also clonal expansion was lower, and the durations of therapeutic benefit were short (with most hematologic malignancy patients relapsing within months). Their efficacy was further diminished in solid tumors like ovarian cancer and neuroblastoma, where tumor engagement was documented but had no meaningful clinical translation. Such failures fundamentally showed that CD3ζ activation could induce T-cell responses, but it was far from sufficient to defeat the immune evasion mechanisms of tumors-measurement that led, on the one hand, to the development of second-generation CARs including crucial co-stimuli

domains. Clinically antiquated today, they have great value as research tools for informing studies into basic T-cell signaling and for benchmarking next-generation designs against. Of course, the most important lesson they impart on co-stimulation for T-cell persistence and efficacy is still inspiring today's therapeutic optimizations for CAR-T. [15]

4.2. Second-Generation CAR-T Cells

Representing a milestone in adoptive cell therapy, second-generation CAR-T cells serve as the clinical gold standard. Collectively, these enhancements introduce a co-stimulatory signal (usually either CD28 or 4-1BB) that will accompany the CD3ζ activation domain-the inclusive activation signal for T cells that approaches more physiological equivalence. The distinction generated by choicing between these costimulatory domains creates different functional profiles: CD28-based CARs (as in axicabtagene ciloleucel/Yescarta) allow rapid, very strong activation kinetics and significant initial tumor killing capability, while 4-1BB designs (like tisagenlecleucel/Kymriah) have a slower but more persistent activity with more sustainability and longer potential control of the disease.

This generation's vindication is most vigorously manifest in the area of B-cell malignancies, with therapies targeting CD19 achieving, as measured by the criteria of an unprecedented 70-90% response rates in previously treated patients, not only large amounts of FDA approvals. The optimized signaling structure attains effective balance between rapid induction of cytotoxic activity and long-lasting persistence, with some patients retaining detectable functional CAR-T cells years after infusion. Nevertheless, there are significant caveats-response rates in solid tumors remain dismal (typically <30%), with significant impediments including antigen heterogeneity, immunosuppressive microenvironments, and physical barriers to tumor infiltration.

Toxicity remains an important concern, as 50 to 90 percent of patients develop cytokine release syndrome (grade > 3 in 20-30%) and 20-40 percent suffer from immune effector cell-associated neurotoxicity syndrome (ICANS). Although these adverse events can often be effectively managed with tocilizumab and corticosteroids, they still require specialized monitoring, as they can be fatal. Additionally, the problem of antigen escape variants developing occurs in 30-50% of responding patients, causing relapse after initially effective therapy. Current studies are focused on making better co-stimulatory domain combinations, safety profiles that are safer through either suicide switches or gentler activation designs, as well as deep penetration into solid tumors through microenvironment alteration strategies. Second-generation CAR-T successes have brought harrowing revolutions in hematological malignancies treatment and a vital groundwork for future third- and fourth-generation advances. [16, 17]

4.3. Third-Generation CAR-T Cells

Integrating synergistic signals, these constructs comprise CD3ζ plus two co-stimulatory domains (e.g., CD28+4-1BB). Although such design outperformed preclinical models to improve functionality, clinical trials failed to show tangible differences from their second-generation counterparts. Paradoxically, in some instances, the exaggerated signaling may hasten T-cell exhaustion. Current research propounds on domain combinations and improvement in activation kinetics, particularly with interest towards hard-to-treat cancers where increased signaling might probably help. [18]

4.4. Fourth-Generation CAR-T Cells (TRUCKs/Armored CARs)

The cutting edge of clinical development today, these "smart" cells also integrate additional therapeutic payloads among those that may be mainly immunomodulatory cytokines (IL-12, IL-15), checkpoint inhibitors (anti-PD-1), or safety switches. Cross-linked features to transcend the immunosuppressive tumor microenvironment have shown promise in early solid tumor trials (e.g., IL-12-secreting CARs remodeling stroma in tumors and safety systems of inducible caspase-9 that heighten controllability) [19].

