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REVIEW

Targeted gene delivery systems for T-cell engineering

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Abstract

T lymphocytes are indispensable for the host systems of defense against pathogens, tumors, and environmental threats. The therapeutic potential of harnessing the cytotoxic properties of T lymphocytes for antigen-specific cell elimination is both evident and efficacious. Genetically engineered T-cells, such as those employed in CAR-T and TCR-T cell therapies, have demonstrated significant clinical benefits in treating cancer and autoimmune disorders. However, the current landscape of T-cell genetic engineering is dominated by strategies that necessitate in vitro T-cell isolation and modification, which introduce complexity and prolong the development timeline of T-cell based immunotherapies. This review explores the complexities of gene delivery systems designed for T cells, covering both viral and nonviral vectors. Viral vectors are known for their high transduction efficiency, yet they face significant limitations, such as potential immunogenicity and the complexities involved in large-scale production. Nonviral vectors, conversely, offer a safer profile and the potential for scalable manufacturing, yet they often struggle with lower transduction efficiency. The pursuit of gene delivery systems that can achieve targeted gene transfer to T cell without the need for isolation represents a significant advancement in the field. This review assesses the design principles and current research progress of such systems, highlighting the potential for in vivo gene modification therapies that could revolutionize T-cell based treatments. By providing a comprehensive analysis of these systems, we aim to contribute valuable insights into the future development of T-cell immunotherapy.

Keywords Targeted gene delivery system · T-cell based immunotherapy · Viral vector · Nonviral vector

Abbreviations

aAPC	Artificial antigen-presenting cell
AAV	Adeno-associated virus
ABE	Adenine base editor
BCMA	B-cell maturation antigen
CAR-T therapy	Chimeric antigen receptor T-cell therapy
CBE	Cytosine base editor
CD19	The cluster of differentiation 19
CPPs	Cell-penetrating peptides
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
DARPins	Designed ankyrin repeat proteins
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
FDA	Food and drug administration
HDR	Homology-directed repair
HIV	Human immunodeficiency virus
ITR	Inverted terminal repeat
LDLR	Low-density lipoprotein receptor
LNP	Lipid nanoparticle
LV	Lentiviral vector
MHC	Major histocompatibility complex
PB	PiggyBac transposon system
RME	Receptor-mediated endocytosis
SB system	Sleeping Beauty transposon system
TAA	Tumor-associated antigen
TCR-T therapy	T-cell receptor engineered T-cell therapy
TIL therapy	Tumor-infiltrating lymphocyte therapy
VSV-G	Vesicular stomatitis virus G protein

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1 Introduction

T lymphocytes are the primary components of the adaptive immune system, and in the past ten years, T-cell based immunotherapy has shown tremendous success in the treatment of autoimmune disorders and cancer [1–3]. Initial T-cell immunotherapy involved isolating and expanding tumor-specific T cells in vitro, followed by their reinfusion into patients through therapies such as tumor-infiltrating lymphocyte (TIL) therapy and cytolytic T-lymphocyte (CTL) therapy [4–6]. Genetically modified T-cells use patient autologous or allogeneic T cells to enhance immune function [7], which primarily focuses on redirecting T cells to tumor cells by genetically introducing specific antigen receptors through diverse gene delivery systems, leading to transient or stable expression [8], such as chimeric antigen receptor T (CAR-T) cells and T-cell receptor T (TCR-T) cell therapy.

The delivery of therapeutic genes to specific tissues and cells is extremely crucial, as it offers a potential treatment method for refractory diseases. Vector systems that deliver targeted genes are typically divided into viral and nonviral vector systems in nature, and are chosen based on cell type, gene length, and experimental purpose. Viral vectors are currently widely used in the genetic engineering of T cells, with promising results in the treatment of hematological diseases [9]. Notably, a total of six CAR-T cell therapy products have been approved by the U.S. Food and Drug Administration (FDA), which include Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, and Carvykti [10]. These immunotherapies employ lentiviral or retroviral gene delivery systems for the genetic modification of T cells. In addition to conventional treatments, the tissue tropism of adeno-associated viral vectors of different serotypes is effective in the treatment of hereditary retinopathy [11], hemophilia [12], and neuromuscular diseases [13]. Moreover, the vast majority of gene therapy clinical trials of have utilized engineered viral vectors, including lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses (AAVs), to deliver therapeutic genes. Despite significant progress in T-cell immunotherapy, viral vectors have several inherent limitations, including their oncogenic potential, immunogenicity, and restricted gene packaging capacity [14–16]. Consequently, nonviral vector systems, such as nanoparticles (NPs), transposons, and clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 (Cas9) systems, have emerged as promising alternatives due to their potential to mitigate these drawbacks, making them viable contenders for targeted gene delivery [17]. For instance, nonviral vector systems offer the benefits of accommodating larger gene sizes and exhibiting lower immunogenicity [18]. Nonetheless, their principal limitation lies in their reduced transfection efficiency [19]. Therefore, there is a pressing need to focus on vector systems capable of targeted gene delivery to T cells, streamlining the process, minimizing costs, preserving the functional integrity of T cells to the fullest extent, and overcoming the technical challenges that currently limit the broad application of T-cell based genetic engineering (Fig. 1).

In this review, we provide a comprehensive overview of the various vector systems employed for gene delivery to T cells, encompassing both viral and nonviral vector platforms. We will also delve into current strategic designs, research progress, identified limitations, and future prospects.

2 Viral vector systems

Viral vectors have been extensively used in the field of gene therapy, with integrative and nonintegrative vectors representing two distinct classes of viral gene delivery systems [20]. For example, lentiviral vectors (LVs) can integrate foreign genes into the host genome to achieve stable expression, while adeno-associated viruses (AAVs) and adenoviruses are nonintegrated viral-like vectors. In general, viral vector are composed of three components: (1) a protein capsid or envelope that encapsulates genetic information; (2) the desired transgene, which can be expressed in cells of interest; and (3) the regulatory cassette that regulates the transgene's stable or transient somatic expression [9]. Each viral vector possesses unique components that are meticulously engineered to address specific considerations and fulfill the requirements for efficient gene delivery to target cells. Commonly employed viral vectors, including retroviruses, adeno-associated viruses, and lentiviruses, form the foundation for the majority of gene therapy applications [21]. Targeted viral vectors are crucial for gene therapy [22], particularly for the precise delivery of genetic material to T lymphocytes, and are pivotal in the treatment of cancer and autoimmune disorders. The strategic design of viral vectors for targeted gene delivery to T cells is emerging as a dominant trend in gene therapy. Although the specific engineering techniques for vector targeting vary among different virus types, the underlying principle is consistent: to exploit the tropism of various viruses or serotypes, or to incorporate ligands, peptides, factors, or single-chain antibodies that possess specific recognition capabilities. In this section, we provide an overview of the current rational design and research advancements in viral vectors tailored for T-cell-directed gene delivery.

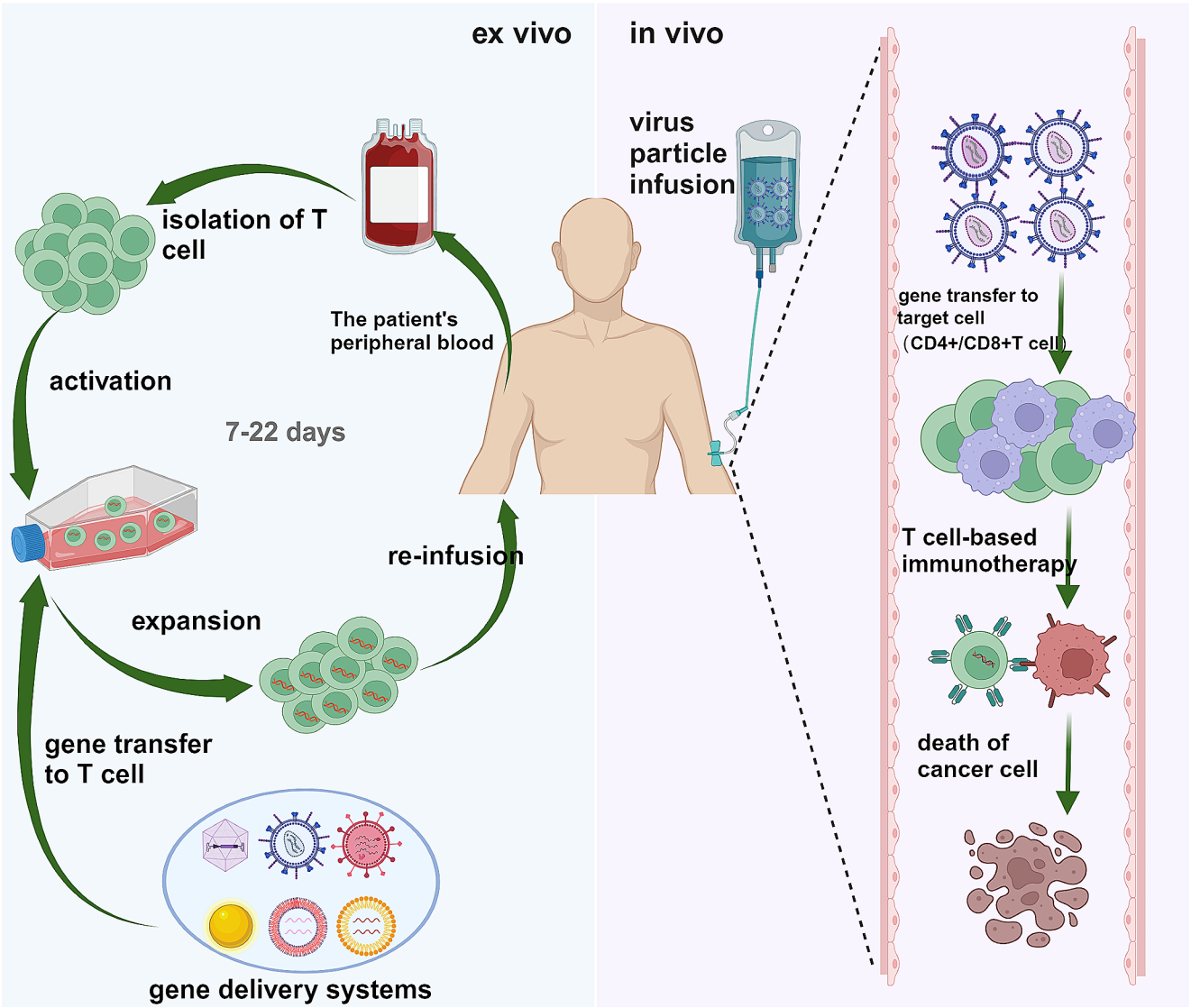

Fig. 1 Overview of T-cell gene therapy in vivo and ex vivo. Ex vivo T-cell therapy entails isolating T cells from patient's peripheral blood, activating in vitro, using a gene delivery system to genetically engineer T cells, and then infusing the T cells with therapeutic genes back into the patient. The therapeutic vector can also be delivered to patients locally or systemically, which will then transduce the target cells in vivo and rectify the condition

