Journal of Hematology & Oncology

REVIEW

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In vivo gene editing and in situ generation of chimeric antigen receptor cells for next-generation cancer immunotherapy

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Abstract

Chimeric antigen receptor (CAR) cell therapy has achieved groundbreaking success in treating hematological malignancies. However, its application to solid tumors remains challenging due to complex manufacturing processes, limited in vivo persistence, and transient therapeutic effects. In vivo CAR-immune cells induced by gene delivery systems loaded with CAR genes and gene-editing tools have shown efficiency for anti-tumor immunotherapy. In situ programming of autologous immune cells avoids the safety concerns of allogeneic immune cells, and the manufacture of gene delivery systems could be standardized. Therefore, the in vivo editing and in situ generation of CAR-immune cells might potentially overcome the abovementioned limitations of current CAR cell therapy. This review mainly focuses on CAR structures, gene-editing tools, and gene delivery techniques applied in anti-tumor immunotherapy to help design and develop in situ CAR-immune cell therapy. The recent applications of in vivo CAR-immune cell therapy in both hematologic malignancies and solid tumors are investigated. To sum up, the in vivo editing and in situ generation of CAR therapy holds promise for offering a practical, cost-effective, efficient, safe, and widely applicable approach to the next-generation anti-tumor immunotherapy.

Keywords CAR-immune cell therapy, The next-generation immunotherapy, In situ generation, Gene-editing tools, Gene delivery techniques

Introduction

The treatment of malignant tumors remains a critical global issue. Over the past decade, immunotherapy has emerged as a promising approach, offering significant advantages in anti-tumor treatments [1]. Unlike traditional methods like surgery, chemotherapy, or radiotherapy, immunotherapy harnesses activated immune cells to selectively identify and attack tumor cells, minimizing side effects [2]. Adoptive cell therapy or anti-tumor immune cell therapy, stands out as a primary strategy in anti-tumor immunotherapy. This technique involves extracting immune cells from the patient's body, amplifying the desired immune cells, and reintroducing them into the body to elicit an immune response against tumor cells [3]. Even after surgery, radiotherapy, or chemotherapy, many patients with malignant tumors still have residual tumor cells. This persistence of tumor cells is a significant factor for post-treatment recurrence [4]. Cellular immunotherapy could activate the immune system to eliminate residual tumor cells, thereby preventing metastasis and recurrence. Whereas, early cell therapies

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encounter several challenges, including non-specificity, which can reduce efficacy due to inadequate recognition of tumor cells. Other issues include tumor immune evasion, loss of major histocompatibility complex (MHC) expression, and the immune tolerance of host cells.

Immune cell therapy involves infusing either autologous or allogeneic immune effector cells that have been activated and expanded in vitro into the patient. These cells are tasked with identifying tumor cells and executing selective killing, thereby disrupting immune tolerance and bolstering the body's immune response. A key challenge in traditional T-cell receptor (TCR) therapy lies in its dependence on the recognition of antigens presented by MHC molecules, coupled with sufficient co-stimulatory signals for T-cell activation. However, tumor cells often evade immune detection by decreasing MHC class I molecules [5]. To circumvent this limitation, chimeric antigen receptor (CAR) therapy has been developed. The current clinical-scale manufacturing of CAR cells including CAR-T requires an assortment of elaborate protocols to isolate, genetically modify, and selectively expand the redirected cells before infusing them back into patients [6, 7]. These complex procedures entail dedicated equipment and considerable technical expertise, which remain as obstacles for implementing them as a standard of care in the treatment of tumors [8]. CAR-immune cell therapy has shown promising results in hematologic malignancies by enabling tumor cell tracking and specific killing. For example, CAR-T therapy requires cumbersome ex vivo T-cell expansion and poses safety concerns such as cytokine release syndrome. However, its efficacy in solid tumors is limited [9]. Therefore, exploring safer and more efficient CAR-immune cell therapy is a focal point and frontier in anti-tumor immunotherapy.

In this review, we aimed to introduce the commonly used CAR-immune cell therapy such as CAR-T, CAR-NK and CAR-M according to the types of immune cells. Moreover, the comparison between ex vivo and in vivo CAR-immune cell therapy were also conducted. We further investigated the common targets in the design process of CARs, gene-editing tools and gene delivery techniques for potential in vivo gene editing of CARs. According to recent researches, the applications of in vivo CAR-immune cell therapy in both hematologic malignancies and solid tumors were concluded. To sum up, the in vivo editing and in situ generation of CAR therapy holds promise for offering a practical, cost-effective, and widely applicable approach to the next-generation anti-tumor immunotherapy.

Chimeric antigen receptor therapy

CAR-immune cell therapy involves the engineering of the autologous immune cells of patient to express antigen receptors that specifically target antigen molecules present on tumor cell surfaces (Fig. 1). The fundamental structure of CAR is shown in Fig. 1 [10]. The CAR structure comprises three essential domains: the extracellular domain, the transmembrane domain, and the intracellular domain [11]. The extracellular domain encompasses antigen recognition domains, commonly single-chain variable fragments (scFv), and hinge domains. The scFv forms the basis for the specific binding of CAR to tumor antigens, constructed from the variable light chain (VL) and variable heavy chain (VH) of monoclonal antibodies linked by polypeptides. Presently, antibodies are primarily developed to target tumor-associated antigens such as CD19, CD20, CD22, CD30, CD33, BCMA, and other targets. The hinge region connects the scFv to the transmembrane domain, with its length determined by the location and accessibility of the target cell epitope. Transmembrane domains serve to link the extracellular domain of CAR to the intracellular signal transduction domain, typically sourced from CD4, CD8α, CD28, or CD3ζ. The intracellular domain comprises the co-stimulatory domain and the signal transduction domain. The co-stimulatory domain commonly originates from the CD28 receptor family (CD28, ICOS) or the tumor necrosis factor receptor family (4-1BB, OX40, CD27), facilitating the dual activation of co-stimulatory molecules and intracellular signals to drive T cell proliferation and cytokine release, thereby enhancing the anti-tumor capabilities of T cells. The signal transduction domains typically consist of the T cell receptor TCR/CD3ζ chain or the immunoglobulin Fc receptor FcεRIγ chain, containing immune receptor tyrosine activation motifs essential for T cell signal transduction [12]. According to the types of immune cells, CAR-immune cell therapy could be divided into CAR-T, CAR-NK and CAR-M [13].

CAR-T

CAR-T therapy is a typical type of precision targeted anti-tumor therapy based on T cells. Through genetic engineering technology, T cells are activated and equipped with a guidance system known as CAR. This transformative process elevates ordinary T cells into "super soldiers" CAR-T cells, which could finely attune to identifying and effectively eliminating tumor cells within the body. Through this approach, the objective of treating malignant tumors is pursued with enhanced efficacy and specificity [14].

CAR-T therapy typically involves engineering the patient's own T cells in vitro and subsequently reintroducing them into the body. This approach is highly personalized, tailoring the therapy to each individual patient, albeit at a considerable cost. As a result, researchers developing fifth-generation CAR-T technology are prioritizing breakthroughs to address these individual limitations. Their goal is to achieve large-scale production and

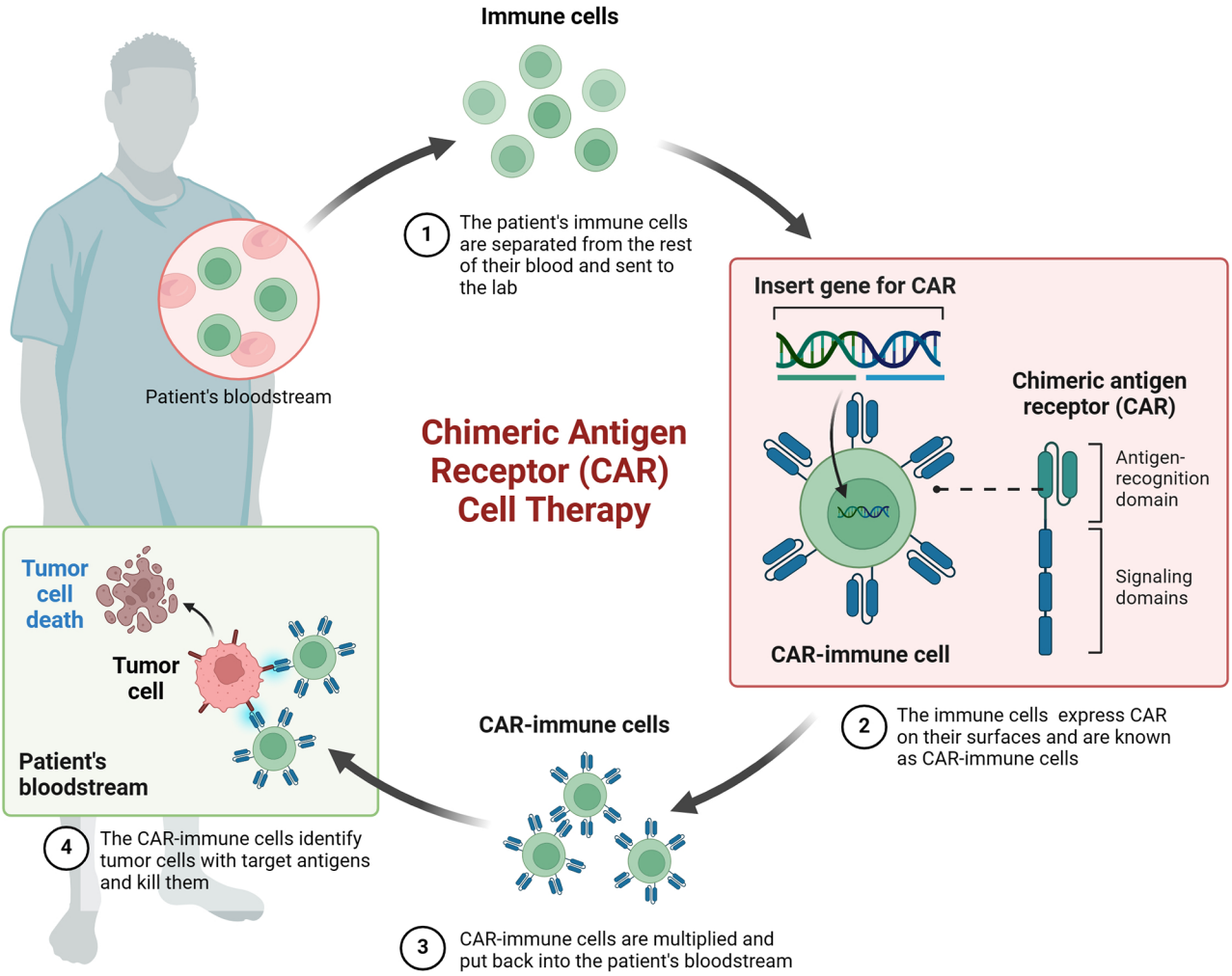

Fig. 1 The basic structures, generating process, and anti-tumor functions of CAR-immune cells

treatment capabilities, ultimately aiming to reduce costs and improve accessibility. The universal CAR uses two systems [15, 16], the BBIR CAR (biotin-binding immune receptor) or the SUPRA CAR (split, universal, programmable), to separate extracellular antigen-targeting domains and T cell signaling units, thereby giving CAR-T cells the ability to recognize multiple antigens. Meanwhile, T cells obtained from allogeneic healthy receptors can eliminate graft-versus-host disease (GVHD) by destroying TCR genes and HLA class I genes in T cells through gene editing techniques in vitro [17]. However, at present, universal CAR-T therapies face significant technical barriers and heightened safety requirements.