4.5. Fifth-Generation CAR-T Cells

The latest frontier involves universal CAR platforms that have engineered cytokine receptors (e.g., IL-2Rβ), which activate both CAR signaling and endogenous JAK-STAT pathways. These designs aim to create "off-the-shelf" allogeneic products through CRISPR editing to eliminate TCR and HLA molecules. Early prototypes demonstrate resistance to exhaustion and improved adaptability to tumor evolution. While still preclinical, fifth-generationapproaches may solve critical challenges pertaining to manufacturing, persistence, and broad-spectrum anti-tumor activity. [20]

5. CAR-T Functions

Currently available CAR-T cell therapies, although considered landmark innovations in cancer therapy, continue to have significant limitations, limiting their acceptance in a wider clinical practice. First-generation constructs demonstrated very poor clinical failures - less than 20% for response rates and around 30 days median persistence due to fast T-cell exhaustion. In second-generation products, improvements can be noted; however, results remain variable - such as CD28-based constructions that showed rapid expansion but rapid contraction, while in 4-1BB, products were noted to have better persistence but probably lower peak efficacy. The toxicity profile of therapy remains one of the challenges, with severe cytokine release syndrome occurring in 22-46 percent of patients and neurotoxicity in 13-31 percent of cases, most often requiring intensive care and compounding costs that can exceed $1 million per patient when including hospitalization. Manufacturing hurdles are substantial, with 5-10 percent of patients failing to receive their therapy due to production failures or disease progression during the 3-6 week manufacturing period. Specific barriers to solid tumors include the immunosuppressive microenvironment (including hypoxia, acidic pH, and high concentrations of TGF-β); a dense extracellular matrix (up to 20 percent collagen); and poor CAR-T cell infiltration (with less than 0.1 percent reaching tumor sites). Access is at an even lower level because only about 0.5 percent of cancer centers around the world can deliver these therapies. However, there are promising approaches, such as point-of-care manufacturing systems potentially shortening production time to 48-72 hours, allogeneic approaches that can reduce costs by 60-80 percent, armored CARs demonstrating 3-5 fold better results in preclinical models, dual-targeting CARs having 70-80 percent effectivity rates in initial trials, and microenvironment-modifying approaches showing 2-3 fold greater tumor infiltration. Although these advancements show that many current limitations may disappear, certain basic challenges must be resolved, such as tumor heterogeneity, T-cell exhaustion, and scope for improvement in preclinical models to hasten clinical translation and bring more accessible and effective cures against a wider variety of malignancies, what they remain [21, 22].

6. Current Challenges in CAR-T Cell Therapy: A Comprehensive Perspective

However, CAR-T cell therapy can transform the whole paradigm in oncology; yet, at the moment, it exists within a multitude of limitations, including all aspects of safety and efficacy at the clinical level, biological efficacy, manufacture complexity, economic viability, and equitable access, which together restrict any broader application. The most immediate of these challenges are treatment-related toxicities, which range from severe cases of cytokine release syndrome (CRS), manifesting in 50-90% of all patients, to immune effector cell-associated neurotoxicity syndrome (ICANS), which affects 20-40% of recipients. These conditions can lead to life-threatening dysfunctions in multiple organs or persisting neurological deficits despite management with alternative therapies such as tocilizumab and corticosteroids. Other safety issues include on-target/off-tumor effects, B-cell aplasia, and theoretical insertional oncogenesis risks. Beyond the management of those toxicity effects, it also demonstrates prohibitively low efficacy against solid tumors (response rates <20% vs. 50-90% for hematological malignancies) due to interrelated biological barriers, including tumor antigen heterogeneity enabling the development of escape variants and an immunosuppressive tumor microenvironment, comprising inhibitory cells (Tregs, MDSCs), cytokines (TGF-β, IL-10), and