2.1 Fundamental structure and function of viral vector systems

In the field of engineered T cells, retroviruses represent a prevalent choice of viral vectors. These viruses constitute a varied group of enveloped RNA viruses known for their capacity to reverse transcribe their RNA genome during replication. Specifically, lentiviruses and γ-retroviruses fall under the Orthoretrovirinae subfamily of retroviruses [23]. Infectious viral particles engage specific cellular receptors through membrane-anchored glycoproteins, leading to either fusion of the viral envelope with the cell membrane or internalization of the particles via endocytosis into the cytoplasm [24]. Viral RNA is transformed into proviral double-stranded DNA through an intricate series of reverse transcription processes. The resulting proviral DNA is subsequently complexed with viral proteins to enable nuclear translocation and integration into the host genome [25]. Retroviral integration is not random across classes; instead, it exhibits characteristic preferences. For instance, lentiviruses, such as human immunodeficiency virus type I (HIV-1), tend to preferentially integrate within transcriptional units [26, 27]. Lentiviral vectors facilitate the stable integration of foreign genes, thereby enabling long-term gene expression. Their notable characteristics, such as a large viral genome capacity, ability to deliver multiple genes, and ability to transduce both dividing and nondividing cells, have led to their extensive use for transgene delivery

[28–30]. The gag, pol, and env genes are essential for retroviral and lentiviral survival and function. The gag gene encodes structural proteins, pol gene controls the expression of enzymes required for reverse transcription and integration into the host cell genome, and the env gene is responsible for the synthesis of viral envelope glycoproteins [31]. Three major modifications have been made to HIV-based lentiviral vectors to increase their effectiveness and safety. The initial generation of lentiviral vectors included a substantial portion of the HIV genome, comprising the gag and pol genes alongside several accessory viral proteins. Subsequently, safer second-generation vectors that lack the virulence factors vif, vpr, vpu, and nef associated with AIDS were engineered. The third-generation lentiviral vectors improved safety even further by dividing the viral genome into separate plasmids, with the gag and pol genes encoded on different plasmids than the rev or env gene, resulting in vectors composed of three distinct plasmids, creating a self-inactivating viral vector system [32]. The γ-retroviral vector derived from Moloney murine leukemia virus has a structure similar to that of a lentiviral vector. It is capable of encapsulating up to 8 kb of genetic material and integrating stably into the host genome, and the fundamental genes necessary for this process are the gag, pol, and env genes. The structural (gag) and enzymatic (pol) proteins are encoded by a single helper plasmid, while the envelope (env) proteins are specified by another plasmid, avoiding recombination sequence overlap and reducing the risk of forming a replication-competent retrovirus. Moreover, this design mitigates immune responses that can be triggered by residual retroviral proteins [33, 34]. Retroviral vectors can be engineered to achieve transient (mRNA or protein delivery), semipermanent (episomal DNA maintenance in nondividing cells over extended periods until cell division), or permanent (stable genomic integration with heritable DNA) cellular modification, aligning with specific therapeutic requirements [35].

Adeno-associated viruses belong to the dependent parvovirus genus of the Parvoviridae family and consist of an icosahedral protein capsid ~26 nm in diameter and a ~4.7 kb single-stranded DNA genome [36]. The AAV capsid consists of three types of subunit proteins, VP1, VP2, and VP3. Current AAV vectors retain the same sequence and structure as the wild-type AAV (WT AAV) capsid, yet they have been engineered to remove most of the protein-coding sequences. This modification preserves T-shaped inverted-terminal repeat (ITR) sequences, which are necessary for direct genome replication and packaging during vector production, facilitating the delivery of genomes up to approximately 5 kb in size [37]. The molecular interactions between the shell of the AAV and the target cell surface receptor, as well as the subsequent processes following particle internalization, are crucial for ensuring the efficacy of AAV-mediated gene delivery. Notably, different serotypes of AAV recognize distinct receptors, which results in their specific tissue or cellular tropism [38]. To date, at least 12 natural serotypes and more than 100 AAV variants have been used in gene delivery research, and AAV mutants have been continuously generated from these vectors to optimize and expand the use of AAV for gene delivery [39, 40].

Lentiviral vectors, a type of retrovirus, are particularly effective for the in vitro genetic engineering of T cells. This involves the incorporation of synthetic chimeric antigen receptors (CARs) or other synthetic receptors that enable T cells to recognize tumor-associated antigens (TAAs) in an MHC-independent manner. Alternatively, T cells can be transduced with T-cell receptor (TCR) sequences that specifically target tumor antigens, thereby enabling them to recognize and eliminate tumor cells [41, 42]. CAR-T cell therapy using lentiviral/retroviral vector engineering has demonstrated noteworthy clinical success in patients with B-cell malignancies, with regulatory approval of the first genetically engineered cell therapy using a lentiviral vector [43]. As of June 2022, the FDA has approved six CAR-T cell products for patients with relapsed and/or refractory B-cell malignancies [44]. Four of the products (KYMRIAH, BREYANZI, ABECMA, and Carvykti) were generated using lentiviral vectors, while the last (TECARTUS and YESCARTA) products were generated using retroviral vectors. Globally, lentiviral vectors are being tested in more than 100 ongoing clinical trials for both in vitro cell modification and in vivo therapy [25, 45]. The procedure typically involves extracting peripheral blood mononuclear cells (PBMCs) from the patient, followed by in vitro processes such as T-cell isolation, activation, transduction, and amplification, before the cells are reintroduced into the patient. This pipeline has several drawbacks in that it adds complexity to the overall process and involves additional steps before cell transduction, which increases the duration and cost of the manufacturing process. The stimulation used for activation in conjunction with prolonged ex vivo culture may alter cells, which may harm the quality of the final products; for example, naive cells can differentiate into a less preferential phenotype, exhibiting a greater degree of exhaustion, lower proliferative capacity, shorter in vivo persistence, and less functionality. Thus, minimizing the manipulation of lymphocytes during genetic modification is of enormous clinical relevance [46]. A potential solution involves engineering a viral vector to deliver T-cell specific genes directly in vivo. This approach can minimize the need for extensive ex vivo manipulation, reduce the associated processing time, and help maintain the integrity and quality of T cells.

2.2 Rational design engineering for viral vector-based targeted gene delivery systems

Viral targeting can be achieved through two primary strategies: leveraging the tissue tropism of various viral vector envelope proteins or by incorporating ligand/antibody sequences into the viral envelope glycoprotein that can specifically recognize molecules on the target cell surface, a technique referred to as receptor targeting engineering (Fig. 2) [47]. Different retroviral envelope proteins are responsible for recognition and attachment to specific cell populations during virus infection. Pseudotyping involves the strategic incorporation of heterologous envelope glycoproteins into the surface of original viral vectors to modify their tropism as required [48, 49]. HIV-based lentiviruses are frequently pseudotyped with the envelope protein of another virus, which provides different characteristics for the vector and influences its orientation and intended cellular target; genetic engineering of T cells that use viral vectors has been designed based on this concept [45].

The natural receptors for most viral vectors are either not present on T cells or are ubiquitously expressed across many cell types, which precludes targeted gene expression specifically in T cells. In this case, a ligand or an antibody sequence is incorporated into the viral envelope glycoprotein to serve as a virus entry receptor that specifically recognizes molecules expressed on the surface of the target cell [46, 50]. Usually, this process involves two steps: either destroying the natural receptor of protein or adding a binder for target recognition. Commonly utilized ligands include single-chain variable fragments (scFv) and designed ankyrin repeat proteins (DARPins) (Fig. 2). DARPins are