CAR-T therapy faces several challenges primarily related to side effects, toxicity, T cell depletion, and the malignant tumor microenvironment (TME) [18]. Additionally, the manufacturing process for mass production is currently time-consuming and costly, posing a significant barrier to making CAR-T cell immunotherapy accessible to a broader patient population. Two notable clinical toxicities associated with CAR-T therapy are cytokine release syndrome (CRS) and neurotoxicity (NTX) [19]. Relapse after CAR-T therapy commonly occurs due to a reduction or loss of antigen density on tumor cells, while CAR-T cell failure can lead to drug resistance and tumor recurrence. Moreover, the inhibitory TME may induce resistance to CAR-T therapy. Selecting the appropriate antigen target is a crucial challenge in CAR-T cell immunotherapy, with the exploration of new targets offering additional possibilities for CAR-T design and selection. Experimental evidence suggests that certain T cell subsets may exhibit greater efficacy than others; for instance, γδT cells provide a quicker initial defense, $CD26^{high}$ CAR-T cells secrete higher cytokine levels, and central memory T cells display increased persistence [20]. Hence, the choice of T cell types represents a key area for innovation in CAR-T therapy.

CAR-NK

Despite the rapid advancements in CAR-T therapy, several limitations remain. These include challenges in effectively treating solid tumors and variability in patient responses, which can lead to complications such as CRS and NTX. Despite these challenges, the remarkable success of CAR-T has spurred researchers' interest in applying CAR engineering techniques to other types of immune cells. This exploration holds promise for expanding the scope of immunotherapy beyond T cells, potentially addressing current shortcomings and broadening the therapeutic landscape.

CAR-NK therapy presents a promising avenue for anti-tumor treatment [21]. Firstly, NK cells can be derived from various sources, including peripheral blood, cord blood, induced pluripotent stem cells (iPSCs), and NK cell lines. iPSC-based NK cell generation requires minimal seed cells, facilitates large-scale culture, offers cost-effectiveness, enables auto-supply, and exhibits low immunogenicity [22]. Moreover, the NK92 cell line exhibits indefinite proliferation capability and high resistance to freeze-thaw cycles. Secondly, CAR-NK therapy boasts a favorable safety profile, primarily attributable to the short lifespan of CAR-NK cells. CRS, commonly associated with CAR-T therapy, is predominantly triggered by factors like INF-γ, TGF-α, IL-1, and IL-6 released by CAR-T cells. CAR-NK therapy holds promise in mitigating CRS and NTX, prevalent side effects in CAR-T therapy. Furthermore, unlike CAR-T cells, which are inhibited by PD-1 expression in the TME, NK cells exhibit low PD-1 expression and minimal immunosuppressive effects. Finally, allogeneic NK cell infusion is well-tolerated and does not elicit immune rejection. Consequently, CAR-NK therapy is poised to become a "universal" product, offering a potent approach for treating solid tumors [23].

CAR-NK therapy harnesses genetic engineering to introduce a chimeric antibody capable of recognizing tumor cells and activating NK cells for simultaneous tumor cell killing. This approach significantly enhances the specificity of NK cells, akin to CAR-T construction. CAR design includes extracellular recognition domains, such as scFv, for the specific recognition of tumor antigens. Additionally, it features transmembrane and intracellular signaling domains, such as the CD3ζ chain, to facilitate NK cell activation. For widespread clinical application of CAR-NK therapy, several developmental challenges must be addressed [24]. Firstly, to mitigate GVHD, complete elimination of T cells is necessary in NK cell therapy. Obtaining sufficient NK cells from diverse sources like peripheral blood, cord blood, human embryonic stem cells (hESCs), human-induced pluripotent stem cells (hiPSCs), or NK cell lines remains challenging. However, obtaining a substantial number of NK cells from hiPSCs holds promise. Secondly, in vitro expansion of NK cells presents a hurdle due to their short half-life of 7–10 days, necessitating large-scale expansion for therapy. This process typically takes 2–3 weeks, emphasizing the critical need for efficient expansion and activation methods. Thirdly, selecting an optimal CAR design is crucial, as existing CARs are primarily tailored for CAR-T cells and may not be ideal for NK cells. The location of CAR-binding epitopes and their proximity to CAR-NK cell surfaces impact antigen binding and CAR-NK cell activation. Fourthly, effective CAR transfer to NK cells is vital. Both viral and non-viral vectors have been employed for CAR transduction. While retroviral vectors offer high transfection efficiency, they pose risks of insertion mutations and carcinogenesis. Lentiviral vectors have lower insertion mutation rates but exhibit lower transfection efficiency. The applicability of transposon-based systems for CAR-NK remains uncertain. The messenger RNA (mRNA) transfection emerges as a safer and feasible method. Lastly, NK cells are sensitive to cryopreservation, with decreased survival and cytotoxicity post-thaw. Supplementation with interleukin-2 (IL-2) partially restores frozen NK cell activity. Optimizing cryopreservation protocols is essential for effective cell preservation. Addressing these challenges is pivotal for advancing CAR-NK therapy into clinical practice, offering a promising avenue for anti-tumor treatment [11].

NK cells demonstrate unique anti-tumor capabilities, such as MHC-independent cytotoxicity, cytokine secretion, and immune memory functions, making them essential in both innate and adaptive immune responses. However, many aspects of immune cell functionality remain unclear. For instance, it is uncertain whether NK cells infiltrate solid tumors more effectively than T cells. Additionally, NK cells can secrete cytokines that attract other immune cells, potentially enhancing the overall anti-tumor response. Despite their crucial role in immune surveillance and tumor defense, the complexities of NK cell behavior within the TME require further investigation to fully realize their therapeutic potential.

CAR-M

The advancement of CAR-T cell therapy for solid tumors has encountered significant hurdles, contributing to a slower progress. One major obstacle is the complex and aberrant tumor vasculature, characterized by reduced adhesion molecules, which impedes the migration of CAR-T cells into tumor tissues. Additionally, there exists a chemokine/chemokine receptor mismatch, further hampering CAR-T cell infiltration into solid tumors. Once inside the TME, CAR-T cells confront hostile conditions such as hypoxia, acidity, upregulated immune checkpoint ligands, and a plethora of immunosuppressive cells. Even if CAR-T cells manage to survive within

the TME, the heterogeneous expression of antigens on solid tumor surfaces poses a challenge, allowing tumor cells to evade detection and thwart complete eradication. These multifaceted barriers highlight the complexity of solid tumor immunotherapy and emphasize the need for innovative strategies to improve CAR-T cell efficacy in this challenging environment.

The main focus of CAR research has been peripheral T lymphocytes, recent years have witnessed an expansion of chimeric receptor application to other lymphoid immune cells, including γδT cells, NK-T cells, and NK cells. Nevertheless, the efficacy of cell therapies derived from these lymphocytes for solid tumor treatment remains elusive. Myeloid cells, such as monocytes and macrophages, present a promising avenue to address these challenges. These cells exhibit robust accumulation within tumors and possess the ability to penetrate dense stromal tissue surrounding the tumor, offering a unique advantage. Myeloid cells have significant potential for direct tumor killing and enhancing endogenous immunity through effective antigen presentation, making them a promising candidate for anti-tumor cell therapies [25]. Like CAR-T and CAR-NK cells, CAR-M cells are engineered with extracellular signaling domains recognizing specific tumor antigens, transmembrane regions, and intracellular activation signaling regions. Current research on extracellular signaling domains primarily targets common tumor antigens such as CD19 and HER2. CAR-M therapy focuses on harnessing the capabilities of macrophages, which are sourced from patients themselves and genetically engineered for tumor killing. Compared to T cells and NK cells, macrophages may have enhanced tumor infiltration capabilities within the immunosuppressive TME, offering novel opportunities for anti-tumor immunotherapy [26].

Saar Gill and Michael Klichinsky, experts in CAR-T cell therapy, initiated the development of CAR-M therapy for anti-tumor treatment [27]. By 2020, they established the modification of macrophages with a HER2-targeted CAR. Using mouse models, they validated the potent tumor-killing efficacy of CAR-M cells. Furthermore, they observed that HER2-CAR-M cells could convert M2 macrophages into M1 macrophages, fostering an inflammatory TME that enhanced the cytotoxicity of T cells. This underscores CAR-M's primary advantage of instigating a pro-inflammatory milieu within tumors [28]. In 2020, a team from Zhejiang University reported the pioneering application of iPSC-derived CAR-expressing macrophages (CAR-iMac) in immune cell therapy against tumors [29]. Their findings demonstrated that CAR-iMac cells displayed strong anti-tumor activity in various hematological and solid tumor models in mice, representing a significant advancement in anti-tumor immunotherapy.

The comparison among CAR-T, CAR-NK, and CAR-M

The features, differences and similarities of CAR-T, CAR-NK, and CAR-M are summarized in Table 1. CAR-T therapy is mainly used for hematologic malignancies, and CAR-M therapy is mainly used for solid tumors because of the capability of penetrating solid tumors. Moreover, the features of blood circulation time, in vitro culture amplification, sensitive to freeze-thaw, and killing mechanisms are also compared among CAR-T, CAR-NK, and CAR-M. As for the risk of toxicity and GVHD, CAR-NK and CAR-M are relatively safer than CAR-T (Table 1).

Compared to CAR-T and CAR-NK therapies, CAR-M exhibits unique advantages. Unlike CAR-T cells, CAR-M cells possess three main advantages: (a) T cells often encounter physical barriers formed by the tumor matrix or encounter immunosuppressive conditions within the TME, hindering their infiltration and efficacy. In contrast, macrophages can readily infiltrate the TME; (b) Tumor-associated macrophages (TAMs) play pivotal roles in tumor progression, metastasis, immunosuppression, and angiogenesis. CAR-M therapy can reduce TAM proportions, modulate TAM phenotypes, and positively impact tumor treatment outcomes; (c) Besides tumor cell phagocytosis, CAR-M cells also facilitate antigen presentation and enhance T cell-mediated killing. Moreover, CAR-M therapy offers a shorter cycle time and lower non-tumor targeting toxicity compared to CAR-T therapy [11].

As a novel cellular immunotherapy, CAR-M holds unique advantages over CAR-T and CAR-NK therapies, including its capacity to induce a pro-inflammatory environment within tumors and reverse the inhibitory TME [30, 31]. While preclinical studies have demonstrated promising anti-tumor efficacy, the clinical effectiveness and safety of CAR-M therapy require further validation. Notably, the anti-tumor activity of CAR-M is target-dependent for direct killing but target-independent for inducing the M1 phenotype, suggesting its potential to reprogram the TME and effectively combat tumors with heterogeneous antigen expression. Engineered macrophages endowed with tumor migration abilities, a sustained pro-inflammatory M1 phenotype, CAR-mediated targeted anti-tumor effects, and specialized antigen presentation capabilities can initiate a multifaceted anti-tumor immune response.