metabolic inhibitors (hypoxia), thereby compounding or unfortunately adding up to physical barriers (dense extracellular matrix, abnormal vasculature) that together inhibit T-cell infiltration and promote T-cell exhaustion. In addition, all these challenges are exacerbated due to the highly complex autologous process associated with such therapy: blood leukapheresis, genetic modification, and ex vivo expansion over 2-4 weeks, at more than $300,000 per patient, coupled with a 5-10% manufacturing failure rate and batch-to-batch variability, thus creating production bottlenecks, while personalized procedures preclude economies of scale and demand specialized infrastructure, including apheresis centers, cGMP facilities and tertiary care hospitals with ICU capability for toxicity management. Rising from the massive burden-of-cost product costs between $373,000 and $475,000, not including hospitalization and long-term monitoring expenses, the entry barriers further interact among healthcare system constraints involving restrictive reimbursement policies, prior authorization requirements, inpatient/outpatient billing disputes to form significant access disparities that predominantly affect the rural populations, developing nations, elderly patients, those with comorbidities or rapid disease progression, and underserved demographic groups, while further concentration of treatment expertise at urban academic centers aggravate geographic and socioeconomic inequities in care delivery. Fundamental scientific challenges underlie these practical constraints, including a lack of tumor-specific antigens, challenges associated with optimization of CAR designs (affinity/specificity balancing, costimulatory domains, safety switches), and challenges regarding long-term maintenance of persistence without exhaustion, all occurring in an evolving regulatory landscape for these living drugs, which must address unique ethical considerations in clinical trial design and long-term safety monitoring. Some novel potential solutions to these problems include more advanced CAR designs (dual-targeting, armored), allogeneic products, combination strategies, and innovative manufacturing techniques; however, achieving the full promise of CAR-T therapy will require coordinated advances in biological innovation, process optimization, healthcare infrastructure building, and policy transformation to address this very complex web of interconnected challenges [23, 24].

7. Comprehensive Solutions to Current Challenges in CAR-T Cell Therapy: An Integrated Approach Towards Clinical Optimization

The remarkable yet constrained potentials of CAR-T cell therapy have accelerated the multifactorial creative approaches that are being employed to address the clinical, biological, manufacturing, economic, and accessibility shortcomings of the therapy on an integrated platform of scientific innovations and healthcare system adaptation. The most immediate threat of the therapy to date—severe treatment-related toxicities—is now being addressed through the development of state-of-the-art safety mechanisms such as suicide genes (iPSA, Caspase-9) or pharmacological controls for self-elimination in CAR-T cells; at the same time, advanced engineering strategies like dual-targeting "AND-gate" CAR systems requiring coincident recognition of two tumor antigens and inhibitory CARs (iCARs) responsive to healthy tissue markers are significantly enhancing the tumor specificity and diminishing the on-target/off-tumor adverse effects. Development of predictive biomarker panels alongside inflammatory cytokines (IL-6, IFN-γ), markers of endothelial activation (ANG2, von Willebrand factor), and indicators of neuroinflammation (GFAP, NfL) will ultimately all enable risk-stratified management protocols for patients coupling cytokine-neutralizing antibodies (tocilizumab, siltuximab) with targeted immunosuppressants for early intervention against toxicity. A hard-hitting multipronged strategy is now emerging to enhance the efficacy of CAR-T against solid tumors, where extremely low rates of response (<20%) are unacceptably noted, juxtaposing armored CAR-T cells genetically modified to secrete TME-modulating cytokines (IL-12, IL-15, CD40L) with metabolic engineering methods (PD-1 knockout, adenosine receptor deletion) to counter immunosuppression, while exciting combination regimens with immune checkpoint inhibitors (anti-PD-1, anti-CTLA-4), TME-modulating small molecules (IDO inhibitors, TGF-β traps), and vascular normalizing agents are in clinical trials. The biological roadblocks of poor CAR trafficking versus persistence are being tackled through chemokine receptor matching (forced CXCR3 or CCR2 expression), in conjunction with Wnt/β-catenin signaling-mediated establishment of stem-like memory T cell phenotypes and

serial CAR-T cell "booster" infusions, whereas alternative effector cell platforms such as CAR-NK cells have innate advantages on tumor homing and reduced toxicity.