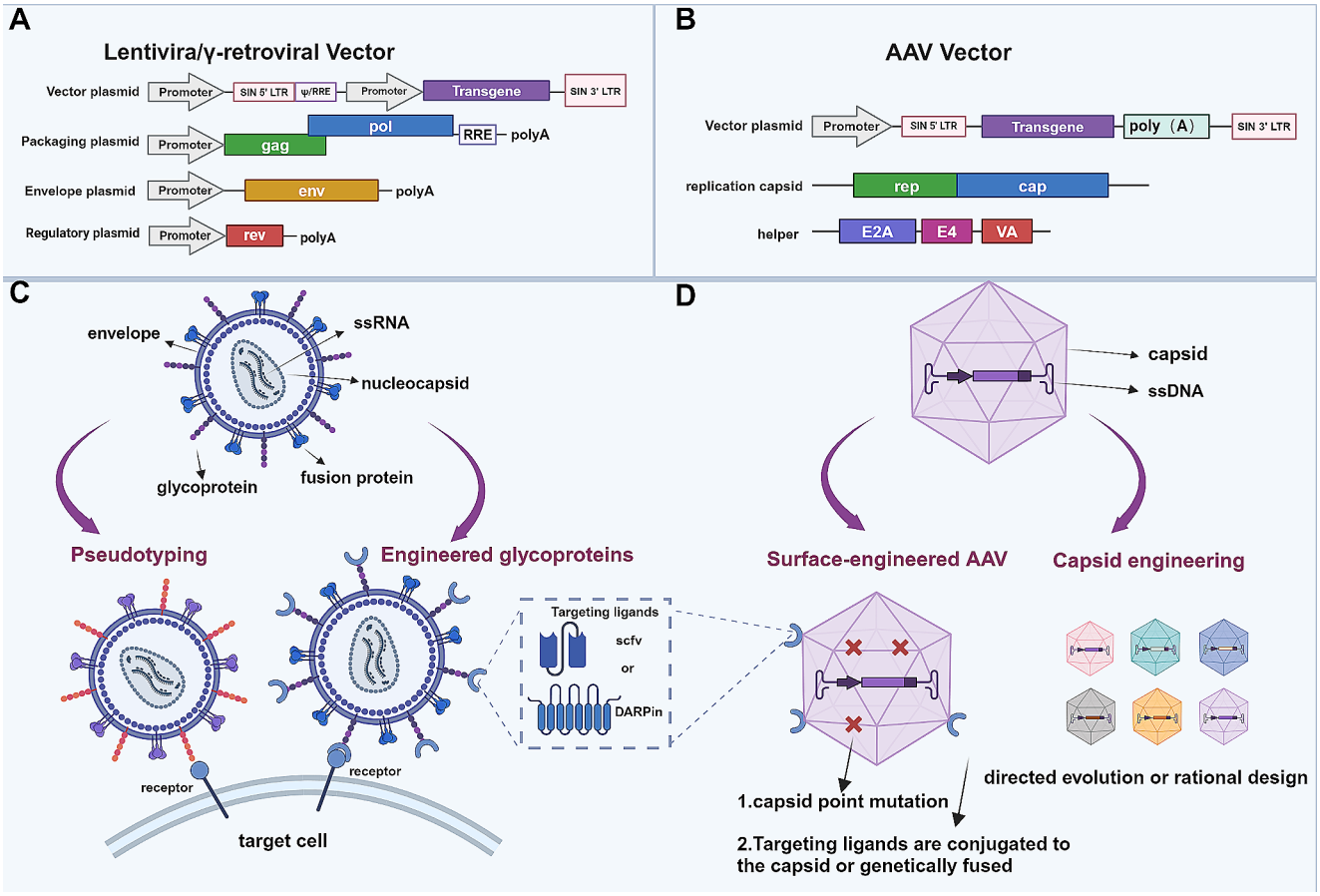

Fig. 2 Rational design engineering for viral vector-based T-cell-targeted gene delivery systems. (**A**, **C**) T-cell-targeted gene delivery engineering of lentiviral vector/retroviral vector. LVs are enveloped particles containing one or more viral glycoproteins and two copies of a ssRNA genome packaged in a nucleocapsid. Targeted delivery of therapeutic genes can be achieved through two strategies: pseudotyping and the utilization of engineered glycoproteins. Retroviral envelope proteins are responsible for the recognition and adhesion of different cell populations, and pseudotyping is the process of incorporating heterologous envelope glycoproteins into the surface of original vectors as needed. An engineered glycoprotein refers to the insertion of a targeting ligand (scFv or DARPin) into the virus envelope glycoprotein so that the ligand may recognize the molecule expressed on the target cell surface as the virus entrance receptor. (**B**, **D**) AAV is composed of a ssDNA genome packaged into an icosahedral protein capsid. AAV vector-specific gene delivery to T cells is mostly based on AAV surface engineering, which includes mutating the capsid to inactivate the native receptor and subsequently adding a target-targeting ligand. Capsid engineering is a suitable design for projected progression

based on naturally occurring ankyrin repeat proteins, a ubiquitously expressed family of proteins that mediate specific protein-protein interactions [51]. These compounds can be tailored to serve as alternatives to scFv through methods such as ribosome display or phage display screening [52, 53]. While DARPins exhibit high affinity comparable to that of scFv, their absence of cysteine residues reduces the likelihood of aggregation, thereby enhancing binding stability and reducing the risk of nonspecific interactions [54].

Lentiviral vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) are widely utilized in T-cell genetic engineering [55, 56]. Low-density lipoprotein receptor (LDLR) and its family members can serve as the primary cellular receptors for VSV-G and are expressed on the surface of the majority of cells [57, 58]. As a result, VSV-G pseudotyped LVs exhibit a broad tropism and can achieve high transduction efficiency in various human cell types, including activated T lymphocytes [58]. The ability of VSV-G LVs to transduce many types of nondividing or slowly proliferating cells provides a rich resource for applications in experimental platforms and is also very attractive for clinical applications. The major drawback of the VSV-G pseudotyped lentivirus is that its genetic manipulation of T cells is limited almost entirely to ex vivo activities, and gene delivery to T lymphocyte subtypes cannot be accomplished by modifying membrane proteins, which are dependent on receptor binding and membrane fusion. Thus, although target cell binding to receptors is accomplished, membrane fusion between cells and viruses is disabled and difficult to achieve [59]. Lentiviral vectors derived from simian immunodeficiency virus (SIV) can be pseudotyped with different envelope proteins to achieve gene transduction in various cells, including T cells. The transduction efficiencies of lentiviruses pseudotyped with different envelope proteins varies, possibly due to differences in receptor expression [60]. Viruses within the Alphaparamyxoviridae family, including Nipah and Measles, possess distinct glycoproteins responsible for recognition and fusion functions. These glycoproteins can be rationally engineered to specifically target and deliver genes to subsets of T lymphocytes (Table 1). Moloney murine leukemia virus-based gamma-retroviral vectors are commonly used as gene delivery vectors for an increasing number of disease-targeting genes, where specific promoter/enhancer elements may lead to increased transgene expression in specific tissues or cells [74], including the use of a human T-cell-specific CD2 enhancer to increase gene expression in T cells [75]. Like lentiviral vectors, γ-retroviral vectors are most commonly used to deliver genes to specific cell types or tissues by receptor targeting engineering: (1) pseudotyping with other enveloped virus glycoproteins [76]; (2) insertion of a retroviral receptor-binding envelope subunit with a ligand, peptide or scFv [77]; and (3) bridging of viral vectors and cells with antibodies or ligands [78, 79].

Among the viral vectors without envelope proteins, the AAV vector has become the most widely used vector for gene delivery in vivo due to its high transduction efficiency, stable transduction, and nontoxicity [80]. However, due to the extensive transduction of cells, it is possible to transfer genes to nontarget cells. Hence, in vivo delivery of these genes via an AAV vector has not reached its full potential. Strategies to address this drawback include cell-surface targeting, transcriptional targeting, and posttranscriptional targeting modifications of AAVs or AAV surface engineering and capsid engineering (Fig. 2). AAV infection of target cells depends on endocytosis triggered by the primary receptor and coreceptor on the cell surface. After entering the endosome, the capsid structure changes, the AAV virion enters the nucleus, its single-stranded genome is released, and transcription and translation begin [81]. Cell surface targeting involves either mutating the AAV so that it no longer recognizes the native receptor or inserting a short peptide (ligand) of the desired cell surface receptor with binding affinity into the capsid protein, which can be stably inherited, or coupling to the viral capsid, which is not heritable. Cell transduction is then mediated by novel ligand-receptor interactions, similar to retroviral vectors targeting envelope proteins [82, 83]. The goal of AAV capsid engineering is to improve viral tissue tropism via directed evolution or rational design. To achieve tissue preference, chimeric viral

Table 1 Overview of the pseudotyped virus and the targeted cell type

Pseudotypes	Envelope protein	Original virus	Natural receptors	Targeting ligand	Targeted cell type
VSV-LV	VSV-G	Vesicular stomatitis virus	LDL-R [58]	–	Activated T lymphocytes [58]
NIV-LV	G, F	Nipah virus	EphinB2/B3 [61]	Scfv/DARPin	CD3 T lymphocytes [62] CD8 T lymphocytes [63]
MV-LV	H, F	Measles virus	SLAM [64] CD46 [65]	Scfv/DARPin	CD4 T lymphocytes [66] CD8 T lymphocytes [67]
SIV-LV	SIVNE1,2	Sindbis virus	NRAMP [68]	OKT3 Anti CD4 MAb	CD3 T lymphocytes [69] CD4 T lymphocytes [70]
BaEV-LV	BaEV	Baboon endogenous retrovirus	ASCT-1 ASCT-2 [71]	–	naive T cells and T-cell progenitors [72]
RD114-SIV	RD114	Feline endogenous retrovirus	ASCT-2 [71, 73]	–	primary blood lymphocytes [60]

particles composed of a mixture of capsids from different serotypes can be used. Tissue-specific promoter or enhancer sequences can also be used during transcription, and after transcription, the physiologically expressed microRNA target sequences can be integrated into the 3'-UTR of the AAV vector cassette to inhibit the expression of the transgene, which does not require tissue [84, 85]. This strategy can limit the production of the transgene product to the desired target cells while not affecting the biodistribution of the vector [86]. Posttranscriptional targeting strategies can be used alone or in combination with cell type-specific promoter and cell surface targeting strategies to avoid the problem of promoter leakage [87]. For T-cell-targeted gene delivery, a cell surface targeting strategy is mostly used. Although AAV is generally considered to be very inefficient at transducing T cells, the use of specific molecules, such as bispecific antibodies [88] or avidin-conjugated ligands, to react with the virus surface and cellular receptors can lead to the targeting of modified AAV to lymphocytes [89, 90].

2.3 Current status of virus-targeted gene delivery specific to T cells

CD3 is the most obvious cell surface marker of targeted T lymphocytes. It is exclusively expressed on T lymphocytes as part of the TCR-CD3 complex [91]. Pseudotyping with envelope proteins from different viruses allowed lentiviral vectors to deliver genes only to T cells in vivo. The first attempt was to use Sindbis virus from the Alphaviridae family to target CD3-expressing T cells via a lentiviral vector containing an anti-CD3 antibody (OKT3) and an engineered shuttle vector containing Sindbis virus glycoprotein [69]. This recombinant vector could specifically transduce gene to human primary CD3-positive T cells and it could also preferential delivery reporter genes to CD3-expressing cell lines in an in vivo xenograft mouse model [69]. The Nipah virus is a member of the Paramyxoviridae family and two key proteins in its cellular entry are receptor attachment protein (G) and fusion protein (F). The former is responsible for the recognition of receptors on the surface of the cell membrane, and the latter mediates the fusion of viruses and the cell membrane when receptors contact each other [92]. A study showed that T-cell activation and targeted gene delivery could be achieved by using a lentiviral vector pseudotyped with the Nipah viral envelope protein while displaying scFvs that specifically bind CD3 on the envelope protein [62]. These pseudotyped lentiviruses were able to activate T cells during transduction and mediate efficient gene delivery to nonactivated T lymphocytes in vitro, even in human whole blood, without any additional external stimulation. In addition, viral particles can directly generate functional CAR-T cells in humanized mice and achieve gene delivery to T cells in vivo.