Common targets in the design process of CAR for different tumors

The advancement of allotherapy outpaced autologous therapy over years, with a growth rate of 33% compared to 23%. This may be attributed to the fact that innate immune cells such as NK cells and macrophages are better suited for the development of allotherapy, indicating that allotherapy is the future direction of development. Consequently, there is an urgent need to identify more

Table 1 The comparison among CAR-T, CAR-NK, and CAR-M

Comparison	CAR-T	CAR-NK	CAR-M
Source cells	T cells	NK cells	Macrophages
Features	• MHC independent • CAR-T cell exhaustion • High cost • Mainly used for hematologic malignancies	• A wide range of sources • Cultured in large quantities • Multiple killing mechanisms • MHC independent • Low cost	• Penetrating solid tumors • Phagocytosis signaling domains • Multiple killing mechanisms • Mainly used for solid tumors
Enter the solid TME	Unable to enter the solid TME	Not clear whether NK cells enter solid TMEs more easily	Easy entry into the solid TME
Blood circulation	Long blood circulation time	Limited blood circulation time	Limited blood circulation time
In vitro culture amplification	High cost for in vitro culture amplification	In vitro culture amplification is a challenge. The half-life of NK cells is about 7–10 days	Low cost for in vitro culture amplification
Sensitive to freeze-thaw	Unknown	Sensitive to freeze-thaw processes. The survival rate and cytotoxicity decreased significantly after thawing	Unknown
Killing mechanisms	• Single killing mechanisms • Might lead to drug resistance, thereby causing tumor recurrence	• Multiple killing mechanisms • Producing cytokines to attract other immune cells and enhance anti-tumor response	• Multiple killing mechanisms • Phagocytosis of tumor cells, promoting antigen presentation, enhancing T cell killing
Effects of TME	TME of solid tumor largely limits its efficacy because of CAR-T cell exhaustion	TME of solid tumor might limit its efficacy	Creating a pro-inflammatory TME for enhancing anti-tumor effects
Risk of toxicity	High risk of CRS and NTX	Relatively safe and low risk of CRS and NTX	Little toxicity
Risk of GVHD	High risk of GVHD	Low risk of GVHD and low immunogenicity	Low risk of GVHD

Abbreviation MHC, Major histocompatibility complex; TME, Tumor microenvironment; CRS, Cytokine release syndrome; NTX, Neurotoxicity; GVHD, Graft versus host disease

suitable targets for designing CAR cell therapy for various tumors. In terms of CAR-T applications in hematologic malignancies, CD19, BCMA, CD22, CD20, and CD123 remain the most commonly targeted antigens. However, in solid tumors, tumor-associated antigen (TAA), HER2, mesothelin (MSLN), GPC2/3, and EGFR are the predominant proteins targeted.

The exploration of novel targets for CAR-NK therapy has led to significant advancements. In 2022, the FDA granted approval for the Investigational New Drug (IND) application of FT536, a pioneering CAR-NK cell therapy derived from iPSC. FT536 is engineered to express a unique CAR designed to specifically target the α3 domains of the Class I MHC associated proteins A and B (MICA/MICB). MICA and MICB, stress proteins prevalent in numerous solid tumors, often evade immune cell detection through proteolytic shedding of their α1 and α2 domains. Studies have demonstrated that antibodies directed against the α3 domain of MICA/B can effectively impede shedding and restore NK cell-mediated immune responses. The approval of FT536 marks a significant milestone, highlighting MICA and MICB as promising targets for anti-tumor immunotherapy across various solid tumors. This novel therapeutic approach aims to counteract the stress-inducing ligands, presenting a potential breakthrough in anti-tumor treatment. A multi-center Phase I clinical trial of FT536 given in combination with a monoclonal antibody following lymphodepletion in participants with advanced solid tumors (NCT05395052) was conducted and was indeed terminated by the sponsor after last update on 2023-09-21. However, another clinical trial on FT536 (NCT06342986) has been registered soon after that. NCT06342986 registered on 2024-03-27, is a single center Phase I clinical trial of FT536 administered intraperitoneally three times for one week for the treatment of recurrent gynecologic cancers. This trial (NCT06342986) focuses on recurrent ovarian, fallopian tube, and primary peritoneal cancer, while the former trial (NCT05395052) was conducted in a wide range of solid tumors.

A recent study unveiled the efficacy of an "off-the-shelf" CAR-NK therapy directed against prostate stem cell antigen (PSCA) in inhibiting pancreatic cancer [32]. Known as CYTO NK-203, this therapy represents an allogeneic CAR-NK cell therapy derived from umbilical cord blood, offering enhanced safety and killing potential of NK cells. Notably, CYTO NK-203 exhibited remarkable effectiveness in mouse models of human metastatic pancreatic cancer, demonstrating sustained presence for over 90 days without inducing treatment-related toxicity, and significantly prolonging the lifespan of the subjects. The research team underscores the promise of this CAR-NK therapy for pancreatic cancer treatment, citing two key factors. Firstly, the therapy adopts a precision medicine approach, targeting the specific marker PSCA present in pancreatic cancer patients. Engineered human natural killer cells are tailored to selectively attack tumor cells, exemplifying a targeted immunotherapeutic

strategy. Additionally, given the high expression of PSCA in stomach cancer and prostate cancer, this CAR-NK cell therapy holds potential for treating these malignancies as well. In 2021, a clinical trial on MSLN CAR-NK injection for the therapy of advanced epithelial ovarian cancer has been approved by the Drug Evaluation Center of the National Medical Products Administration, China (CXSL2101267). Marking the first "off-the-shelf" allogeneic CAR-NK product in China, this development is poised to enhance ovarian cancer treatment efficacy and elevate patient quality of life. Moreover, the scalability and standardization of "off-the-shelf" products enable mass production, thereby expanding treatment accessibility, reducing costs, and fostering widespread clinical adoption. Another trial (NCT05410717) is an open, exploratory clinical study to evaluate the safety and preliminary efficacy of Claudin6, GPC3, Mesothelin, or AXL targeting CAR-NK cells in patients with Claudin6, GPC3, Mesothelin, or AXL-positive advanced solid tumors (ovarian cancer and others). This trial has been registered on 2022-06-05 and is continuously recruiting.

As for CAR-M, in 2022, Novartis initiated a preliminary strategic collaboration agreement with Carisma Therapeutics for the clinical development of CT-0508, a product candidate for HER2-targeted CAR-M cell therapy. The above trial of CT-0508 (NCT04660929) is also still active and has already enrolled 48 subjects. Clinical trials of CAR-M for solid tumors have demonstrated high expression of CAR activity and good tolerability in macrophages that administered CAR-M to patients. Preliminary data suggests that CAR-M has the potential to modulate the solid TME, bone marrow cell composition, and T cell composition. Currently, Carisma Therapeutics is advancing three CAR-M research pipelines targeting HER2 (for solid tumors), MSLN (for treatment of MSLN-positive solid tumors), and PSMA (for treatment of metastatic castration-resistant prostate cancer).

The advantages of in situ generation of CAR

Currently, most applications of gene editing technology are focused on ex vivo cell editing, where cells collected from the patient's body are genetically modified outside the body and then reintroduced into the patient as a therapeutic drug. It is important to note that ex vivo gene editing is limited in terms of applicable cell types. While certain cells such as hematopoietic stem cells exhibit high survival rates after genetic modification ex vivo, others have low survival rates or may not survive at all post-editing. Neurons represent a typical example of this phenomenon, often experiencing death or loss of function following gene editing. Consequently, ex vivo gene editing may not be suitable for some neurological genetic diseases. In contrast, in vivo gene editing involves directly modifying genes within the body's cells. Although currently linked to higher risks, successful implementation of in vivo CAR-immune cell therapy would provide access to a wider range of target cells and organs, enabling the treatment of various medical conditions.

For instance, CAR-immune cell therapy (such as CAR-T, CAR-NK, CAR-M) has traditionally involved extracting cells from patients and modifying them ex vivo to express specific CARs targeting particular antigens before reinfusion into the patient for treatment (Fig. 2). One significant challenge facing these therapies is their highly personalized nature; this leads to extended production cycles and correspondingly high costs. The development of allogeneic or "off-the-shelf" cell therapies has opened new possibilities for future advancements in this field. Furthermore, recent developments have introduced another avenue: in vivo CAR-immune cell therapies [33].

Pioneering research by mRNA vaccine expert Drew Weissman's team [34] has yielded remarkable results in developing in vivo CAR-T cell therapies using mRNA and lipid nanoparticles (LNPs) technology [35]. Therapeutic CAR-T cells generated through mRNA and LNP technology successfully reduced fibrosis in disease mouse models while restoring heart function [36]. Similarly groundbreaking is Moderna's pioneering attempt at utilizing mRNA and LNP technology for in vivo CAR-M therapy which brings hope for further expansion of different types of in vivo cell therapy [33]. Compared with ex vivo CAR therapy, in situ CAR-immune cells could simplify the process and avoid extracting immune cells to transform genes in non-primary environmental conditions (Fig. 2). They could be easily mass-produced in a stable form, freeze-dried, stored, and used directly when needed. It could not be denied that in situ CAR-immune cells is challenging, but clinically valuable, which could transform CAR-immune cell therapy from autologous cell-based drugs into "off-the-shelf" drugs [37, 38].

Gene-editing tools for potential in vivo gene editing

Site-specific integration of CAR transgenes is crucial for improving clinical applicability and optimizing the CAR-immune cell phenotype. Studies have shown that integrating the CAR encoding sequences in the TCR locus, helps to reduce tonic signaling, prevent rapid T cell exhaustion, and enhance the efficacy of CAR-T cells [39, 40]. Kinetic analyses of antigen-induced CAR internalization and degradation suggest that CAR expression and cell surface CAR dynamics are regulated by enhancer/promoter elements [39]. Thus, integrating CAR transgenes in the TCR locus not only reduces the risks of tumors, TCR-driven autoimmunity, and alloreactivity, but also contributes to a safer and more effective CAR-T

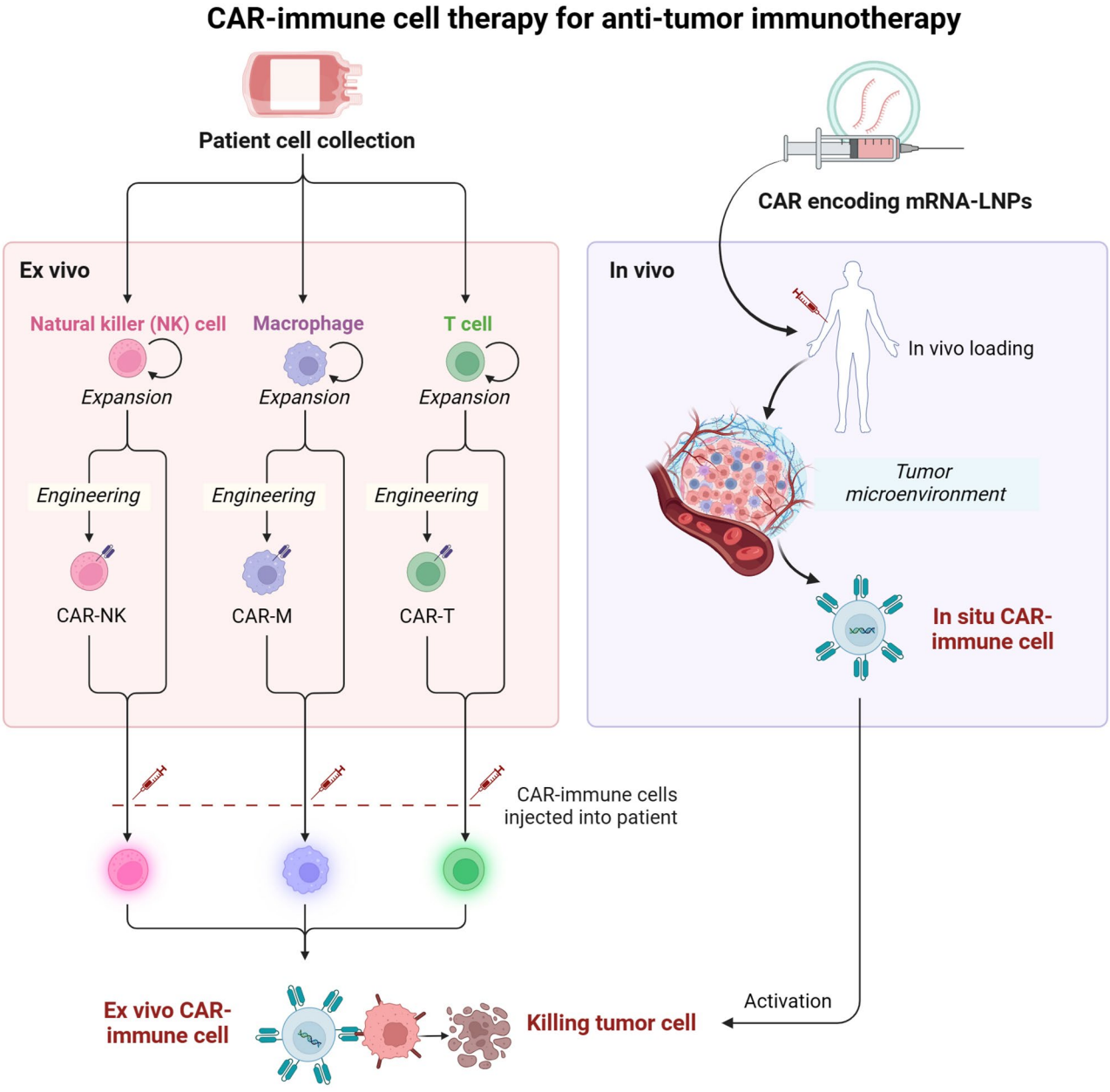

Fig. 2 Ex vivo and in vivo CAR-immune cell therapy for anti-tumor immunotherapy

therapy. Moreover, there appears to be a strong connection between CAR immunobiology and the potential of gene-editing technology in developing safer and more efficient CAR-based therapy.