The complex autologous manufacturing process became an important contributor to therapy's prohibitive costs and limited accessibility-have the potential to be revolutionized through allogeneic "off-the-shelf" platforms utilizing gene-edited healthy donor cells (TCR and HLA knockout to prevent GVHD and rejection) combined with standardized bioreactor systems and non-viral gene delivery methods (transposon systems, CRISPR-Cas9); collectively, these processes would enable cryopreserved master cell banks for immediate clinical use while reducing production cost by 60-70%. Parallel developments in automated closed bioreactors with integrated quality control monitoring and decentralized manufacturing models with regional production hubs are addressing the scalability issues, and the emerging point-of-care systems may facilitate same-day CAR-T product generation. To ameliorate any significant economic burden (that presently stands at $373,000-$475,000 per treatment), the implementation of value-based pricing schemes inclusive of outcome-based reimbursements and indication-specific pricing tiers will roll hand in hand with process intensification measures focused on the reduction of the requirements of viral vectors and shortening the timelines of manufacturing, while the WHO's Technical Expert Panel on Cell and Gene Therapy is engendering a global environment for technology transfer to low/middle-income ones.

Issues of equitable access are being resolved by introducing innovative healthcare delivery models that include mobile apheresis units, telemedicine-based toxicity management networks, and specialized training programs that expand treatment center capacity, complemented by clinical trial designs expressly incorporating underserved populations and a fast-tracked regulatory pathway for underserved regions. These translational efforts are bolstered by fundamental advances in CAR design bioinformatics that incorporate machine learning algorithms to optimize antigen-binding domains and signaling architectures; high-throughput screening platforms allow rapid assessment of novel costimulatory domain combinations (CD28/4-1BB hybrids, ICOS-containing constructs), while synthetic biology methods permit the development of logic-gated circuits that respond to tumor microenvironmental cues. Concerted efforts to launch international CAR-T registries and real-world evidence platforms will aid in the long-term safety monitoring and outcome assessment among diverse patient populations, which will in turn inform iterative improvements on both product design and clinical management protocols." [25, 26]

Next-generation product engineering, manufacturing innovation, healthcare adaptation, and global accessibility initiatives constitute this very broad solution set as part of a coordinated translational medicine agenda to tackle the multidimensional limitations of current CAR-T therapy. Significant synergistic integration of biological insights, engineering advances, clinical optimization, and health policy development will leave the field progressing toward realizing CAR-T as a widely accessible, economically sustainable treatment modality-sustaining durable remissions in hematological malignancies and solid tumors-indeed a paradigm shift in cancer immunotherapy. Time for progress suggests that these technology and operational advancements will be able to fulfill the dream of CAR-T therapy from an expensive boutique therapy to a general treatment modality in the future. In fact, with innovations emerging in ongoing developments in gene editing, cellular engineering, and health delivery, in due time, CAR-T will create a vast therapeutic reach and a significant clinical impact. [27]

8. In Vivo CAR-T Cell Therapy: Addressing Conventional Limitations Through Advanced Delivery Platforms

In vivo CAR-T Cell therapy represents a paradigm shift in adoptive cell therapy, fundamentally rethinking the traditional ex vivo manufacturing process by enabling direct genetic engineering of a patient's T cells in their own body. The manipulation also avoids the complex, time-consuming, and expensive ex vivo manipulation envisaged for the conventional CAR-T therapy involving leukapheresis, genetic modification, expansion, and reinfusion, processes littered with manufacturing

failures, product heterogeneity, and logistical delays that severely limit access. In contrast, in vivo mechanisms use either systemic or localized delivery systems to introduce CAR-encoding genetic material directly to the T cells in the body, reprogramming these cells in situ. Critically, the field, in its active pursuit, is advancing mainly through two technological pathways, one being non-viral lipid nanoparticle (LNP)-based systems and the other being viral vector-based platforms, which possess unique mechanisms and advantages. [28]