The selective and specific delivery of transgenes into specific types of lymphocytes is extremely desirable for immunotherapy and gene therapy. CD4+ T cells play important roles in both innate and adaptive immunity and are critical for gene modification in basic research and immunotherapy. In two patients with chronic leukemia who were treated with CAR-T cell therapy, CAR-T cells were still detectable and achieved sustained remission more than a decade later; both of these patients had highly activated CD4+ CAR-T cells, suggesting a role of CD4+ CAR-T cells in long-term remission of leukemia [93]. The Sindbis pseudotyped lentivirus was used for specific transduction via antibody conjugation and binding to a specific antigen on the surface of target cells. It has been shown that pretreatment of Sindbis pseudotyped HIV vectors with anti-CD4 antibodies can preferentially infect CD4+ T cells [69, 70]. The replacement of the VSV-G envelope protein with the hemagglutinin protein and the fusion protein of the Measles virus has also been achieved in PBMCs to deliver the gene only to CD4+ T cells. This resistance is achieved by the presence of mutations that prevent the virus from recognizing its natural receptor and the addition of scFv sequences that specifically recognize CD4 or DARPIN sequences that bind CD4 with high specificity [94]. In mice systemically originally administered human PBMC-NSG or CD34-NSG (hematopoietic stem cell recombination), reporter gene expression was detected mainly in lymphatic organs. Flow cytometry analysis of reporter genes in lymphoid organs revealed that pseudotyped lentiviral vectors delivered the genes to CD4+ T cells, indicating that pseudotyped lentiviral vectors could serve as a viable tool for the sustained in vivo reprogramming of CD4+ T cell [66].The research team published a study later in 2020 demonstrating that the pseudotyped lentivirus could specifically and efficiently target gene delivery to CD4+ T cells in huPBMCs and in CD34 hematopoietic stem cell of recombinant immunodeficient mice. The results highlighted enhanced antitumor effects in vivo and circumvented the biases introduced by in vitro culture conditions, indicating promising clinical application potential for the pseudotyped lentivirus [95].

CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), are among the most crucial immune cells capable of directly recognizing and eliminating tumor cells. In preclinical and clinical studies, CD8+ cytotoxic T lymphocytes (CTLs) have been demonstrated to have potent antitumor activity [96, 97]. The targeted delivery of genes to CD8+ T cells has yielded promising results using the Measles virus and Nipah virus. The selectivity of CD8-LV for CD8+ T cells relies on scFv antibody fragment derived from the monoclonal antibody OKT8 and displayed on the H protein

of the Measles virus. The lentivirus pseudotyped by Measles virus envelope glycoprotein was highly selective for CD8+ cytotoxic T cells present in human PBMCs [67]. The in vivo targeting potential of the virus was investigated in NSG mice transplanted with human PBMCs. The results revealed that the therapeutic transgene could be delivered to CD8+ T cells with high efficiency and specificity, thus effectively killing tumor cells [67, 98]. In a recent study, the Nipah virus glycoprotein was exchanged with the Measles virus glycoprotein for gene delivery to CD8+ T cells via CD8-specific DARpin displayed on the virus surface as a receptor for CD8-specific recognition. The virus was found to have a titer of more than 10^8 TU /ml without the effect of neutralizing antibodies in the population, which will be an important step in the clinical translation of this promising vector type [63]. A study also demonstrated that the Nipah pseudotyped CD8-targeting lentiviral vector could directly and selectively deliver CD19-CAR to human cytotoxic CD8+ T cells in PBMC-NSG and CD34-NSG mouse models. In both models, large numbers of CD8 CAR-T cells were present in the spleen and blood, whereas CD8 negative cells remained devoid of the CAR gene [99]. Cytokine release syndrome (CRS), characterized by weight loss; wrinkling fur, apathy, ataxia, and circular movement; and lymphocyte infiltration in the spleen, liver, and brain, was observed in this animal model, similar to what has been observed in some patients treated with CAR-T cells [100]. These findings demonstrated the possibility of therapeutic CD8 CAR-T cell generation in vivo. In subsequent research, the team provided the first evidence for anti-tumoral activity mediated by in vivo generated human CAR-T cells [101]. A single injection of Nipah virus-modified CD8-targeting lentiviral vector was sufficient to generate CD19 CAR-T cells in mice and eliminate tumor cells in the bone marrow and spleen; unexpectedly, CAR-positive NK and NKT cells were detected in the injected mice.

For the pseudotyped lentiviral vectors employing in directly targeted gene delivery to T cells, the translation into a clinical setting appears to be straightforward, since they transduce human T cells without requiring a strong T cell activation signal and have been derived from LV vectors for which profound clinical experience is available [102]. Nevertheless, further studies in primates may be needed before real human clinical trials can commence. Moreover, as an immunogen, the immune response should be taken into account in clinical applications. Direct in vivo injection of targeted lentiviral vectors to generate specific therapeutic T cells also requires dose-dependent and long-term antitumor efficacy consideration, which should be addressed in subsequent in vivo animal studies. The presence of neutralizing antibodies in humans should also be considered when pseudotyping with different virus-type envelope proteins, such as Measles virus glycoprotein.

Retroviral vectors are the longest studied and the first viral vectors used in human gene therapy. These vectors play a pivotal role in engineering T cells. A series of clinical trials investigating primary immune deficiency have demonstrated the benefits of retroviral-based gene therapy [103–105]. Although serious adverse events caused byγ-retroviruses insertion mutagenesis have been reported in subsequent studies [106–108], the study of retrovirus gene delivery to T cells is still meaningful. This section provides a brief overview of the use of γ-retroviruses for delivering genes to T cells, as well as the significant benefits and notable drawbacks associated with retroviral vectors.

In 1990, the first report emerged on the use of gamma retroviruses to genetically modify T cells for human immunotherapy, specifically for the treatment of advanced melanoma using retrovirus-modified tumor-infiltrating lymphocytes (TILs). The viability and safety of retroviral gene transfer for human gene therapy were demonstrated for the first time in this pioneering work [109]. Patients with adenosine deaminase severe combined immunodeficiency (ADA-SCID) who have been treated with T lymphocytes transduced with a γ-retrovirus expressing the ADA gene to reestablish the immune system have sustained integrated vector and ADA gene expression in T cells after gene therapy [110]. Since then, retroviral vectors have been widely used in gene therapy, especially for treating T-lymphoid malignancies. In the case of adoptive T-cell therapy, two CAR-T cell therapeutic products (Yescarta, Tecartus) using γ-retroviral vector have been approved by the FDA for relapsed or refractory large B-cell lymphoma and mantle cell lymphoma.

However, utilizing retroviral vectors in T-cell gene engineering carries inherent risks, including the potential for insertional mutagenesis and the reactivation of dormant endogenous viruses. A notable example of these risks is seen in the widely-discussed application of retroviral vectors in CAR-T cell therapy. Recent FDA warnings have highlighted the potential risk of T-cell malignancies in patients treated with CAR-T therapy. Although a direct causality between CAR-T cell therapies and malignant transformation has not been definitively established after more than a decade of clinical application, there have been cases where the CAR gene was inadvertently integrated into leukemic B-cell during the manufacturing process, resulting in treatment resistance [111]. An analysis conducted at the University of Pennsylvania involving 449 treated patients revealed that over a median follow-up period of 10.3 months, 16 patients (3.6%) had developed secondary primary malignancies (SPMs). However, CAR-T cell products have not yet been shown to play a direct role in inducing malignant

transformation [112]. Consistent with the assessment of FDA, the benefits of CAR-T cell therapies are currently considered to outweigh the potential risks. However, it is crucial to implement long-term patient monitoring following CAR-T cell therapy and to apply heightened scrutiny in the design of CAR constructs and the selection of infusion protocols. Additionally, reports indicate that reactivation of human herpesvirus 6 (HHV-6) can occur in patients receiving CAR-T cell therapy. This reactivation is not attributed to the CAR construct itself but is associated with factors such as T-cell activation, proliferation, and culture duration. Moreover, an increase in the incidence of lytic-active HHV-6 has been observed over time [113]. Overall, the advantages of CAR-T cell products are considered to outweigh the potential risks. It is essential to conduct long-term monitoring of patients after CAR-T cell therapy and to exercise increased vigilance in the design of CAR configurations and strategies for infusion treatments. These observations further highlight the significance of targeted direct in vivo CAR gene delivery systems. However, achieving precise targeted delivery remains a paramount challenge.

Adeno-associated viruses have become increasingly popular as viral vectors for the in vivo delivery of gene editing agents. The majority of current research focuses on employing AAVs to treat monogenic diseases such as Zolgens syndrome [114], hemophilia B [115], and Lebe's congenital amaurosis type 2 [116–118]. Moreover, promising studies are exploring the precise delivery of genes to T cells using AAVs. Christian J. et al has developed a modular approach for efficient targeted gene delivery wherein DARPins-specifically designed to recognize CD4 as the targeting ligand—were genetically fused to the AAV capsid protein VP2 [119]. Two arginine residues within the 60 capsid monomers were mutated to abrogate binding to the native receptor [120]. Specific gene delivery to CD4+ lymphocytes using AAV has been demonstrated in a mouse model after systemic administration, enabling genetic modification of these cells both in vitro and in vivo without detectable off-target effects [119]. The team also inserted the nanoantibody into the GH2/3 loop of the AAV capsid VP2 to demonstrate that AAV-targeted gene delivery to murine CD8+ T lymphocytes was greater than 99%, indicating that the blockade of mouse spleen cell transduction could be overcome by receptor targeting [121]. Nawaz, W. et al. described a novel method for generating CAR-T cells in vivo, wherein the injection of an AAV vector harboring a CAR gene yielded CAR-T cell suspensions adequate for inducing tumor regression in a murine leukemia model [122]. Using an AAV vector and a Sleeping Beauty (SB) transposon, Chen, Sidi, et al. developed an AAV-Sleeping Beauty hybrid vector system screening for membrane protein targets in CD8+ T cells in a mouse model [123]. A CRISPR library was integrated into the Sleeping Beauty transposon, which is positioned between the inverted terminal repeats of AAVs, facilitating the successful identification of membrane protein targets amenable to direct T cell editing and the enhancement of T cell function [123]. Systemic delivery of the exosome-associated AAV8 (exo-AAV8) vector in mice enables transgene expression across diverse immune cell lineages [124]. The use of AAV as a vector for RNA-based nucleases achieves efficient site-specific gene knock-in in T cells, resulting in targeted recombination in primary human T cells [125].