A comparison between random integration and site-specific integration in CAR-immune cell therapy was investigated [41]. In random integration, viral vectors often rely on strong viral or non-viral promoters to drive constant CAR transgene expression, especially when inserted into highly active genomic regions. This approach can lead to excessive exogenous gene expressions in T/NK cells and trigger antigen-independent tonic signaling because of CAR clustering. The pressure and tonic signaling lead to CAR-T cell exhaustion characterized by a reduction in central memory phenotypes and ultimately poor clinical outcomes. In contrast, site-specific integration of CAR transgenes under active promoters of T/NK cells eliminates external pressure. This allows the CAR transgene to leverage endogenous regulatory elements and chromatin dynamics, ensuring that CAR expression is modulated in a more natural and dynamic manner. Therefore, site-specific integration promotes the expansion of long-lasting memory T cells with reduced exhaustion and enhanced anti-tumor functions,

improving the therapeutic potential of CAR-immune cell therapy for tumor eradication.

In order to achieve in vivo gene editing to generate in vivo CAR cells, a variety of gene-editing tools are required. These gene-editing tools are utilized to introduce targeted CAR gene into corresponding immune cells in the TME, thereby contributing to the in vivo editing and in situ generation of CAR therapy [42]. The most commonly used gene-editing tools are concluded as follows (Fig. 3; Table 2).

TALEN-based genome editing

Transcription activator-like effector nucleases (TALEN) technology targets specific DNA sites by utilizing DNA recognition modules to bind TALEN components to their respective DNA sequences [43]. Subsequently, under the action of FokI endonuclease, specific DNA sites are cleaved. Repair of these cleaved sites, including insertion, inversion, deletion, and gene fusion, is then accomplished through intrinsic cellular mechanisms such as homology-directed repair (HDR) or non-homologous end joining (NHEJ). The core principle of TALEN technology lies in the sequential execution of three distinct functions by the same protein (TALEN): guiding entry into the cell nucleus, specific recognition of target DNA sites, and cleavage of target DNA sites [44]. With the gradual maturation of TALEN technology, laboratories worldwide have extensively employed it to perform gene targeting operations. Through integration with techniques such as microinjection into stem cells, gene therapy, neural networks, and lentiviral infection, TALEN finds applications in various fields including animal and plant breeding, thereby catalyzing advancements in life sciences. Depleting cancer-associated fibroblasts (CAFs) in stroma-rich solid tumors offers a promising approach to convert immune-evasive tumors into ones susceptible to CAR-T cell-mediated cytotoxicity. Das et al. [45] utilized a TALEN-based gene editing platform to engineer non-alloreactive, immune-evasive CAR-T cells (termed UCAR T cells) that specifically target the CAF marker fibroblast activation protein (FAP). In an orthotopic mouse model of triple-negative breast cancer (TNBC),

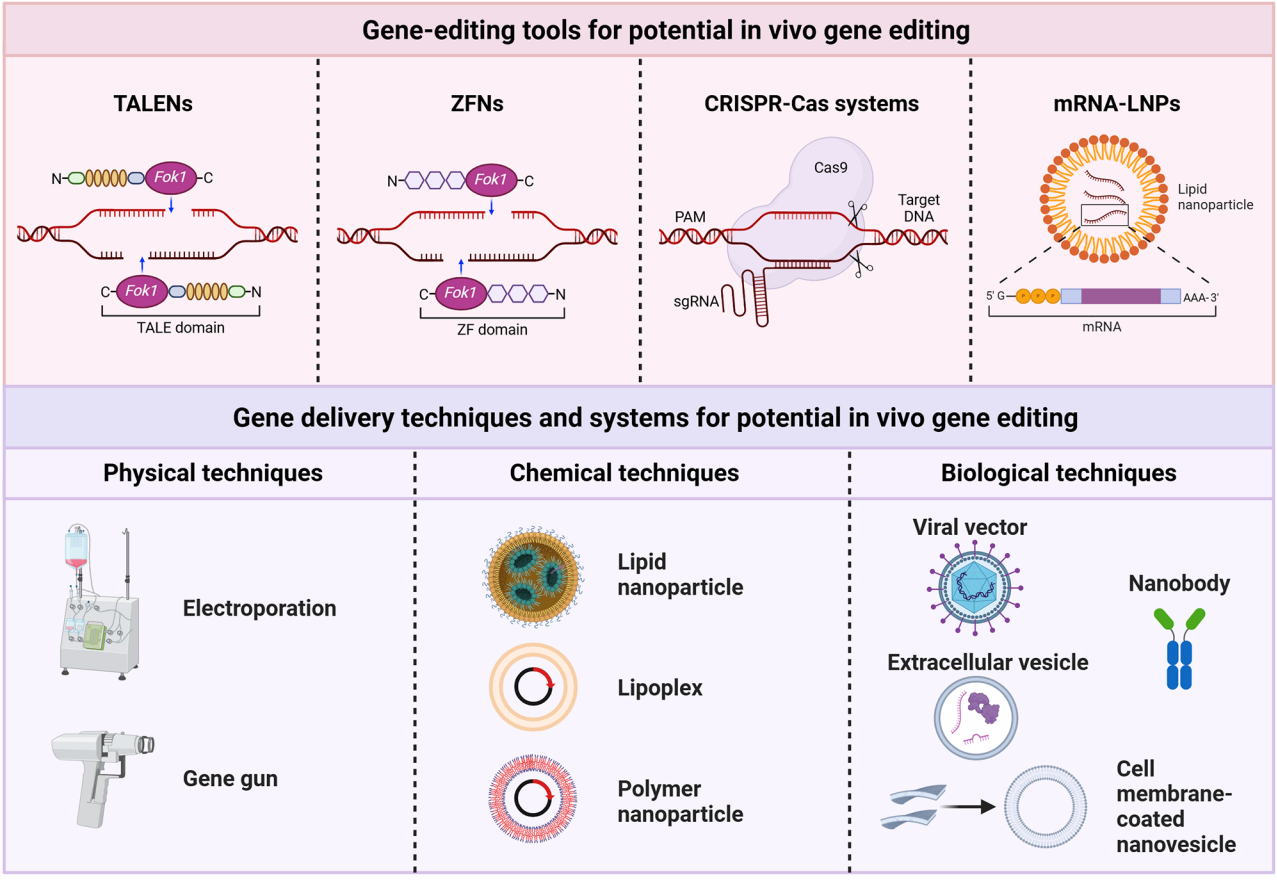

Fig. 3 During the process of in vivo editing and in situ generation of CAR therapy, both gene-editing tools and gene delivery techniques and systems are of great importance. As for gene-editing tools, TALENs, ZFNs, CRISPR-Cas systems, and mRNA-LNPs are widely used for potential in vivo gene editing of CAR therapy. There are three common types of gene delivery techniques and systems for potential in vivo gene editing: physical techniques, chemical techniques, and biological techniques

Table 2 Gene-editing tools for potential in vivo gene editing

Gene-editing tools	Components	Advantages	Disadvantages
TALEN	The specific DNA binding protein TALE and FokI endonuclease	• Simple design with gene sequencing • Good controllability of cleaved sites • High specificity	• Complex manufacturing process • Relatively high cytotoxicity • Difficult to deliver across cell membranes
ZFN	ZFP with specific recognition sequence and FokI endonuclease	• High gene editing efficiency • High specificity	• Complex manufacturing process • High off-target rate • Relatively high cytotoxicity
CRISPR-Cas	CRISPR sequences and Cas nucleases	• Accurate targeting • Low off-target rate • Low cytotoxicity • Cheap and easy to operate	• Specificity is not high, and mutations unrelated to the target may be produced • Difficult to deliver across cell membranes
Base editor	The nickase Cas9 (nCas9) fuses with nucleobase modifying enzyme	• High specificity and efficiency • Avoid unnecessary DNA cutting and repair processes, thus reducing unnecessary mutations	• Difficult to deliver across cell membranes • Only for single nucleotide mutations • sgRNA independent off-target editing
mRNA-LNP	LNPs-coated mRNA encoding sequences	• High gene editing efficiency • Facilitating intracellular mRNA delivery • Protecting mRNA from extracellular ribonuclease	• Poor stability and difficult to store • Risk of toxicity and immune-related adverse reactions

Abbreviation TALENs, Transcription activator-like effector nucleases; ZFNs, Zinc-finger nucleases; ZFP, Zinc finger protein; CRISPR, Clustered regularly interspaced short palindromic repeats; sgRNA, single guide RNA

these FAP-targeting UCAR T cells demonstrated effectiveness in depleting CAFs, reducing desmoplasia, and facilitating tumor infiltration. Another study [46] has similarly highlighted the potential of TALEN-mediated gene editing to create allogeneic, IF/THEN-gated dual CAR-T cells. These dual CAR-T cells can effectively target immunotherapy-resistant solid tumors while minimizing safety risks, underscoring the clinical potential of this approach for solid tumor treatment.

ZFN-based genome editing

Zinc-finger nucleases (ZFNs) are a class of artificially synthesized restriction endonucleases. They are formed by fusing zinc finger DNA-binding domains with the DNA cleavage domains of restriction endonucleases [47]. Upon designing and synthesizing ZFNs targeting specific gene sequences, they induce site-specific DNA cleavage, creating double-stranded DNA breaks (DSBs). Through disruption of NHEJ, ZFNs can inactivate target genes, or facilitate DNA repair via homologous recombination (HR), thereby rejoining the broken DNA strands. The combination of these two steps accomplishes general genome editing operations. Additionally, by integrating ZFN technology with intracellular DNA repair mechanisms, researchers can adeptly edit the genome in vivo. This technology can be utilized for gene knockout, gene insertion, gene activation, gene silencing, or artificial modification of gene sequences to meet specific requirements. In the medical field, plasmids or stem cells containing therapeutic genes modified by ZFN technology can be introduced into the human body for gene therapy. Moreover, ZFN technology can directly repair, replace, or delete harmful genes for therapeutic purposes. ZFN technology offers excellent specificity and efficiency, minimizing the risk of genetic/genomic errors. Theoretically, researchers can perform ZFN operations on cells from any species at any stage of growth, enabling seamless gene modification without disrupting cellular status.

Despite its simplicity and practicality, ZFN technology has certain limitations [48]. ZFN-mediated DNA cleavage requires dimerization of two FokI cleavage domains and at least one recognition unit binding to DNA. While the DNA recognition domain possesses strong specificity, the cleavage process by ZFNs does not entirely depend on homodimer formation. Thus, the formation of heterodimers can lead to off-target effects, ultimately resulting in DNA mismatches and sequence alterations, causing significant cytotoxicity. Accumulation of these adverse effects beyond the capacity of cellular repair mechanisms can lead to apoptosis. Furthermore, ZFN-induced mutations in relevant genes may lead to unforeseen consequences, potentially triggering immune reactions or even tumors if used in vivo. Existing methods cannot predict whether introduced ZNF proteins will provoke attacks from the immune system. Moreover, to date, ZFN technology is limited to in vitro operations. It requires extraction of cells from the human body, manipulation, and subsequent reintroduction into the patient's body. Direct introduction of relevant ZFN components for gene editing into patients carries significant potential risks and low efficiency. These limitations render ZFN operations in humans cumbersome and challenging to widely apply.