8.1. Lipid Nanoparticle (LNP)-Based In Vivo CAR-T Strategies

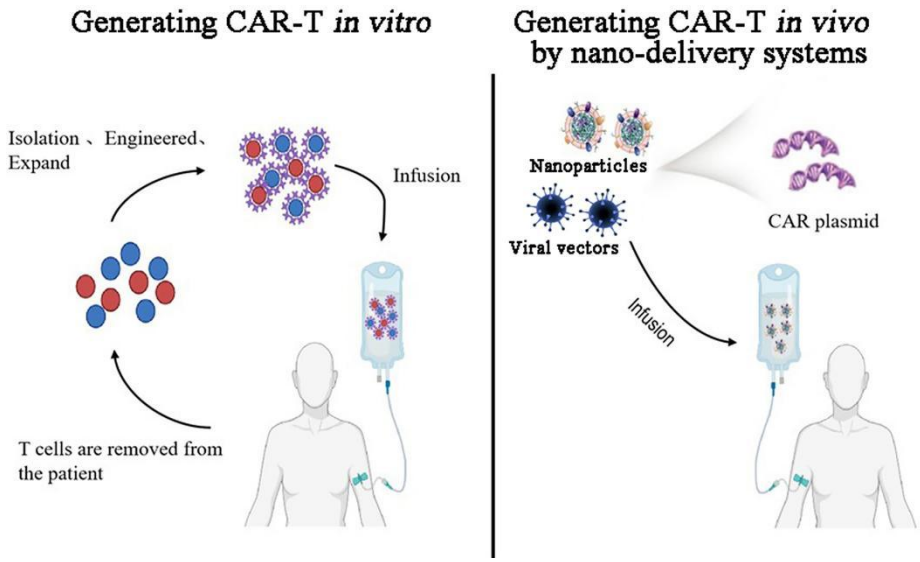

Figure 2. Lipid Nanoparticle (LNP)-Based In Vivo CAR-T Strategies (In-Vivo Induced CAR-T Cell for the Potential Breakthrough to Overcome the Barriers of Current CAR-T Cell Therapy)

Lipid nanoparticles have emerged as a highly promising non-viral platform for in vivo CAR-T cell generation, leveraging their modularity, safety profile, and potential for repeat dosing.

8.1.1. Mechanism, Delivery, and Advantages.

Lipid Nanoparticle (LNP) is an injectable carrier of a short message RNA encoding the CAR construct. Wherein, LNPs are infused-systems that target specific T cells only by using antibodies or ligands conjugated at the surface to T-cell markers (eg anti-CD3, anti-CD8). Here, mRNA comes out in the cytosol via endocytosis, hence the former requirement for the nuclear entry is avoided, thus allowing rapid and transient expression of CAR on the surface of T cells within few hours. Recently developed and optimized lipid compositions (e.g., ionizable lipids such as SM-102 or ALC-0315, PEG-lipids, cholesterol, helper phospholipids like DSPC) use sophisticated targeting ligands (e.g., CD45-targeting antibodies) to significantly enhance specificity of transfection into T cells. The mRNA delivery allows for very fast CAR protein expression within 24-48 hours which is critical for aggressive malignancies. The transient meaning-days to weeks-can be beneficial for safety in that it can reduce chances of long-term uncontrolled CAR-T cell activity and permit flexible repeat dosing to maintain therapeutic levels or capture emerging tumor antigens. LNPs lack viral components; therefore, there are no risks from either insertional mutagenesis or pre-existing or induced anti-vector immunity. Gradual in vivo generation of CAR-T cells yields a more 'natural' mode of immune homeostasis, lessening the severity of CRS and neurotoxicity compared with the huge bolus of pre-activated cells infused in conventional therapy; preclinical data consistently show significantly milder levels of pro-inflammatory cytokines (IL-6, IFN-γ). These are inherently off-the-shelf LNP formulations (lyophilized or frozen) much simpler and cheaper to manufacture under GMP conditions as compared to autologous cell products or viral vectors. This would dramatically reduce production costs (estimates suggest 80-90% reduction potential) and eliminate the need for complex cell-processing facilities and logistics (e.g., cryopreservation, shipping live cells), enabling effective administration in community oncology settings and expanding global access, particularly to resource-