Given the current in vivo gene therapy methods employing adeno-associated viruses [126], further research, which should focus on evaluating the specificity, biocompatibility, and in vivo safety of the engineered viruses, is essential to refine the targeted gene delivery of adenoviruses to T cells. In addition, the large number of preexisting neutralizing antibodies against AAV due to natural infections in childhood and the generation of neutralizing antibodies induced by AAV vector-mediated gene transfer in the human population will affect the use of these types of viral vectors for additional therapy [127]. A range of approaches to this situation are being tested, such as immunosuppression, hybridization of the AAV capsid, mutation [128], rational design and directed evolution [129], or early administration of the IgG-cleavage proteases IdeS and IdeZ in AAV recipients [130, 131]. The next step in the targeted gene delivery of T cells by adenovirus vectors can be combined with site-specific DNA endonucleases to customize the AAV.

3 Nonviral vector systems

Despite the proliferation of investigations into the application of viral vectors for gene therapy, concerns regarding the immunogenic reactions elicited by these vectors and the risks associated with unintended transgene integration have spurred a quest for alternative methodologies within the scientific community. The appeal of nonviral vector systems lies in their reduced immunogenic and cytotoxic profiles, coupled with the simplicity of their design and manufacturing processes (Table 2). Therapeutic genes can be delivered specifically to T cells using transposons, nanoparticle vectors, or CRISPR systems.

3.1 Transposons in T-cell-targeted gene delivery

As nonviral vectors, transposons or mobile genetic elements can also be used to achieve durable and efficient transgene expression. Transposons can be broadly divided into two classes: class I elements or retrotransposons that migrate through RNA intermediates via a "copy and paste" mechanism and class II or DNA transposons that migrate via a

Table 2 Comparison of viral versus nonviral delivery systems for targeted gene transfer to T cells

	viral vector system			nonviral vector system		
	Lentiviral vector	gama-retroviral vector	AAV	nanoparticle	transposon	CRISPR/Cas9 system
Maintenance	Integration	Integration	Non-integration	Non-integration	Integration	Non-integration
Cargo size	<8 kb	<8 kb	<5 kb	As needed	increased cargo size decreased efficiency	As needed
Genotoxicity	Insertional mutagenesis possible	Insertional mutagenesis possible	Insertional mutagenesis possible	inflammatory response	Insertional mutagenesis possible	Off-target toxicity
Immune response	+	+	+	+	+/−	+
Preclinical evidence	Mouse model	Primary T lymphocytes	Mouse model	Mouse model	Mouse model	Mouse model

DNA-mediated "cut and paste" mechanism [132]. Class II transposons are genes encoding transposase proteins in the middle flanked by inverted terminal repeats (ITRs) containing transposase binding sites. Typically, a transposon consists of two functional components: a transposase and a gene. Together with the inverted terminal repeats (ITRs) that flank the gene, they form a two-component vector system. During transposition, the transposase mediates the excision of the element from its donor plasmid, followed by reintegration of the transposon into the chromosomal locus to deliver the gene specific to a specific type of cell. Translocation-based gene delivery systems have the following advantages: (1) the transposition mechanism enables the stable integration of a gene into the host genome, ensuring long-term expression without the risk of gene loss and resulting in efficient transgene expression as demonstrated in preclinical models [133]; (2) the production of plasmid DNA is straightforward and cost-effective; and (3) it provides a higher transgenic capacity and superior safety profile compared to viral vectors [133, 134]. The types of transposable elements that are commonly used for targeted gene delivery in T cells are Sleeping Beauty transposons and PiggyBac (PB) transposons, and their applications in T-cell gene delivery are described in this section.

3.1.1 PiggyBac transposon system

PB, which was originally isolated from the cabbage trocar moth Ni [135] and is known for its precise excision ability, has undergone a series of optimizations over the years [136]. PiggyBac elements comprise two main components: the piggyBac transposases, responsible for facilitating gene integration, and 5' and 3' inverted repeat sequences that encompass the transgene. Transposase and transposons are introduced into the genome, resulting in efficient "cut and paste" transposition of the transgene into the genome at the TTAA nucleotide element [137, 138]. A series of optimizations of transposases, such as the unique hyperactive piggyBac transposase obtained by mutation, excision, and integration of a PBase optimized with WT mammalian codons (mPBase), which is more than 10-fold greater, have been performed to improve transposition efficiency [139]. The excision ability/integration-deficient (Exc (+) Int (−)) transposases have also been generated by mutagenesis for transient gene transfer in mammalian cells, such as the transient introduction of transcription factors for induced pluripotent stem cell production [140]. A prominent feature of the piggyBac transposon system is that there is no DNA synthesis involved in PB transposition, and the target gap caused by transposition is closed only by ligation (the 3'OH end of the transposon directly attacks the interlacing position of the 5' end of the TTAA target sequence), making PB transposition precise and unambiguous [136, 141]. Furthermore, PB transposons exhibit the most efficient transposition in mammalian cells, have a larger payload (up to 14 kb) than viral vectors, and allow multiple transgene deliveries via the design of polycistronic cassettes [142]. The PB transposon system has been widely used in mammalian transgenics, mutagenesis, in vitro modification of clinically relevant cell types, and in vivo mammalian gene transfer [143].

The PB transposon system has been successfully applied in the gene transfer of T cells; the expression level of the reporter gene is approximately 40%, and the system can also stably express multiple transposable genes in T cells for a long time. Moreover, integration site profiling in human T cells has shown that the piggyBac transposon does not exhibit a preference for integrating near known proto-oncogenes; instead, it demonstrates site specificity [144]. PiggyBac can also deliver a large 14 kb reporter gene to T cells without affecting cell viability. It can deliver the caspase 9 (iCasp9) gene and successfully express it in T cells [145]. The PB transposon system is recognized for its potential to revolutionize gene therapy by enabling the development of CAR-T cell therapies that precisely target specific tumor antigens. When T cells were genetically modified with PB to generate hGMR-CAR-T cells, no off-target effects or organ toxicities were found during the adoptive transfer of autologous hGMR-CAR-T cells into cynomolgus monkeys

[146]. Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) can be modified with PB to express HER2-CAR. Truncated CD19 expressed as a second transgene can be used to select transgenic CTLs, and HER2-CAR-modified EBV-CTLs (HER2-CTLs) can eliminate HER2-expressing tumor cells both in vitro and in a NOD-SCID xenograft model [147]. Several phase I clinical trials in which specific CAR genes, such as EGFR, CD19, and BCMA, are targeted for therapy via the PB transposon system have preliminarily demonstrated the safety and efficacy of CAR-T cells generated via the piggyBac transposon genetic engineering system [148–150]. However, utilizing the PB transposon system as a vector for gene delivery in CAR-T cell therapy presents potential risks. A clinical trial involving the treatment of ten patients with relapsed and refractory CD19+ B-cell malignancies using CAR-T cells engineered with the piggyBac system resulted in the development of lymphomas from the CAR-T cells in two cases, with one patient dying due to disease progression [151]. The malignant transformation of the modified T cells is suspected to be due to multiple genetic alterations, with the manufacturing process of the CAR-T cells possibly playing a significant role in this adverse outcome. Although the PB transposon system offers cost-effectiveness and a greater transgene capacity than viral vectors in the field of gene engineering, the associated risks highlighted by recent findings mandate a cautious approach in its therapeutic applications [151, 152].

3.1.2 Sleeping Beauty transposon system

The Sleeping Beauty gene is a transposase gene of the Salmoninae subfamily of fish element reconstructed from phylogenetic data and can be used for genetic transformation and insertional mutagenesis. Named after the famous fairy tale, the Sleeping Beauty transposon was the first transposon to be able to be transposed efficiently into vertebrate cells [153]. Because transposable elements coexist within the host, the transposition activity needs to be modulated to avoid insertional inactivation of essential genes, and the two components of the SB system need to be optimized to achieve the most efficient molecular tools [154]. Transposases like SB10 and SB11 have been subjected to a series of optimizations to enhance transposition efficiency. The most recent breakthrough is the creation of SB100X, a hyperactive transposase identified through extensive genetic screening. SB100X surpasses the efficacy of nonviral vectors, facilitating gene transfer to primary cell types with efficiency matching that of integrated viral systems [155, 156]. Furthermore, a hyperactive variant, hySB100X, was generated by mutating SB100X, resulting in increased transposition activity [157, 158]. The transposon DNA sequence has also been optimized to express transgenes with highly complex structures (e.g. repetitive motifs, multiple genes, various regulatory sequences) [159, 160]. The mutation, addition, or deletion of nucleotide residues in the ITR sequence has produced an improved version based on the original transposon vector (pT), such as pT2, pT3, pT2B, or pT4 [161]. The use of Sleeping Beauty transposon-based gene delivery offers outstanding potential for innovative and potentially curative treatments for a range of genetic diseases [154, 162, 163]. Major examples of gene therapy for various diseases include cancer [164–167], immunologic diseases [168], pulmonary diseases [169], neurological diseases [170], and muscular diseases [171].

The implementation of Sleeping Beauty transposon-targeted gene delivery in T cells needs to solve two problems. First, the targeted insertion of transposons is required because the insertion site of SB transposons is non-specific and the delivery strategy is inefficient for primary T cells. Second, direct integration of transposons into the nucleus as DNA plasmids is difficult and requires the use of vectors such as AAVs or nanoparticles. In theory, these hybrid vectors can be used as alternatives to established viral/nonviral vectors, as described above, for T-cell type specific genetic engineering [172]. At least one component of the transposon system, transposon vector DNA, or transposase must bind to a defined site in the human genome to achieve targeted transposon insertion. Proof-of-principle studies have shown that by combining a site-specific DNA-binding domain (DBD) with SB transposase, transposons can be integrated into predetermined genomic sites [134, 173] or via site-specific integration of adeno-associated viruses [174] and that mutagenesis of specific amino acids in the SB transposase alters target site selection in human cells [175].