Using ZFN genome-editing technology, researchers successfully disrupted the expression of the TCRα constant (TRAC) or TRBC chains in T cells, resulting in a loss of TCR function. These modified T cells became anergic, meaning they were unable to respond

to TCR-specific stimulation, and importantly, showed no imbalance in T cell subsets [49]. TCR-negative T cells have since been utilized to generate universal CAR-T cells, providing a promising platform for "off-the-shelf" immunotherapies [50]. These universal CAR-T cells may offer a more readily accessible and scalable form of immunotherapy for anti-tumor treatment [16].

CRISPR-Cas systems

Both TALEN and ZFN technologies rely on the synthesis of DNA sequence-specific binding protein modules for targeted genome editing, a process that is cumbersome and time-consuming. However, Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) gene-editing technology, as a newly emerged genome editing tool, achieves RNA-guided DNA recognition and editing. CRISPR/Cas technology utilizes a sequence-specific guide RNA molecule to guide the nucleases to the target site, thereby accomplishing genome editing. The development of the CRISPR/Cas system provides a novel platform for constructing more efficient gene-targeting modification techniques [51].

The CRISPR/Cas system consists of CRISPR sequences and Cas gene families. CRISPR is composed of a series of highly conserved repeat sequences interspersed with equally highly conserved spacer sequences. In the vicinity of CRISPR, there also exists a subset of highly conserved CRISPR-associated genes (Cas genes). The proteins encoded by these genes possess functional domains with nuclease activity, enabling them to perform specific cleavage of DNA sequences [52]. As a ubiquitous system in prokaryotes, the initial function of the CRISPR/Cas system is to identify and degrade foreign nucleic acid sequences, thereby exerting antiviral effects. This process occurs in two steps: biological synthesis of crRNA and RNA binding and cleavage. Moreover, the CRISPR platform focuses on ultra-small Cas enzymes such as Cas14, which are the core components of the CRISPR gene editing system. Smaller-sized Cas enzymes are easier to deliver and hold the promise of expanding the scope of in vivo gene editing. CRISPR gene editing tools are focused on their potential for promoting CAR-immune cell therapy [53]. One study [54] developed TCR-knockout CAR-T cell by combining a self-inactivating lentiviral platform via CRISPR/Cas systems. They showed that TCR-knockout CAR-T cell showed greater potency than traditional TCR-positive CAR-T cell. A further promising strategy to reduce the allogeneic immune response involves eliminating MHC class I expression by targeting the B2M gene. Multiplex knockout CAR-T cells have been created to reduce alloreactivity while enhancing anti-tumor efficacy. These modified CAR-T cells, targeting antigens like CD19 or PSCA, exhibited both diminished immune rejection and improved therapeutic activity.

Base editors

Base editors (BEs) are tools that enable single-base substitutions at the level of individual nucleotides without generating DNA double-strand breaks [55]. They hold immense potential in both basic research and gene therapy fields. Approximately one-fourth of pathogenic mutations responsible for genetic diseases in humans require the swapping of adenine bases (A-to-T and A-to-C; or their complementary strand counterparts, T-to-A and T-to-G). Base editing represents a more precise form of gene editing, with its primary advantage being the ability to achieve edits without breaking the DNA double helix. Currently, the most widely used DNA base editors are cytosine base editors (CBEs) and adenine base editors (ABEs) [56]. CBEs convert C·G to T·A base pairs, while ABEs convert A·T to G·C base pairs. However, there has not been a base editor capable of achieving swaps between adenine bases, such as A-to-T and/or A-to-C (i.e., purine to pyrimidine). The development of novel DNA base editors capable of adenine base swapping holds significant potential value in the field of gene therapy.

The team of Tong et al. [57] introduced a novel DNA base editor, which achieved efficient adenine base swapping editing for the first time. Through the comprehensive utilization of protein engineering, flow cytometry, deep sequencing, and other techniques, the study conducted a series of redesigns, engineering modifications, protein evolution, mutation screening, and validation of ABE, leading to the development of a novel DNA base editor named adenine-to-Y base editor (AYBE, where Y = C or T). This research holds significant importance for establishing disease models in the field of basic research and for gene therapy. It marks a new breakthrough in the development of next-generation gene editing tools.

Targeting T cell malignancies with CAR-T cells is often complicated by T cell fratricide, where CAR-T cells attack each other due to shared antigens such as CD3 and CD7. Base editing offers a novel solution by precisely disrupting the expression of problematic antigens through the introduction of stop codons or removal of splice sites, without causing double-strand DNA breaks. In one study [58], researchers created fratricide-resistant CAR-T cells by sequentially eliminating TCR/CD3 and CD7 using base editing, prior to lentiviral-mediated CAR expression targeting CD3 or CD7. Molecular analysis of these base-edited cells confirmed the absence of chromosomal translocations typically found in conventional CRISPR-Cas9 treated cells. In vitro, these engineered 3CAR/7CAR cells exhibited potent cytotoxicity against CD3 + CD7 + T-acute lymphoblastic leukemia cells, and

their efficacy was validated in an in vivo human-murine chimeric model. This base editing approach enhances the safety and effectiveness of CAR-T therapies for T cell malignancies.

mRNA-LNPs

mRNA holds certain advantages over DNA, leading to superior transfection efficiency and longer protein expression duration. The core principle of mRNA vaccines involves encoding antigenic information within mRNA and delivering it to the host cell cytoplasm, where it expresses and induces antigen-specific immune responses in vivo [59]. mRNA vaccines could produce antigens targeting protein target of pathogens [59].

However, mRNA faces challenges such as stronger immunogenicity and instability, which have historically hindered its progress compared to DNA. mRNA molecules, being long-chain polymers with negative charges, experience electrostatic repulsion from the negatively charged cell membrane, making it difficult for them to penetrate cells. Moreover, mRNAs are inherently fragile single-stranded structures and are rapidly degraded by enzymes in vivo. The information encoded in mRNA for ribosome-mediated protein synthesis should be delivered into cells to encode proteins. Accordingly, there are two barriers to delivering mRNA into cells: enzymatic degradation during delivery and membrane barriers due to electrostatic repulsion. It was only with the introduction of modified nucleosides into mRNA sequences and the development of delivery systems capable of encapsulating and delivering mRNA that these technical challenges were largely addressed. Special modifications or encapsulation delivery systems are needed to promote intracellular mRNA expression, altering mRNA's intracellular distribution, cell targeting, and uptake mechanisms to enhance delivery and vaccine efficacy. Common delivery techniques include electroporation, protamine, cationic nanoemulsion, and cationic polymer liposomes. Electroporation involves the formation of membrane pores through high-voltage pulses, enabling direct transfection of mRNA molecules into human cells. The other methods either protect mRNA molecules from extracellular degradation or promote their fusion with the cell membrane to enhance transfection. With the approval of mRNA vaccines for preventing COVID-19, LNPs have emerged as the most popular delivery technology [60]. Golubovskaya et al. [61] introduced an innovative CAR mRNA-LNP technology for efficiently transfecting NK cells, which were expanded from primary peripheral blood mononuclear cells (PBMCs), to develop CAR-NK cells. In this approach, CD19-CAR mRNA and BCMA-CAR mRNA were incorporated into LNPs, resulting in CAR expression in NK cells. Both BCMA-CAR-NK and CD19-CAR-NK cells exhibited remarkably higher levels of cytotoxicity and secretion of IFN-γ and Granzyme B compared to normal NK cells. Additionally, CD19-CAR-NK cells were shown to remarkably inhibit Nalm-6 tumor growth. To sum up, CAR mRNA-LNPs can be a powerful method for generating functional CAR-immune cells with enhanced anti-tumor activity, providing a promising alternative for anti-tumor immunotherapy.

To sum up, the most commonly used gene-editing tools such as TALEN, ZFN, CRISPR-Cas systems, Base editors, and mRNA-LNPs have been widely applied in the CAR therapy. Each gene-editing tool has its own advantages and disadvantages (Table 2). For example, mRNA-LNPs are characterized by high gene editing efficiency. LNPs could protect CAR mRNA from extracellular ribonuclease, and further facilitate intracellular mRNA delivery. However, the poor stability of mRNA is warranted to be resolved and be carefully stored. Moreover, the risk of "off-target" toxicity and immune-related adverse reactions of these gene-editing tools should be further investigated before clinical applications. Accordingly, it is necessary to choose the most suitable gene-editing tool to generate functional CAR-immune cells based on clinical needs.

Gene delivery techniques and systems for potential in vivo gene editing

With the appropriate gene delivery techniques and systems, the above gene-editing tools could be delivered into immune cells, thereby introducing CAR genes for in situ generation of CAR-immune cells. There are three common types of gene delivery techniques and systems for potential in vivo gene editing: physical techniques, chemical techniques, and biological techniques (Fig. 3). Physical techniques include electroporation and gene guns, which are equipped with certain devices. As for chemical techniques, the most commonly used gene delivery methods are LNPs. When compared with physical techniques or chemical techniques, biological techniques such as viral vectors, exosomes, cell membrane-coated technology are regarded as the safest delivery systems with great potential for clinical applications.

Physical techniques

Electroporation, also known as electrotransfection, is a technique that utilizes a high-intensity electric field to temporarily increase the permeability of cell membranes, allowing the absorption of exogenous molecules from the surrounding medium [62]. This method enables the introduction of nucleic acids, DNA and RNA, proteins, carbohydrates, dyes, and viral particles into both prokaryotic and eukaryotic cells. Compared to other physical and chemical transformation methods, electroporation is a valuable and effective alternative.

Needle-free injection (NFI) technology utilizes a high-pressure source to generate a high-velocity liquid jet, which penetrates the epidermis and dermis layers to deliver drugs subcutaneously or intramuscularly [63]. In contrast to traditional needle injection (NI) methods that involve the penetration of the skin layers with a needle and gradual absorption of a large volume of liquid, NFI delivers drugs in a more dispersed form, significantly increasing the contact area between the injected liquid and capillaries, thereby promoting drug absorption and reducing injection pain. Recently, NFI technology has been explored for various medical purposes, such as vaccination, insulin injection, and medical aesthetics. Both intradermal and intramuscular administration routes of NFI have been employed for different types of vaccines. However, the application of NFI in mRNA-based vaccines, is still being explored [64].

In genetic engineering, the gene gun or biolistic particle delivery system is a device used to transfer exogenous DNA, RNA, or proteins into cells [65]. This apparatus involves coating target genes onto heavy metal particles and using mechanical force to propel these microprojectiles into cells, thereby integrating the desired genetic information into the target cells. This technique of delivering DNA via microprojectiles is commonly referred to as "biological ballistics". This device is capable of transforming nearly any type of cells and is not limited to nuclear transformation; it can also transform organelles, including plastids and mitochondria.

Chemical techniques

The most commonly used mRNA vaccine delivery methods, besides LNPs, include cationic lipoplexes (LPXs), lipopolyplexes (LPPs), polymer nanoparticles (PNPs), inorganic nanoparticles (INPs), cationic nanoemulsions (CNEs), and so on.

LNPs carrying mRNA contain four additional components besides the negatively charged mRNA: ionizable lipids, helper lipids, cholesterol, and PEGylated lipids [66]. Before entering cells, cationic lipids can electrostatically complex with negatively charged mRNA molecules, forming complexes that enhance mRNA stability. Upon reaching the cell membrane, cationic lipids fuse with the negatively charged cell membrane, destabilizing it and facilitating mRNA delivery. Once engulfed by cells through endocytosis, LNPs encounter acidic conditions within endosomes, which contain various hydrolytic enzymes. The decreased pH protonates ionizable lipids, causing disruption of LNP's bilayer structure and mRNA release. Moreover, mRNA binds to ribosomes responsible for protein production and is translated into proteins [67].