limited regions without stringent cold chain requirements, with lyophilization offering particular advantages for stability and distribution. Such "self-armoring" CAR-T cells in vivo could be thus generated, by co-delivery of multiple mRNA species at once, from CAR mRNA encoding together with several immunomodulatory proteins. These proteins [e.g., dominant-negative TGFβ receptor, membrane-bound IL-15, secreted anti-PD-L1 scFv, or cytokines like IL-12] would counteract the immunosuppressive tumor microenvironment (TME); co-delivery of tumor antigen mRNA could efficiently act as an in situ vaccine, thus rallying the endogenous immunity with that of the CAR-T activity regarding the tumor-associated antigens. This leverages on the endogenous T cells that have not undergone the stress of leukapheresis and ex vivo manipulation, hence likely to retain intrinsic fitness and expansion potential; particularly useful in cases of heavily pre-treated, lymphopenic patients, whereas clinical observations suggest it has better expansion kinetics than cells made ex vivo [30, 31].

8.1.2. Current Challenges and Solutions.

To effectively transfect T cells in vivo, it is necessary to deal with two major hurdles: achieving sufficient in vivo transfection efficiency and reducing off-target delivery. Some solutions currently under investigation include new targeting ligands with improved efficacy (like optimized anti-CD5, anti-CD7), different ionizable lipids more efficiently activating endosomal membrane escape in lymphocytes, and specific targeting strategies designed on T cell activation states. The antibody-conjugated LNPs (anti-CD3e, anti-CD8a) target >80% specificity in preclinical models. These strategies apply T-cell homing signals (mRNA encapsulated for CXCR3 or CCR5) which are co-delivered with the CAR [32].

8.2. Viral Vector-Based In Vivo CAR-T Strategies

Viral vectors, particularly adeno-associated viruses (AAV) and lentiviruses (LV), represent the other major pillar of in vivo CAR-T delivery, leveraging their high transduction efficiency and potential for stable CAR expression.

8.2.1. Mechanism, Delivery, and Advantages.

Recombinant viral vectors (principally AAV due to safe profile; however, LV is being investigated) are designed to insert DNA encoding the CAR construct. Systemic or localized infusion of these vectors into the target tissue then occurs. For transduction of T-cells with vectors, either their natural, often ineffective, want to tropism are resorted to for entering the T-cells or particular capsid modifications (such as AAV6.2FF or AAV-LK03) or attachment of surface ligands are engineered to gain improved binding and internalization by specific T-cells. After entering, the vector genome traffics to nucleus where CAR transgene is expressed; AAV mostly forms as episomes while LV become integrated into host genome enabling long-term expression but raises safety considerations. Locoregionally administering (e.g., intra-tumoral, intra-lymphatic) is frequently done to boost T-cell transduction on the ground and minimize systemic exposure/toxicity. Engineered viral capsids, such as AAV variants with heightened T-cell tropism, can attain quite high transduction efficiencies in monkeys (30-50% of CAR+ T-cells reported). Hence, stable and long-lasting CAR expression (for months or possibly years) by DNA transfer through strong viral promoters will have durable remission without the need for redosing repeatedly, unlike the short-lived mRNA-LNPs. These vectors, especially LV, have a very huge payload capacity as compared to LNPs, which may include in combination with the CAR complicated circuits of genetic material. This involves an expression of several transgenes (such as constitutive or inducible safety switches like iCasp9, chemokine receptors like CCR2b or CXCR3 for enhanced homing, cytokines like IL-15 or shRNAs against immune checkpoints like PD-1 or immunosuppressive enzymes like IDO) from a single vector toward the aim of creating highly engineered, multifunctional CAR-T cells resistant to TME suppression, with integration facilitating permanent genetic modification. Given the even more patent improved delivery routes, viral vectors hold promise in transducing both migrated and tissue-resident memory T cells -- the population inhabiting such difficult sites which serve as chronic surveillance and control