At present, the most widely used method is to use SB transposons to modify T cells in vitro and introduce a second-generation CAR targeting CD19 by electroporation to genetically modify T cells to redirect T cells to tumors expressing CD19 [172, 176]. Several clinical trials for B-cell lymphoma have also evaluated the safety and effectiveness of this method [177–179], and the first virus-free CAR-T cell clinical trials for multiple myeloma are underway [180]. With the advantages of being cost-effective and easy to perform, the Sleeping Beauty transposition system can be used to engineer TCR genes from the T cells of cancer patients. Transposable T cells can specifically produce multifunctional responses to homologous mutant neoantigens and tumor cell lines. The transposition of mutation-specific TCRs by Sleeping Beauty can promote the use of personalized T-cell therapy for unique neoantigens [164]. The Sleeping Beauty transposon system combined with methotrexate selection allows for the efficient transfer of multiple genes to T cells [181]. CD19-specific CARs can

be directly expressed in memory and effector T cells by introducing a DNA plasmid with SB. When cocultured with CD19+ artificial antigen-presenting cells (aAPCs), massive expansion of CAR+ T cells can be achieved [182]. With the use of the mini-circle (MC) vector as a vector for SB transposon components, MC is the minimal expression cassette; without a bacterial origin of replication or antibiotic resistance genes, the MC achieves more efficient transgene expression in T cells, and the survival rate of T cells after electroporation is also greater. The antitumor effect of these cells is comparable to that of lentivirus-transduced CAR-T cells, suggesting that these cells have superior clinical application potential [183]. SB transposon systems can be delivered to target cells by loading them into targeted ligand-modified nanoparticle lipid prototype cells (a closed neutral lipid bilayer), with folate (FA) serving as the cancer cell-targeting motif and dexamethasone (DEX) serving as the nuclear localization signaling molecule. In vivo studies have shown that the transfection efficiency of FA-modified native cells in tumor tissues is much greater than that in other tissues, indicating that the SB transposon system can be delivered to T cells in vivo by targeted modification of delivery vectors [184].

T-cell genetic engineering using transposons as delivery vectors, such as CAR-T cells, CTLs, and TILs, has been shown to have antitumor effects, lower production costs and greater genetic capacity than the use of viral vectors, demonstrating the potential of transposons for application in gene delivery to T cells. However, targeting immune cells in vivo remains a nascent endeavor, particularly for the delivery of small molecules and the intrinsic issues of transposons. In the practical application process, intrinsic issues related to transposons, such as their safety for clinical gene transfer, potential genotoxicity, self-resistance to the plasmids they are carried on, the influence of enhancer or promoter elements on transgene transcription, the number of copies integrated, the pattern of vector insertion sites, and the precision of insertion site targeting, must be considered. One approach to risk assessment of vectors used for gene delivery is to approximate the maximal tolerated cancer dose before cancer is initiated (or accelerated) in model animals [133]. These potential risks must be carefully evaluated by investigators and regulators alike, against the potential benefit that delivery of a therapeutic transgene can provide.

3.2 Nanoparticles

NPs are granular dispersions or solid particles with sizes in the 1–100 nm range that have been well-studied for cancer therapy in recent decades, and a large number of NPs are currently being used for therapeutic delivery, diagnostic, and imaging applications [185–187]. Based on their chemical composition, nanoparticle systems can be divided into three broad categories: polymer-based, lipid-based, and inorganic nanoparticles. Each system has advantages and limitations concerning drug load, stability, biocompatibility, and biodegradability, whereas the main types of therapy for delivering T cells are hydrophobic small molecules, proteins, and nucleic acids [188].

3.2.1 Rational design engineering for nonviral vector-based targeted gene delivery systems

The targeted delivery of nanoparticles to T lymphocytes is a greater challenge than targeting tumor cells. This is due to the smaller size of T cells compared to tumor cells, the increased nuclear-to-cytoplasmic ratio, decreased phagocytic activity, and the lower endocytic rate [188]. Moreover, different nanoparticle types and materials may induce immune responses or affect the function of T cells [189]. It is generally believed that the targeted gene delivery of nanoparticles to T cells in vivo needs to meet several criteria: (1) The size of the nanoparticles should be limited. The diameter of nanoparticles delivered to T cells should be between 10 and 100 nm, with 50 nm being ideal [190]. Nanoparticles smaller than 10 nm are at risk of being cleared by the glomerulus [191], chemical molecules with a diameter in the range of 100 nm can leak from the blood and accumulate in the tumor tissue (EPR effect), which is effective in both human cancer and animal tumor models [192], and too large a size is not compatible with clathrin-coated vesicles inducing their own endocytosis (RME) [193]. (2) The entry of nanoparticles does not cause an immune response in the body, and the recognition and clearance of monocytes, tissue macrophages and other immune cells triggered by nanoparticles in the blood should be avoided, further limiting the size of nanoparticles [194–196]. (3) The most efficient route for T-cell uptake is to induce its own endocytosis (RME) through clathrin-coated vesicles (Fig. 3); thus, nanoparticles are ideally designed to interact with specific receptors on the T-cell surface and, for T-cell targeting, to be conjugated with antibodies or other RME ligands; therefore, the intracellular signaling resulting from such receptor ligation is harmless to cells [193, 197]. (4) A negatively charged coating can shield the nanoparticles and diminish their surface charge to reduce off-target binding. (5) A properly designed carrier matrix can protect nucleic acids from nuclease degradation after cellular endocytosis [198].

In general, successful gene delivery requires the design of nanoparticles to overcome both extracellular and intracellular barriers without affecting cell function. Polymers containing primary amines, such as poly(L-lysine) (PLL), can bind anionic DNA and compress it into positively charged

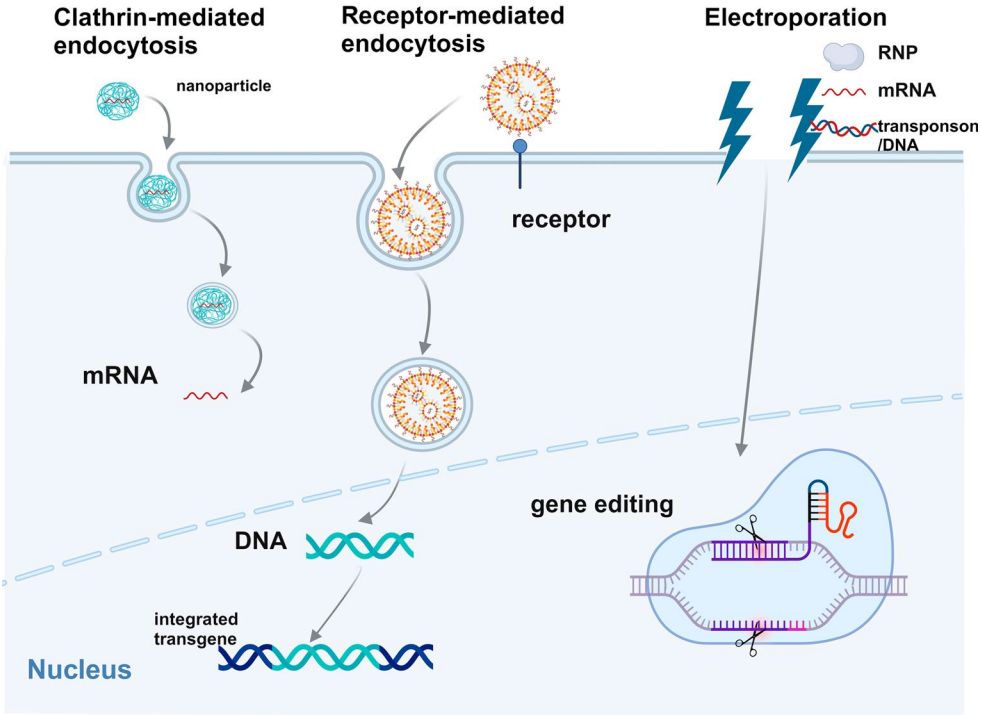

Fig. 3 Pathway of nonviral vectors into T cells. There are two types of nonviral vectors for T cells: clathrin-dependent pathways and receptor-mediated pathways, the main difference of which is the sizes of the vectors. Electroporation, such as the use of the CRISPR system and transposon system for gene knock-in, is the most essential approach for editing T cells

nanoparticles, similar to cations. Tertiary amine polymers with high buffering capacity, such as polyethyleneimine (PEI), can undergo endocytosis and subsequently escape the endosome via a proton sponge mechanism [199–202]. The latest generation of cationic polymers, such as chitosan and polyβ-amino ester (PBAE), have also been proven to be safe and effective DNA delivery vectors because they shield the positive charge of DNA to reduce off-target binding during DNA delivery [203–205]. Targeting is generally achieved by the addition of appropriate ligands to nanoparticles. Antibodies and antibody fragments are the most suitable candidates for the conjugation of nanoparticles and antibody molecules through adsorption, direct covalent linkage, or the use of adaptor molecules [206–210]. The majority of investigations pertaining to in vivo gene delivery predominantly employ antibodies or their derivative fragments that are designed to specifically recognize and bind to the extracellular regions of cell-surface proteins [211].

3.2.2 Nanoparticles based on different material types for T-cell-targeted gene delivery

To achieve effective gene delivery to T cells, cationic polymers must meet nanoparticle design requirements (Fig. 4), such as surface-anchored ligands that selectively bind nanoparticles to T cells and initiate rapid receptor-induced endocytosis to internalize them, negatively charged coatings, and suitable carrier matrices. In a recent report, researchers used T-cell-targeted anti-CD3E $F(ab')_2$ fragments conjugated to the surface of biodegradable poly(β-amino ester)-based nanoparticles to codeliver plasmids encoding CD19 4–1BBz CAR and piggyBac transposase to CD3+ T cells in vivo via CD3e $F(ab')_2$ displayed on the nanoparticle surface [199]. Nuclear localization and microtubule-associated sequences are loaded with DNA cargo to promote nuclear entry to improve gene transfer [212]. In vitro validation showed that CD3-targeting nanoparticles could selectively bind to T lymphocytes, and lymphocytes transfected with nanoparticles were fully functional and secreted at levels similar to those of T cells transduced with lentiviral vectors encoding the same CAR. In vivo studies have shown that targeted nanoparticles can reprogram circulating T cells in situ with leukemia-specific CAR genes. Although phagocytes in the reticuloendothelial system remove a small fraction of the injected nanocarrier, nanoparticles carrying a CD19-specific CAR gene can specifically and selectively rapidly modify CD3+ T cells in vivo to cause tumor regression in a mouse model that is comparable in efficacy to adoptive T-cell therapy, providing strong evidence that synthetic nanoparticles can be engineered to program antigen recognition into lymphocytes [200]. Similarly, by combining synthesized mRNA with a positively charged PBAE polymer, followed by the addition of PGA, which shields the positive charge of PBAE-mRNA particles and enhances lymphocyte targeting, the resulting mRNA nanoparticles can mediate efficient genome editing of CAR-T cells without interfering with their function [213].