LPPs are a type of double-layered structure where polymers encapsulate mRNA as the core, while lipids form the outer shell. Compared to traditional LNPs, they exhibit superior mRNA encapsulation and protection, and can gradually release mRNA molecules as the polymer degrades [68]. The excellent dendritic cell targeting of the LPP platform enhances antigen presentation, activating T cells' immune response more effectively, thus achieving the desired immunotherapeutic effect.

INPs are synthesized from inorganic particles and biodegradable polycations, such as golden NPs, silica NPs, quantum dots, and carbon nanotubes. The most common type is mesoporous silica NPs [69], which are characterized by uniform pores, ease of functionalization, biocompatibility, high surface area, large pore volume, and biodegradability. To enhance the delivery and transfection efficiency of mRNA, cationic polymers are typically encapsulated on their surface or within their pores.

Biological techniques

Viral vectors play a crucial role in the field of gene delivery, offering unprecedented opportunities for gene therapy and gene editing by efficiently transferring genetic material into target cells. These viral vectors are often engineered for safety and efficacy, such as by attenuating their pathogenicity or introducing receptor specificity [70]. Commonly used viral vectors include adenoviruses, retroviruses, and adeno-associated viruses, each with specific advantages and applications. As research on viral vectors advances, a clearer understanding of their potential in treating various diseases emerges, laying a solid foundation for the future development of gene therapy.

Nanobodies (VHHs) are small, stable, camelid-derived single-domain antibody fragments that serve as the variable regions of heavy-chain-only antibodies. They exhibit affinities comparable to traditional scFvs [71, 72]. Due to their properties, VHHs can be effectively utilized as antigen recognition domains in CAR-immune cells. Xie et al. [73] focused on creating VHH-based CAR-T cells designed to target the TME and inhibit the growth of solid tumors in immunocompetent mice. These VHH-based CAR-T cells specifically target the tumor stroma and vasculature through the EIIIB+fibronectin splice variant, which is expressed by various tumor types and on neovasculature. Moreover, another study [74] humanized a CD19-specific VHH, referred to as H85, to assess how humanization of the antigen-recognition domain influences CAR expression, density, cytokine secretion, and cytolytic reactivity in CAR-T cells. The humanized version, named HuH85, was shown to be expressed on the surface of transduced T cells at levels comparable to H85. Both H85CAR-T cells and HuH85CAR-T cells exhibited similar anti-tumor effects against Ramos and Namalwa cells, secreting comparable amounts of IFN-γ, IL-2, and TNF-α after co-cultivation. These findings suggest that HuH85 can be effectively used to develop

VHH-based CAR-T cells targeting CD19-associated hematologic malignancies, highlighting the potential of VHHs in enhancing CAR-immune cell therapy.

Exosomes or small extracellular vesicles secreted by various cell types, have garnered significant interest as potential vehicles for gene delivery [75]. These naturally occurring nanoparticles possess several advantages, including stability in biological fluids, low immunogenicity, and the ability to cross biological barriers, such as the blood-brain barrier [76]. Moreover, exosomes can be engineered to encapsulate specific nucleic acids, including small interfering RNA (siRNA) [77], microRNA (miRNA) [78], or plasmid DNA [79], making them promising candidates for targeted gene therapy. Harnessing the therapeutic potential of exosomes for gene delivery holds great promise for treating a wide range of diseases, from tumor to neurodegenerative disorders, offering a novel approach with potential clinical applications.

Cell membrane-coated technology represents an innovative method for gene delivery, utilizing the natural properties of cell membranes to improve the delivery efficiency and biocompatibility of therapeutic cargo [80–82]. By encapsulating nucleic acids or gene editing tools within cell membrane-derived nanovesicles, this technology can mimic the surface characteristics and targeting abilities of the original cell type. This not only shields the cargo from degradation and immune recognition but also facilitates specific targeting to desired cell populations. Furthermore, the use of autologous cell membranes reduces the risk of immune rejection, enhancing the safety profile of the delivery system. Cell membrane-coated nanovesicles could enhance drug delivery and immune evasion, thereby serving as the promising platforms for the effective and safe delivery of mRNAs [59]. With ongoing advancements in this field, cell membrane-coated technology holds immense potential for revolutionizing gene therapy and regenerative medicine [83], offering a versatile platform for the treatment of various diseases.

The applications of in situ generation of CAR therapy in tumors

Hematologic malignancies

CAR-T cell immunotherapy is revolutionizing the treatment paradigm for patients with B-cell lymphoma (BL). Nevertheless, the current methodology for CAR-T production is intricate and costly. The field is in dire need of a more cost-effective approach to CAR-T cell production directly within the body. In a groundbreaking study [84], the Spleen Selective ORgan Targeted (SORT) LNPs were utilized to produce CAR-T cells in situ, presenting a streamlined alternative to the current labor-intensive process (Fig. 4A; Table 3). Optimized Spleen SORT LNPs exhibited remarkable efficacy in transfecting T cells post intravenous injection, achieving up to 5.8% CD8+T cells. These LNPs effectively delivered Cre recombinase mRNA

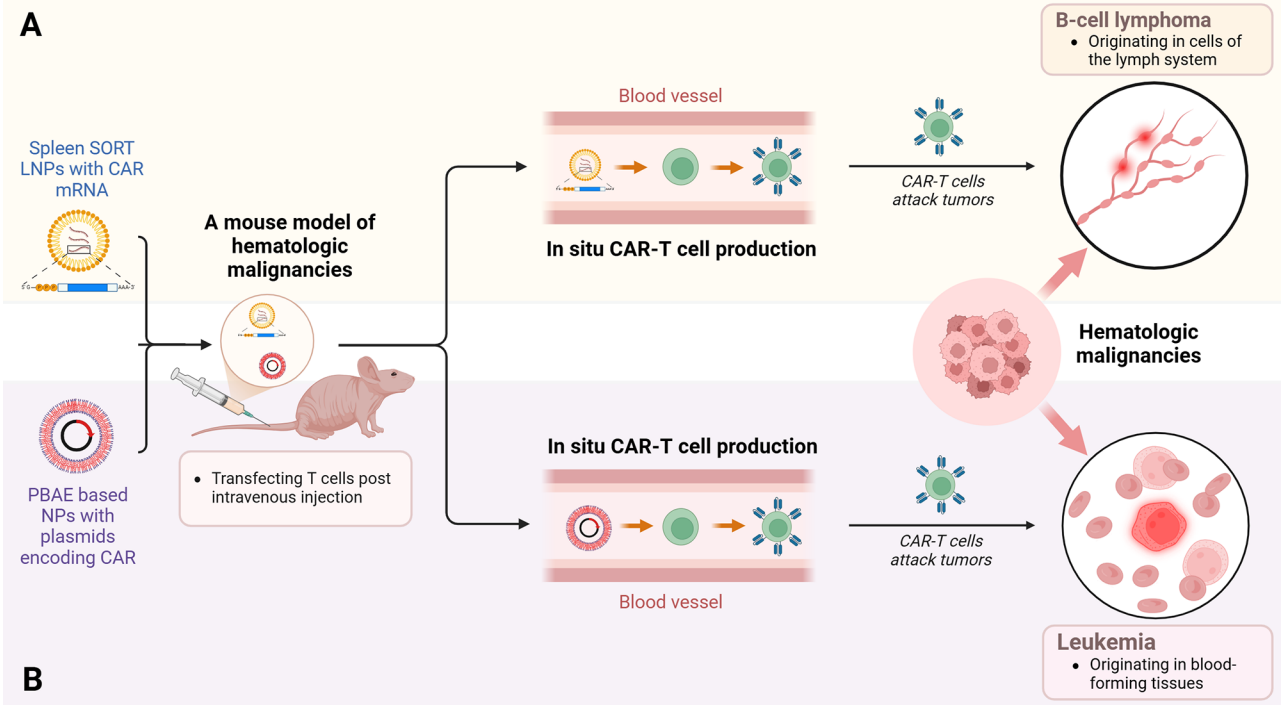

Fig. 4 The applications of in situ generation of CAR therapy in hematologic malignancies. (**A**) The Spleen SORT LNPs with CAR mRNA were utilized to produce CAR-T cells in situ, serving as a potential alternative for the treatment of B-cell lymphoma [84]; (**B**) PBAE-based NPs as safe and effective DNA delivery vectors, could deliver plasmids encoding CAR to produce CAR-T cells in situ for the anti-tumor immunotherapy of leukemia

Table 3 The applications of in vivo editing and in situ generation of CAR therapy in tumors

Study	Tumor type	Gene delivery system	Gene-editing tool	In vivo CAR cell	Citation
Hematologic malignancies					
Álvarez-Benedicto et al.	B-cell lymphoma	Spleen SORT LNPs	Cre recombinase mRNA and CAR encoding mRNA incorporating both CD28 and 41BB co-stimulatory domains	CAR-T	[84]
Smith et al.	Leukemia	PBAE polymer functionalized with MTAS and NLS sequences	Plasmids encoding an all-murine 194-1BBz CAR and the hyperactive iPB7 transposase	CAR-T	[8]
Solid tumors					
Chen et al.	Glioblastoma	The pCAR-laden nanomicelle coated with citraconic anhydride-modified dextran	Macrophage-specific promoter-driven anti-CD133 CAR plasmids (pCARs) encoding the CD3ζ intracellular costimulatory domain	CAR-MΦ	[94]
Gao et al.	Brainstem gliomas	Couple macrophage-cell-targeting and phenotype-switching RP-182 peptide onto the surfaces of biodegradable PBAE-based NPs	Plasmid DNA encoding the ErbB2-specific CAR with the CMV promoter or macrophage specific CD68 promoter	CAR-MΦ	[95]
Yang et al.	Hepatocellular carcinoma	Ionizable lipid PPZ-A10-formulated LNPs	GPC3-specific CAR mRNA and Siglec-GΔITIMs mRNA	CAR-M	[96]

Abbreviation SORT, Selective ORgan Targeted; LNPs, Lipid Nanoparticles; mRNA, messenger RNA; CAR, Chimeric Antigen Receptor; PBAE, Poly(β-amino ester); NPs, Nanoparticles

and CAR encoding mRNA to T cells in both reporter mice and a lymphoreplete BL model, eliminating the necessity for active targeting ligands. Furthermore, in situ-generated CAR-T cells significantly prolonged the overall survival of mice with a less aggressive form of BL and attenuated tumor metastasis to the liver by enhancing anti-tumor T cells. Collectively, these findings underscore the potential of Spleen SORT LNPs as a platform for in situ CAR-T cell therapy in BL. Notably, the choice of co-stimulatory molecule within the CAR emerged as a pivotal factor in determining in situ CAR-T cell efficacy. While CARs containing 41BB exhibited prolonged survival, substituting the co-stimulatory molecule with CD28 failed to confer the same benefit. The production of CAR-T cells in situ correlated with an increase in T cells within liver lesions, thereby explaining the observed reductions in abdominal circumferences and metastatic liver lesions in mice. These findings suggest that harnessing more potent third and fourth generation CARs could yield greater benefits in aggressive tumors. Third generation CARs, incorporating both CD28 and 41BB co-stimulatory domains, demonstrate enhanced expansion and prolonged persistence of CAR-T cells [85]. Similarly, fourth generation CARs integrate cytokines into their structures to promote CAR-T cells and remodel TME, thereby enhancing CAR-T cell efficacy [86].