points for tumor-associated checkpoint restriction at epithelial barriers. With a fairly stringent regulatory pathway, these vectors have favored clinical development for gene therapy. The efficacy of Phase I trials using AAV for CD19-directed in vivo CAR-T has been shown in comparisons with approved ex vivo products in terms of efficacy (70-80% CR in B-ALL); however, there was a significant lowering in toxicity profiles (Grade ≥3 CRS <10%). Research findings using single-cell RNA sequencing prove that such in vivo CAR-Ts generated through viral transduction very often portray better differentiation states (higher proportions of stem-like memory T and central memory T than ex vivo expansion by mostly terminally differentiated effector cells, evidenced by 3-5 fold higher memory-associated transcription factor (such as TCF7, LEF1)-expressing T-cells, which translates to better persistence, expansion potential, and long-term immunity for anti-tumor effects [33, 34].

8.2.2. Current Challenges and Solutions.

Existing challenges include pre-formed humoral immunity against viral capsids, particularly for AAV, the vector immunogenicity itself which can drive immune clearance of transduced cells, insertional mutagenesis risks (mostly with LV), and attaining true T-cell specificity. Among solutions are the engineering of synthetic capsids that are less immunogenic and have better T-cell tropism, immune-evasive capsids, or transient immunosuppression at the time of vector administration. Strategies to improve specificity include using T-cell specific promoters (e.g., CD3, CD2, CD5 promoters) and the incorporation of microRNA target sequences (e.g., miR-142, highly expressed in hematopoietic cells) to restrict CAR expression primarily to T lymphocytes. Also included are regulatable systems that may permit precise dose control (e.g., rapamycin-inducible dimerizers, tetracycline-controlled transactivators). Universal CAR designs disrupting TCR and HLA enable allogeneic in vivo generation without risk of graft-versus-host disease (GVHD) [35, 36]. In vivo CAR-T therapy has emerged as a transformative approach in cancer immunotherapy, addressing the core limitations of conventional ex vivo CAR-T therapy. These limitations include high costs, manufacturing complexities, treatment delays, toxicity, and limited accessibility. Both LNP-based and viral vector-based in vivo CAR-T modalities have made significant strides in overcoming these challenges. LNPs offer unparalleled safety, rapid implementation, and flexibility for scalable global distribution. Viral vectors provide high transduction efficiency, stable long-term expression, and access to critical T-cell populations. Future adaptations may combine the strengths of both platforms to further enhance efficacy and accessibility.

This technological leap fundamentally redefines cellular immunotherapy, positioning in vivo CAR-T as a cornerstone for next-generation, affordable, and potentially curative cancer treatments. The next generation of CAR-T therapies will feature armored CARs, allogeneic "off-the-shelf" products, metabolic engineering, and direct in vivo reprogramming of T cells using lipid nanoparticles or viral vectors. These advancements promise to reduce costs, eliminate treatment delays, improve safety, increase accessibility, and enhance efficacy against solid tumors. By democratizing global access and treating a significantly larger number of patients annually, in vivo CAR-T therapy holds great promise for the future of cancer treatment [37].

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