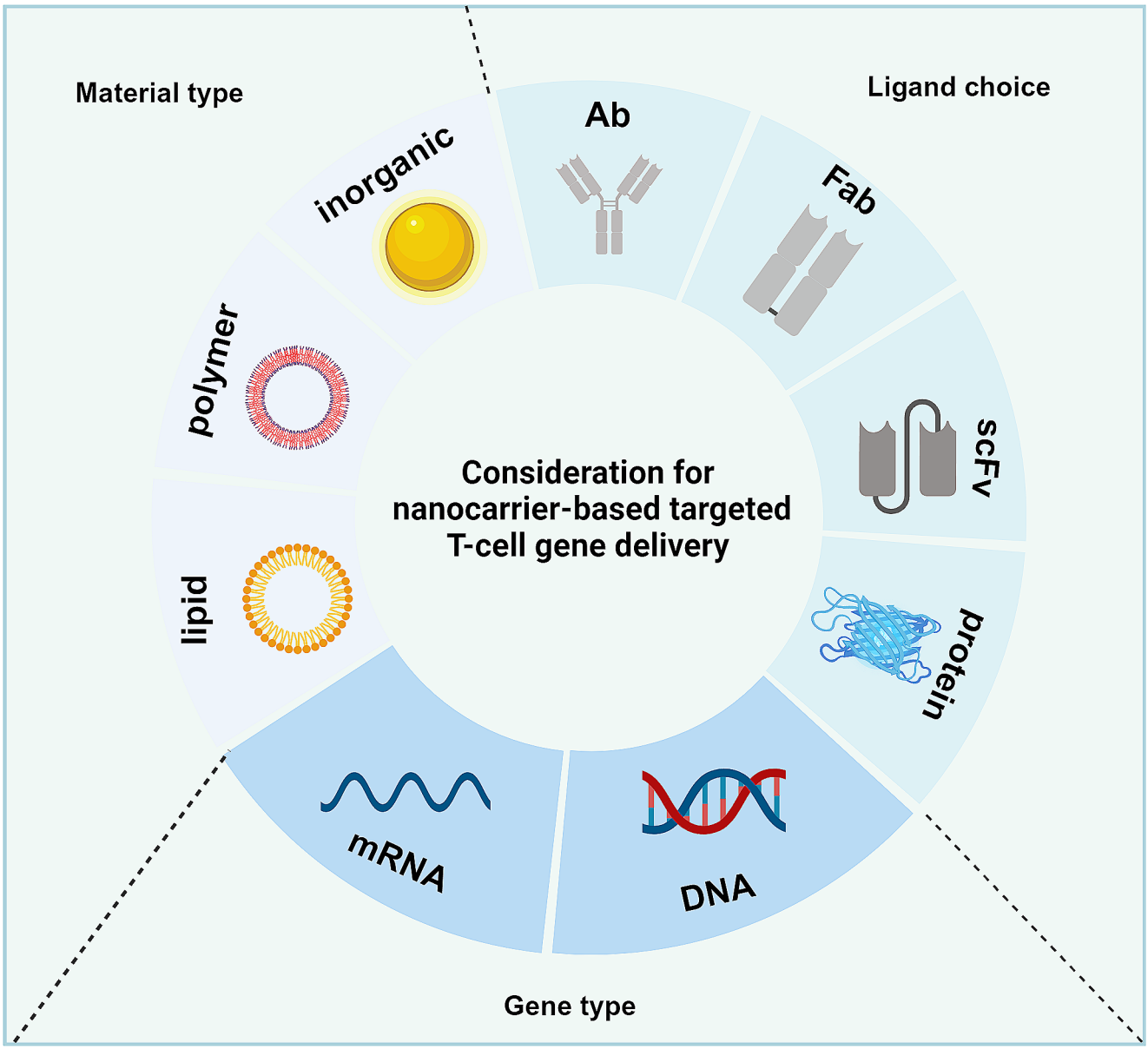

Fig. 4 Considerations for nanocarrier-based targeted T-cell gene delivery. Liposomes, polymers, and inorganic ions are examples of common nanomaterials used to deliver specific genes to T cells. The ligands linked to the nanoparticles could be Ab, Fab, scFv, or protein. NPs can transport both DNA and RNA to T cells as therapeutic agents

Another study induced cell targeting by coupling an anti-CD8 antibody to polyglutamic acid (PGA) to form a conjugate electrostatically adsorbed to the particles using a biodegradable poly(β-amino ester) (PBAE) polymer formulation with a half-life between 1 and 7 h under aqueous conditions [214]. The transient expression of virus-encapsulated nucleic acids (IVT mRNAs) that produce disease-specific CARs or TCRs showed that only nanoparticles functionalized with T-cell-specific (anti-CD8) antibodies were able to efficiently deliver the transgene, while isotype control-functionalized nanoparticles produced gene expression levels close to those of the background levels. Repeated infusions of these polymeric nanocrystals induced sufficient host T cells expressing tumor-specific CARs or virus-specific TCRs to induce tumor regression at levels comparable to those induced by adoptive T-cell therapy in mouse models of human leukemia, prostate cancer, and hepatitis B-induced hepatocellular carcinoma [198]. Similarly, CD7 antibody-conjugated chitosan nanoparticles allow specific delivery of siRNA to T cells [215]. Using transferrin receptor (TfR) expression on activated T cells, Y. Xie et al. designed a lung siRNA delivery system based on transferrin-polyethylenimine (Tf-PEI), which successfully induced the knockdown of related genes in a mouse model [216].

Among nonviral gene vectors, lipid nanoparticles (LNPs) are stable and advanced platforms for systemic gene delivery research, and they are the first nanoparticles that have been used for cancer therapy [217]. Lipid-based nanoparticles are composed of lipids or lipid-like materials for DNA binding, auxiliary lipids for improving transfection efficiency, and DNA vectors encoding therapeutic genes (Fig. 4). The efficiency of lipid-based DNA delivery and subsequent in vitro and ex vivo gene expression depend on various factors, such as nanoparticle size, amount of DNA, and cell line [218]. In summary, LNPs offer the advantages of reduced toxicity and the ability to deliver gene fragments of varying lengths into cells. For example, a team developed and optimized anti-CD3-targeted lipid nanoparticles (aCD3-LNPs) to deliver tightly packed, reporter gene mRNA specifically to T cells [219]. The activation and exhaustion of T cells were linked to the aCD3 antibody coating on the surface of LNPs, and they evaluated the use of LNPs in direct, in situ transfection of T cells. Additionally, therapeutic CAR-T cells could be generated in vivo by injecting CD5-targeting LNPs containing the mRNA required for reprogramming T lymphocytes. Treatment with modified mRNA-targeting LNP diminished fibrosis and restored cardiac function after damage in mice. These findings indicate that CAR-T cells generated in vivo have the potential to be a therapeutic platform for treating a wide range of diseases [220]. Schmid et al. described antibody-targeting nanoparticles that bind to CD8+ T cells in mouse blood, lymphoid tissues, and tumors [221]. The nanoparticles are targeted by conjugating anti-CD8a $F(ab')_2$ antibody fragments generated by the IDeS-mediated cleavage of full-length IgG molecules. The nanoparticles demonstrated specific binding in vitro and in vivo, indicating that antibody fragments on the surface of nanoparticles can be used not only to target specific T-cell subsets but also to functionally neutralize coinhibitory receptors, which is beneficial for preventing immune tolerance and enhancing the efficacy of tumor immunotherapy [221]. Similarly, in a study by another group, conjugation of a CD4 antibody to LNPs was shown to specifically target and deliver genes to CD4+ T lymphocytes. Specific accumulation of CD4-targeted mRNA-LNPs can be observed in the spleens of mice after systemic injection, providing a new idea for HIV-related treatment [222].

Inorganic nanomaterials are widely used in gene delivery, cancer therapy, and imaging due to their easy functionalization, unique electrical, optical properties, biocompatibility, and low cytotoxicity. Commonly used inorganic nanomaterials include gold, silver, calcium phosphate, graphene oxide, quantum dots, and magnetic nanomaterials [223, 224]. Among them, gold nanoparticles (AuNPs) are the most widely used for targeted gene delivery to T cells (Fig. 4). Their size can be easily changed, and their structure can be modified to achieve multiple functions by creating multiple functions through monolayers; moreover, nucleic acids and targeting agents can be placed on the surface to achieve targeted gene delivery [225, 226]. One study reported that they developed a gold nanoparticle carrier that can efficiently deliver small-molecule drugs or genes to targeted lymphocyte populations. The gold nanoparticles (amph-NPs) have a gold core with a diameter of 2–3 nm and are surrounded by an amphiphilic organic ligand shell consisting of a mixed monolayer of alkyl alcohols terminated by hydrophobic methyl and water-soluble sulfonate groups. Lymphocyte targeting was achieved by conjugated anti-CD8 VHH nanobodies. After particle endocytosis, the protein-targeted fraction is degraded, allowing NPs to regain their cell-penetrating ability and enter the cytoplasm of T cells in situ. In vivo experiments have shown that the uptake of targeted T cells by targeting nanoparticles is 40 times greater than that of nontargeting nanoparticles, and the delivery of TGF-β inhibitor-loaded particles to T cells enhanced their cytokine polyfunctionality in a cancer vaccine model [227].

Exosomes are nanoscale membrane carriers secreted by various cell types and are present in most body fluids [228, 229]. A large number of clinical studies have shown that exosomes have high potential in the treatment of human diseases [230–233]. Furthermore, by displaying antibodies against target receptors on exosomes, endogenous exosomes can be used as artificial cellular immune controllers to redirect immune effector cells and regulate their immune reactivity. A recent study showed that the scFv antibodies targeting CD3 and EGFR on exosomes were able to bind to T cells and EGFR-expressing triple-negative breast cancer (TNBC) cells simultaneously. Both in vitro and in vivo studies have demonstrated that exosomes have outstanding antitumor activity [234]. These discoveries may pave the way for the implementation of versatile platform technologies tailored for the advent of next-generation immunotherapeutic strategies.