Smith et al. [8] outlined a novel method for rapidly equipping circulating T cells with tumor-targeting capabilities. Specifically, they demonstrated that DNA-loaded NPs can efficiently introduce leukemia-targeting CAR genes into T-cell nuclei, leading to prolonged disease remission (Fig. 4B; Table 3). These PNPs are straightforward to produce in a stable form, facilitating storage and reducing costs. Effective delivery of nucleic acids into T cells requires uptake by the cells and transport of their DNA cargo into the nucleus. The first step was to couple T-cell-targeting anti-CD3e f(ab′)2 fragments to the surfaces of biodegradable poly(β-amino ester) (PBAE) based NPs [87], enabling receptor-mediated endocytosis by lymphocytes. Subsequently, they functionalized the polymer with peptides containing microtubule-associated sequences (MTAS) and nuclear localization signals (NLS) to expedite nuclear import of the genetic cargo via the microtubule transport machinery [88]. CD3-targeted NPs exhibited selective binding to T lymphocytes, with minimal interaction with off-target cells. Confocal imaging revealed rapid internalization of the particles into the cytoplasm within 120 min, likely due to receptor-induced endocytosis. Within 30 h post-transfection, 194-1BBz receptors were detected on the surfaces of treated cells. The use of PBAE polymer functionalized with MTAS and NLS sequences significantly enhanced gene transfer efficiency, as evidenced by higher nuclear targeting of CAR-transgene expression in primary T cells compared to controls. NP-transfected lymphocytes exhibited full functionality, selectively lysing Eμ-ALL01 leukemia cells and secreting effector cytokines at levels comparable to T cells transduced with a lentiviral vector encoding the same CAR. Integration of piggyBac transposable elements into NP-delivered plasmids ensured sustained high-level expression of 194-1BBz gene in T cells over an extended period through somatic integration.

The clinical adoption of NP-mediated T cell programming will be contingent upon the safety profile of the procedure. To address this concern, they opted for the use of PBAE polymer as the core material for the T cell-targeted nanocarriers. This polymer has a half-life ranging from 1 to 7 h in aqueous conditions, ensuring

biodegradability and minimizing potential long-term effects [87]. Additionally, they modified these nanocarriers to shield their positive charge, thereby reducing off-target binding and enhancing specificity for T cells. PBAE-based NPs have previously been described as safe and effective DNA delivery vectors [89, 90], albeit using local and untargeted rather than systemic application. The inquiry into whether the potential advantages of in situ T cell programming outweigh safety concerns related to gene transfer into off-target cells necessitates further assessment, either through nonhuman primate modeling or direct implementation in a phase-I dose-escalation trial. Given that the signaling domains of CARs are meticulously crafted to mimic T cell-intrinsic stimulatory signals, they deduced that any toxicities stemming from CAR expression in non-T cells would likely be minimal, and could be effectively managed in a clinical context. To mitigate this risk entirely, the CAR transgenes delivered via NPs could be engineered to express under the control of a T cell-specific promoter [91]. In summary, they demonstrate that circulating T cells can be engineered to express leukemia-specific CARs through the delivery of genes by PNPs, empowering them to combat the disease. The simplicity of NP manufacturing and their stability facilitate long-term storage and cost reduction. Consequently, when utilized in clinical settings as a novel form of active immunotherapy, this technology holds promise for offering a practical, cost-effective, and widely applicable approach to anti-tumor treatment.

Solid tumors

CAR-immune cell therapy has shown promising results in hematologic malignancies by enabling tumor cell tracking and specific killing. For example, CAR-T therapy requires cumbersome ex vivo T cell expansion and poses safety concerns such as cytokine release syndrome. Moreover, its efficacy in solid tumors is limited. Therefore, exploring safer and more efficient CAR-immune cell therapies is a focal point and frontier in solid tumor immunotherapy.

Macrophages showcase distinct effector capabilities and demonstrate significant infiltration within the solid TME [81], particularly in the brain. In this organ, macrophages (MΦ) serve as both structural support and the primary immune effector cells of the central nervous system (CNS), constituting approximately 5–12% of brain cells [92]. Unlike T cells, macrophages primarily function in phagocytosis, processing, and antigen presentation, with a stronger ability to compete for oxygen and nutrients. Surgical resection, the cornerstone of CNS tumors clinical intervention, induces the release of inflammatory factors around the surgical cavity, leading to the accumulation of numerous microglia/macrophages in the local vicinity postoperatively. Compared with CAR-T and CAR-NK, CAR-M, as a new cellular immunotherapy, has unique advantages such as strong tumor invasion ability, enhanced antigen presentation ability, enhanced T cell killing ability, and less non-tumor targeting toxicity compared with CAR-T and CAR-NK therapy. Some studies have investigated the applications of in situ generation of CAR in solid tumors.

Glioblastoma (GBM) is the most common malignant tumor of CNS. Surgical resection is the primary intervention for GBM patients, followed by adjuvant therapies such as radiotherapy and chemotherapy to eliminate residual tumor cells. However, glioma stem cells (GSCs) are resistant to conventional treatments and are difficult to eradicate. After surgical resection of the solid tumor component, residual GSCs quickly regenerate and result in rapid tumor recurrence within months [93]. Exploring effective strategies to specifically eliminate GSCs is crucial to preventing postoperative GBM recurrence. Chen et al. [94] stated that in situ modification of these microglia/macrophages into CAR-MΦ capable of specifically recognizing and engulfing GSCs, thereby activating the adaptive immune system and forming immunologic memory, holds promise for addressing the challenge of postoperative GBM recurrence. Utilizing injectable hydrogels as the "drug reservoir" system, they proposed delivering macrophage-targeted pCAR-NPs and CD47 antibodies to the peritumoral local microenvironment postoperatively, facilitating in situ editing of microglia/macrophages and generating CAR-MΦ capable of targeting GSCs (Fig. 5; Table 3). Simultaneously, by blocking the "don't eat me" signal of tumors, they synergistically enhanced the phagocytic efficiency of CAR-MΦ against GSCs, activating the adaptive immune system through antigen presentation, eliminating residual GSCs postoperatively, and forming immunologic memory to prevent GBM recurrence.

The hybrid system of NP-hydrogel constructed in this study offers multiple potential advantages in postoperative tumor treatment: (a) employing a "filling-type" drug delivery method around the surgical cavity, enhancing patient compliance; (b) simplifying ex vivo editing and reinfusion procedures by locally editing CAR structures of microglia/macrophages around the surgical cavity, avoiding potential systemic side effects of systemic administration. Additionally, by delivering drug reservoirs through hydrogel systems into the surgical cavity, sustained inhibition of residual GSCs around the surgical cavity can be achieved; (c) utilizing hydrogel to co-deliver CD47 antibodies around the surgical cavity postoperatively, avoiding potential systemic side effects observed in clinical trials of systemic administration of CD47 antibodies, allowing them to only function in blocking the "don't eat me" signal of tumor cells and enhancing the phagocytic efficiency of CAR-MΦ against GSCs.

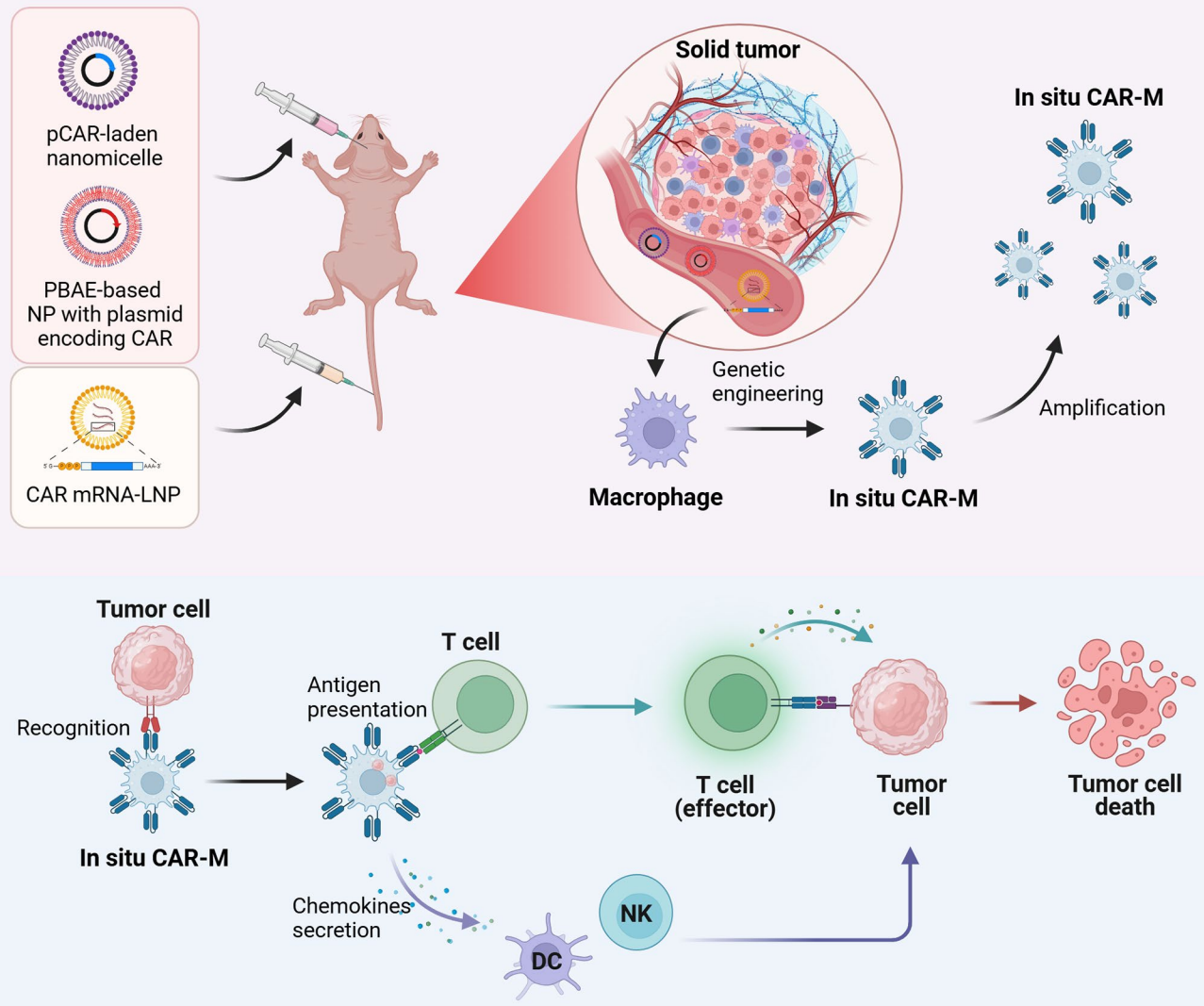

Fig. 5 The applications of in situ generation of CAR therapy in solid tumors. As for glioblastoma or brainstem gliomas, pCAR-laden nanomicelle or PBAE-based NPs with plasmid encoding CAR were utilized to produce CAR-M cells in situ via intracranial injection, respectively. As for hepatocellular carcinoma, CAR mRNA-LNPs were utilized to produce CAR-M cells in situ via intravenous injection. CAR-M cells could recognize tumor cells, present antigens to T cells, and further activate T cells and induce NK cell recruitment to play an anti-tumor role

Gao et al. [95] presented a synthetic universal DNA nanocarrier in their study, aimed at in situ editing of intratumoral MΦ. They utilized an ErbB2-specific CAR to direct the phagocytic activity of these MΦ towards tumors, thereby initiating a locoregional anti-tumor immune response (Fig. 5; Table 3). They showed that the RP-182 peptide, embedded in the NP shell, effectively targeted MΦ and reprogrammed M2-like TAMs into an anti-tumor M1-like phenotype. Moreover, the DNA nanocomplex carrying the CAR gene could introduce ErbB2-targeted CAR into these MΦ, turning them into "living" therapies that sequentially eliminate invasive tumor cells. Their findings suggest a practical approach to anti-tumor immunotherapy for brainstem gliomas (BSGs), with potential applicability to other ErbB2-positive solid malignancies in patients.

Hepatocellular carcinoma (HCC) is a widespread and often fatal illness, with tumor regression being uncommon among advanced HCC patients due to the scarcity of effective treatments. Considering the abundance of macrophages in HCC and their significance in tumor immunity, converting them into CAR-M cells is believed to enhance phagocytosis targeted at HCC cells and promote tumoricidal immunity. In the study of Yang et al. [96], mRNA encoding CARs was enclosed within LNPs designed to target liver macrophages. Remarkably, these LNPs were capable of adsorbing specific plasma proteins, facilitating their targeting of HCC-associated macrophages. Moreover, mRNA encoding Siglec-G lacking

ITIMs (Siglec-GΔITIMs) was codelivered to liver macrophages by LNP to relieve CD24-mediated CAR-M immune suppression. Mice treated with LNPs generating CAR-M as well as CD24-Siglec-G blockade significantly elevated the phagocytic function of liver macrophages, reduced tumor burden and increased survival (Fig. 5; Table 3). This study proposes a promising and adaptable strategy for HCC treatment, underscoring the need for thorough evaluation in rigorous clinical trials.

The in vivo editing technology proposed in the above studies provides a new approach for immunotherapy of solid tumors (Table 3), potentially expanding the application of CAR-immune cell therapy and laying a theoretical foundation and experimental basis for the treatment of tumor stem cells with CAR cells.

Clinical hindrances of in vivo editing and in situ generation of CAR therapy

Although in situ CAR-immune cell therapy is still in its early stages, preclinical data have shown promising potential for treating a wide range of complex diseases. Whereas, to realize its full potential and transition into routine clinical practice, several challenges must be addressed.

Gene delivery systems must ensure that genetic cargo is specifically targeted to the cell type intended for reprogramming within target tissues, in order to prevent unintended off-target genetic modifications. One challenge of in situ cell therapy is the risk of off-target gene transfer during reprogramming. Despite the advanced functionalization of NPs, they can still be internalized by off-target cells. Achieving specific targeting and transfection of the desired cell population in vivo remains highly challenging [97]. To address these safety concerns, a conditional and inducible translation strategy has been proposed. This strategy would limit transgene expression to the intended cell populations while sparing other cells [38]. Additionally, synthetic NPs are prone to interact with proteins under physiological conditions, leading to their recognition and clearance by the reticuloendothelial system (RES) [98]. Most LNPs developed thus far exhibit a natural liver tropism, due to the apolipoprotein E (ApoE) protein corona, which directs their uptake by hepatocytes [99]. To overcome this limitation, Siegwart et al. introduced a SORT strategy to engineer NPs that enable extrahepatic delivery of gene editing systems [100]. This method allows for selective transfection of specific cell types, such as epithelial cells, endothelial cells, B cells, and T cells, thereby expanding the potential for precise gene delivery beyond the liver.

Sustained clinical responses are essential for the long-term success of cell therapies. While lentiviral vectors provide stable integration into the host genome, allowing for continuous transgene expression, NP-based nonviral approaches often require repeated dosing to maintain transgene expression over time. Ensuring the safe, chronic dosing of NPs in humans is therefore critical for advancing in situ CAR-cell therapy. To achieve this, the biocompatibility, biodegradability, pharmacokinetics, and long-term toxicity of synthetic NPs must be carefully evaluated [38]. Another significant challenge with NPs is their limited transfection efficiency. Even with LNPs, which have been used in clinical applications, their ability to escape from endo/lysosomal compartments and enter the cytoplasm is quite limited. This limitation could lead to the degradation of genetic cargo within lysosomes before it can exert its intended effect [101]. To address this issue, several strategies are designed such as using pH-responsive NPs and pore-forming peptides that trigger escape mechanisms [102].

Achieving sustained functionality and persistence of in vivo CAR-immune cells is a critical challenge for optimizing therapeutic outcomes in tumor patients [103]. One approach focuses on refining CAR design by incorporating co-stimulatory domains, which can promote both the longevity and activity of T cells [104]. These co-stimulatory domains modulate intracellular signaling pathways within CAR-T cells, helping to extend their functionality over time [105]. Additionally, efforts are being made to enhance the formation of memory CAR-T cells, which can enable long-lasting anti-tumor responses. This approach is being tested in both hematological malignancies and solid tumors, as memory CAR-T cells are believed to offer sustained therapeutic effects [106]. However, solid tumors often present a more difficult challenge due to the immunosuppressive TME, which might inhibit T cell activity and reduce the efficacy of CAR-T therapy. One strategy to overcome this involves improving CAR-T cell homing to tumor sites, achieved by remodeling the tumor vasculature with specific agents to increase the infiltration of T cells [107, 108]. Furthermore, the heterogeneity of antigens in solid tumors presents significant challenges for CAR-T cells, as it hinders their ability to effectively detect and eliminate tumor cells, thereby limiting their overall anti-tumor activity [109]. This challenge is closely related to the inadequate in vivo production of CAR-T cells that can target the diverse range of tumor-associated antigens. Several strategies have been developed including co-expression of several CARs on a single T cell and expression of a chimeric receptor including more antigen recognition domains [110].

Similar to ex vivo CAR-immune cell therapy, the transition of in vivo CAR-immune cells from preclinical success to clinical applications faces significant hurdles. Ensuring the safety and efficacy of these therapies in clinical applications is paramount [111]. Regulatory agencies such as FDA mandate that viral vectors and

NPs meet stringent quality, safety, and efficacy standards before approval [98]. Additionally, viral vectors used for CAR gene delivery in clinical settings must undergo rigorous testing for purity, safety, stability, and functionality to mitigate potential risks [112]. Another major regulatory challenge involves the safety profile of gene-editing tools like CRISPR/Cas9, which are often employed in the development of in vivo CAR-immune cell therapies [113]. CRISPR/Cas9 systems can sometimes lead to unwanted gene editing events, such as variable length insertions or deletions (indels), at off-target sites in the modified T cells [114]. To minimize the risks associated with off-target effects in CRISPR/Cas9 gene editing, especially during T cell modification both in vivo and ex vivo, high-fidelity Cas9 variants have been developed [114]. Overcoming these regulatory concerns is crucial for advancing in vivo CAR-immune cell therapies into clinical practice.

To summarize, current research is focused on addressing key challenges in the development of in vivo CAR-immune cell therapies. This includes improving the precise targeting of immune cells in vivo and enhancing the transfection efficiency of NPs. It is necessary to boost the long-term efficacy of CAR-immune cells and refine the genetic cargo to reduce off-target effects. Moreover, the use of nonhuman models is essential for evaluating the safety and pharmacokinetics of in vivo CAR-immune cells. In parallel, attention must be given to optimizing manufacturing practices, ensuring quality assurance, and navigating regulatory requirements to successfully transition these therapies into clinical applications.

Future perspective and conclusion

CAR-immune cell therapy represents a significant advancement in anti-tumor immunotherapy. For example, approximately 90% B cell malignancies patients respond positively to CAR-T therapy. Whereas, long-term researches reveal that 40–60% of these patients experience relapse. Moreover, the traditional production methods for CAR-T cells are complex and costly [115]. Ex vivo CAR-T cell requires the collection of patient blood. Before the patient could receive CAR-T, they should undergo lymphodepletion via chemotherapy a few days before CAR-T cell infusion [116]. Because this process utilizes the patient's own T cells, CAR-T cells cannot be produced at large scales or made readily available when needed. Additionally, the facilities for manufacturing this type of cell therapy are limited, and the entire process might take 4–6 weeks. These factors contribute to limited patient access to CAR-T therapy.

Owing to the above limitations of ex vivo CAR-immune cell therapy, novel developments have introduced another avenue as the next-generation anti-tumor immunotherapy: in vivo editing and in situ generation of CAR-immune cell therapy. According to recent researches, the in vivo CAR therapy has been applied in both hematologic malignancies and solid tumors, which contributes to excellent therapeutic effects in animal models. The in vivo editing and in situ generation of CAR therapy holds promise for offering a practical, cost-effective, and widely applicable approach to the anti-tumor immunotherapy. During the process of in vivo editing and in situ generation of CAR therapy, both gene-editing tools and gene delivery techniques and systems are of great importance. As for gene-editing tools, TALENs, ZFNs, CRISPR-Cas systems, base editors, and mRNA-LNPs are widely used for potential in vivo gene editing of CAR therapy. The therapeutic effects of CRISPR-Cas and mRNA-LNPs have been clarified in recent studies for the next-generation CAR immunotherapy. There are three common types of gene delivery techniques and systems for potential in vivo gene editing: physical techniques, chemical techniques, and biological techniques. Physical techniques include electroporation, NFI technology, and gene guns, which are equipped with certain devices. As for chemical techniques, the most commonly used gene delivery methods are LNPs, LPXs, LPPs, PNPs, INPs, and so on. When compared with physical techniques or chemical techniques, biological techniques such as viral vectors, exosomes, cell membrane-coated technology are regarded as the safest delivery systems with great potential for clinical applications. With the appropriate gene delivery techniques and systems, the above gene-editing tools could be delivered into immune cells, thereby introducing CAR genes for in situ generation of CAR-immune cells.

Either ex vivo or in vivo CAR-immune cells, these cells translation to solid tumors faces challenges due to manufacturing complexities, short-lived in vivo persistence, and transient therapeutic impact. In recent years, various novel scaffold biomaterials could be used for cell survival and retention of CAR-immune cells. One study [117] designed an innovative macroporous biomaterial scaffold called "Drydux" for the rapid and efficient in situ generation of CAR-T. CAR-T cells generated using Drydux exhibit prolonged in vivo release, enhanced functionality, and persistence exceeding 150 days, with cells transitioning to memory phenotypes. In contrast to conventional CAR-T with temporary tumor control, equivalent doses of Drydux-generated cells induced lasting tumor remission. It holds promise in revolutionizing solid tumor CAR-T cell therapy by delivering durable, rapid, and cost-effective treatments and broadening patient accessibility to this groundbreaking therapy.

CAR-immune cell therapies or checkpoint immune inhibitors always suffer from immunotoxicity and autoimmune disease [9]. Based on biomaterial-based immunotherapies, the spatial modulation of interactions between immune cells and tumor cells could enhance

the efficacy of immunotherapy by precisely controlling in vivo immune activation within tumor tissues, while also minimizing immune-related adverse events. Zheng et al. [118] designed a rational design of immunomodulating NPs that could in situ modify the tumor cell surface with NK cell activating signals to achieve in situ activation of tumor-infiltrating NK cells, as well as direction of their anti-tumor immunity toward tumor cells without noticeable side effects. CAR-immune cell engineering assisted by properly designed biomaterials has improved therapeutic efficacy and reduced side effects, providing a sustainable strategy for improving anti-tumor immunotherapy. At the same time, the low cost and diversity of biomaterials also offer the possibility of industrial production and commercialization [119]. Finally, more strategies are warranted to enhance the anti-tumor efficacy of in situ generation of CAR-immune cells, and ensure their functional persistence and security in vivo.

To sum up, as the next-generation anti-tumor immunotherapy, the in vivo editing and in situ generation of CAR therapy holds promise in revolutionizing conventional CAR cell therapy by delivering durable, rapid, and cost-effective treatments and broadening patient accessibility to this groundbreaking therapy. However, some limitations are warranted to be resolved to produce a general platform for both hematologic malignancies and solid tumors before clinical trials.

Author contributions

Conceptualization, Methodology, Investigation: WYZ Visualization, Supervision: XH Writing-original draft: XH and WYZ Writing-review & editing: XH.

Funding

National Natural Science Foundation of China (82203059, 82300932). Hubei Natural Science Foundation (2022CFB656).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Received: 24 July 2024 / Accepted: 7 November 2024
Published online: 13 November 2024

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