At present, nanoparticles are mostly used as drug delivery systems in T-cell based immunotherapy to optimize the drug delivery curve [235]. The predominant focus in the field of direct gene delivery to T cells via nanoparticles has been on utilizing cationic polymers and liposomes as nonviral vectors. These vectors are particularly appealing due to their capacity to accommodate genetic fragments of various sizes without any inherent limitations on size [236]. A potential drawback of nano-delivery systems is the possibility that T cells, upon exposure to alien nanomaterials, may elicit an immune response or undergo functional alterations [189]. Furthermore, the activation of T cells is coupled with a multitude of cellular modifications, such as the modulation of surface receptor expression and morphological

reorganization, which could potentially impact the interactions between nanoparticles and immune cells [237]. For example, a study indicated that using nanoparticles to target T cells may be associated with complex immunological consequences, which requires further investigation for potential therapeutic applications [219].

3.3 CRISPR/Cas9 system for T-cell-targeted gene delivery

As a constantly developing gene editing technology, the CRISPR–Cas system has distinct advantages compared with transposons for precise gene editing. Protein-based (zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)) and RNA-based (CRISPR–Cas9) technologies can achieve specific gene knockouts and site-specific knock-ins to varying degrees. The CRISPR–Cas system is an adaptive immune mechanism used by bacteria and archaea to resist the invasion of foreign pathogens by silencing nucleic acids in nature. Jennifer Doudna et al. clarified how CRISPR–Cas works; in brief, once the Cas effector is assembled with a spacer containing a guide RNA molecule, the complex can bind to and cleave a specific sequence near the original protospacer adjacent motif (PAM) [238, 239]. The basic principle is that broken DNA double strands can be repaired by nonhomologous end joining or homology-directed repair (HDR) processes. With the use of nonhomologous end joining, which repairs DNA double-strand breaks without end processing, HDR repair allows the delivery of a transgene to a specific site for integration through the delivery of a DNA repair template. The high flexibility and efficiency of the RNA-guided nuclease CRISPR represent a game-changing technology that has been widely employed in the field of cell therapy [240].

The base editor is an editing system based on the CRISPR–Cas system that can be used for precise base manipulation. It uses nuclease-damaged Cas9 fused with deaminase to introduce specific point mutations into DNA without causing DSBs or relies on a donor DNA template and HDR [241, 242]. The base editor avoids DSBs caused by nucleic acid backbone cleavage by chemically modifying target bases directly, significantly improving product purity and reducing indels. The two main current base editors are the cytosine base editor (CBE) and the adenine base editor (ABE), which mediate all four possible switching mutations, C to T, A to G, T to C, and G to A [243], and can be used to correct more than 70% of disease-associated single-nucleotide polymorphisms (SNPs).

Although base editors can effectively recognize four metastatic mutations, improve the efficiency of correcting point mutations, and can be applied in the treatment of human genetic diseases, they cannot cause eight transforming mutations or the precise insertion or deletion of target gene segments; moreover, effectively installing or correcting these mutations for targeted transposition, insertion and deletion is difficult. Liu et al. reported a gene-editing technique called prime editing that can mediate all 12 possible combinations of base shifts as well as target indels in human cells without DSBs or donor DNA templates [244]. Prime editing adds a new search-and-replace capability to genome editing, broadening its scope.

As a constantly evolving technology, CRISPR–Cas system-mediated gene editing in T cells can not only knock out the αβ TCR in T cells but also prevent graft-versus-host reactions in adoptive cell therapy [245]; additionally, these cells can knock out genes related to immune suppression microenvironments, such as PD-1 [246], and T-cell functions, such as ID3 and SOX4 [126]. This gene-editing system allows for the precise insertion of the CAR site at any point in the T-cell genome, resulting in CAR-T cells with well-defined transgene copy numbers and predictable transgene expression regulation [247]. The use of Cas9 RNPs and an exogenous single-stranded DNA template by homology-directed repair allows the introduction of precisely targeted nucleotide substitutions in primary T cells [248]. The specific delivery of CD19-specific CARs to the T-cell receptor alpha constant (TRAC) site results in CAR-T cells being superior to conventional virus-delivered CAR-T cells in terms of antitumor activity, avoiding tonic CAR signaling and delaying effector T-cell differentiation and exhaustion [249]. A CRISPR–Cas9 genome targeting system that does not require a viral vector has recently been reported, allowing for the rapid and efficient insertion of large DNA sequences (greater than one kilobase) at specific sites in the genome of primary human T cells while maintaining cell viability and function. Using this system to knock in the ectopic TCR targeting the cancer antigen NY-ESO-1 and replace the endogenous TCR, antitumor effects were achieved both in vitro and in vivo [250]. Multiple edits of T cells can be achieved by base editing without affecting cell proliferation or aberrant DNA damage response pathway activation [251].

The role of CRISPR-Cas9 in reprogramming T cells is unquestionable. The optimal T-cell based gene editing method should be cell-specific and can directly perform complex modifications for stable gene addition in vivo, which is not only related to the gene editing target site but also dependent on the delivery method [252, 253]. Currently, the commonly used strategy for delivering the Cas-9 RNP complex into cells for targeted editing is electroporation (Fig. 3), which can be used only for gene editing in ex vivo T cells and induces significant dose-dependent toxicity with increasing template size. Physical damage to cells may also affect their state. The use of clinically validated viral

and nonviral vectors combined with targeted gene editing technology is promising for achieving targeted gene knock-in in T cells. The Cas9 protein (Cas9P LV) was packaged in lentivirus particles, and the Cas9 protein was subsequently delivered into lentivirus particles containing vectors expressing sgRNA to edit target cells, which reduced off-target effects [254]. Moreover, the use of nanoblades, a protein delivery vector based on friend murine leukemia virus (MLV), allows the transfer of Cas9-sgRNA ribonucleoprotein (RNP) into cell lines and primary cells both in vitro and in vivo [255]. The combination of the cell-targeting and cell-fusion capabilities of enveloped viruses with the transient delivery of CRISPR–Cas9 tools can not only mediate gene knockout and CAR transgene integration to produce transgenic CAR-T cells but also use viral pseudotyping (HIV) to target Cas9 RNP-mediated genome editing activity to specific cell types (CD4+ T cells) in mixed cell populations [248]. Similarly, engineered LNPs can be used to effectively deliver Cas9/sgRNA RNPs to specific target tissues for gene editing [256]. Synthetic RNP nanocapsules, customized by surface modification, can effectively achieve targeted gene editing in vitro and in vivo without any significant cytotoxicity [257]. Another delivery approach within the CRISPR framework that holds significant promise is the peptide-based delivery system, which utilizes cell-penetrating peptides (CPPs) to transport ribonucleoprotein complexes into target cells for the purpose of gene editing [258]. CPPs are a class of short peptides typically composed of a maximal length of 30 amino acids [259]. They are characterized by their ability to efficiently translocate across various cellular membranes with minimal cytotoxic effects and without eliciting an immune response [259–261]. A recent study has shown that engineered Peptide-Assisted Genome Editing (PAGE) CRISPR-Cas system can be used to achieve efficient and rapid gene editing in mouse T cells and human primary T cells [262]. This system simplifies the ex vivo engineering process for the development of next-generation CAR T cells, offering significant advantages, including minimal toxicity [262]. Another research indicates that the combination of CRISPR ribonucleoproteins with selected amphiphilic peptides can enhance the editing efficiency of primary T lymphocytes and peptide-mediated ribonucleoprotein delivery paired with an adeno-associated-virus-mediated homology-directed repair template can introduce a chimeric antigen receptor gene at the T-cell receptor α constant locus [263]. These bioengineered cells have shown promising anticancer properties in murine models. The Peptide-Enabled RNP Delivery (PERC) technique for CRISPR engineering presents minimally genotoxicity and less invasive alternative to electroporation for genome editing in primary T cells, without requiring specialized equipment [263]. The peptide-based gene delivery system could potentially streamline the production of engineered T cells. However, the use of peptide-based CRISPR systems for gene editing raises concerns about the possibility of pre-existing immune responses that might restrict their in vivo application. Further research is warranted to thoroughly evaluate the immunogenicity of the peptide-based CRISPR system within primary cells. Overall, the strategic integration of viral and non-viral delivery methods with CRISPR-Cas9 holds promise for achieving precise gene knock-in in T cells in vivo, thus bolstering the potential for sophisticated immunotherapeutic strategies.

As the forefront of gene-editing technology with significant clinical potential, employing the CRISPR/Cas system to modify T cells is crucial for breaking through the limitations encountered in existing T-cell therapies. Beyond facilitating precise in vivo gene knock-in, the targeted integration of specific genes can yield allogeneic T cells that exhibit heightened antitumor efficacy and reduced side effects [264]. While the application of a secure and potent CRISPR/Cas delivery system in clinical settings is not yet widespread, numerous efforts have been directed towards integrating CRISPR with established methods to enhance gene editing in T cells.

4 Conclusion

The continuous update and development of gene delivery systems is a prerequisite for the progress of immune cell therapy. An optimal gene delivery system should not only ensure precise gene targeting and integration but also adhere to the fundamental principle of minimizing cellular damage. A key area of focus in current T-cell based immunotherapy is the optimization of gene delivery vectors, guided by clinical trials. The ability to deliver T-cell-specific genes in vivo could initiate a new trend in the field. The critical features and technical challenges of gene delivery vectors include not only avoiding vector autoimmunity and ensuring the accommodation of the appropriate gene size but also minimizing genotoxicity and host immune responses. Most importantly, precise targeted delivery of genes to desired cells, such as the T cells highlighted in this review, is paramount. This report summarizes the current state of research on potential vectors for targeted gene delivery to T cells. In vivo gene-targeted delivery is still in its early stages, and much work is needed before this approach can be officially applied in clinical trials; nevertheless, once the technological bottleneck of targeted gene delivery vectors is overcome, the entire field of cell therapy may benefit.

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Declarations

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