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Review

Delivery technologies for T cell gene editing: Applications in cancer immunotherapy

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ABSTRACT

While initial approaches to adoptive T cell therapy relied on the identification and expansion of rare tumour-reactive T cells, genetic engineering has transformed cancer immunotherapy by enabling the modification of primary T cells to increase their therapeutic potential. Specifically, gene editing technologies have been utilized to create T cell populations with improved responses to antigens, lower rates of exhaustion, and potential for use in allogeneic applications. In this review, we provide an overview of T cell therapy gene editing strategies and the delivery technologies utilized to genetically engineer T cells. We also discuss recent investigations and clinical trials that have utilized gene editing to enhance the efficacy of T cells and broaden the application of cancer immunotherapies.

1. Introduction

In the past decade, cellular therapies have revolutionized cancer immunotherapy. Notably, adoptive T cell therapies have been widely investigated in preclinical and clinical stages following the FDA approval of five chimeric antigen receptor (CAR) T cell therapies: Kymriah, Yescarta, Tecartus, Breyanzi, and Abecma [1–4,5]. Adoptive T cell therapy involves the isolation and subsequent reinfusion of patient T cells to mediate antitumour, antiviral, or anti-inflammatory effects [6]. Initial adoptive T cell therapy approaches relied on identifying and expanding tumour-reactive T cells, which harnesses the endogenous immune system to act against cancer and viral infection [7–9]. Instead of relying on these rare T cell populations, primary T cells can be genetically engineered to improve their ability to target cancer cells [10].

Genetically engineered therapies, including CAR T cell and engineered T cell receptor (TCR) therapies, involve isolating patient T cells and reprogramming them *ex vivo* to target cancer cells [11–13]. The T cells are engineered to express a receptor, expanded, and transferred back into the patient [14]. These therapies rely on exogenous gene expression induced in primary T cells, resulting in transient or stable expression of the transgenic receptor in a wild type background [15]. By introducing receptors that bind to specific cancer markers, the transgenic T cells can target tumour cells to achieve positive therapeutic outcomes [16]. However, this is typically achieved using viral transduction, which has a smaller cargo capacity, higher immunogenicity, and higher manufacturing cost than non-viral delivery systems [17,18]. These limitations have driven the field to explore alternative gene editing technologies—including transposons, designer nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR–associated protein 9 (Cas9)—to introduce exogenous receptors and precise genetic modifications [19]. The recent growth of gene editing in T cells and FDA-approved CAR T cell therapies have motivated the exploration of novel delivery systems—such as electroporation, cell squeezing, and nanoparticles—to further enhance therapeutic efficacy (Fig. 1) [20].

In addition to introducing exogenous receptors and redirecting T cell function, gene editing has been used to generate T cells with improved antigen responses, enhanced antitumour activity, and potential for use in allogeneic applications. CAR T cell therapies have been successfully applied to treat B cell malignancies. However, treating solid tumours remains challenging, as local immune suppression and prolonged stimulation in the tumour microenvironment lead to T cell dysfunction and exhaustion [21,21–23]. Recent advances in gene editing and delivery technologies could be applied to treat cancers previously resistant to T cell immunotherapies. In this review, we discuss current *ex vivo* T cell engineering strategies and their use in clinical applications.

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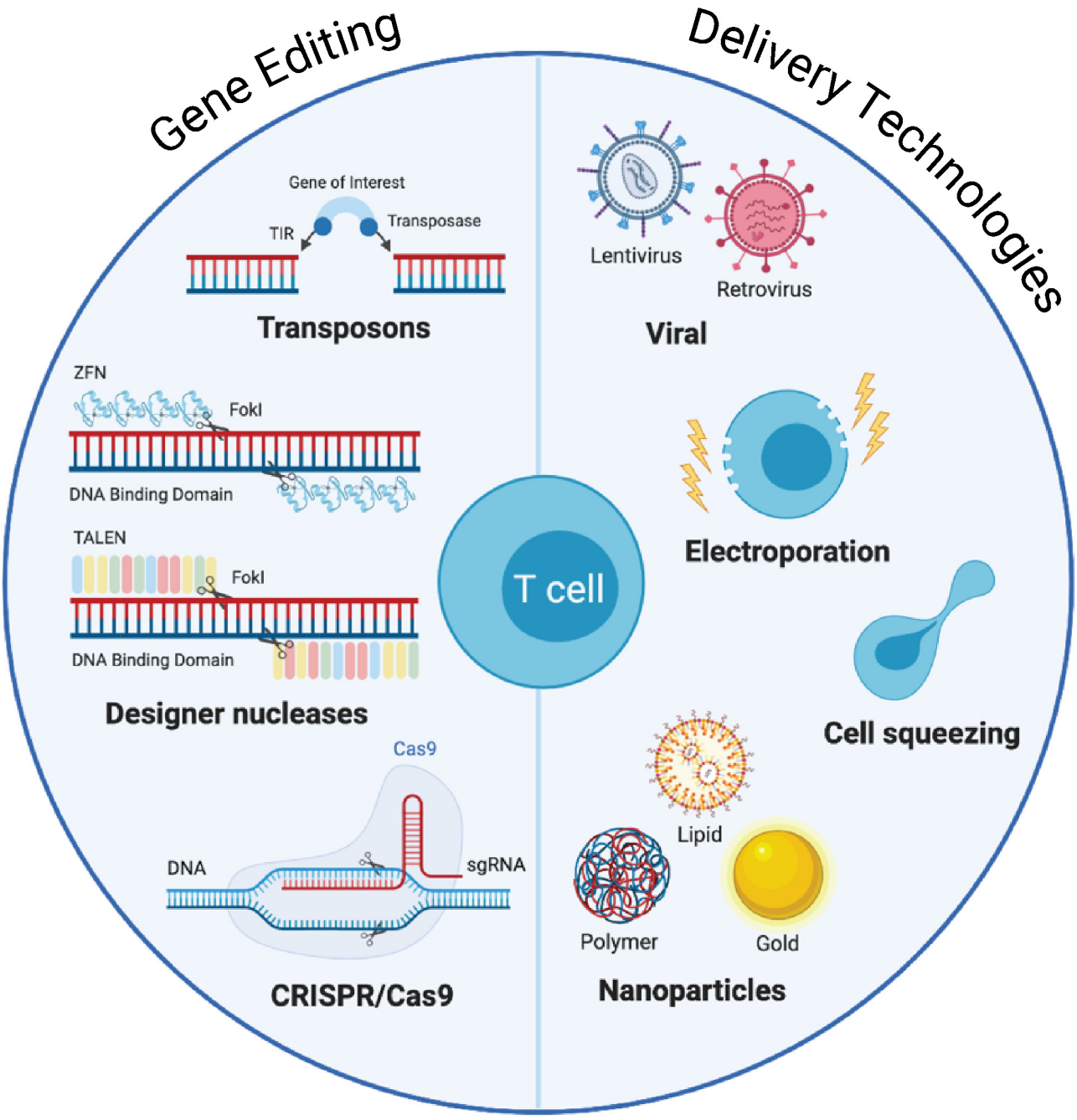

Fig. 1. Delivery technologies for gene editing of T cells. Gene editing strategies that have been explored in T cells for applications in cancer immunotherapy include transposons, designer nucleases like zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. In addition to viral transduction, novel delivery systems—such as electroporation, cell squeezing, and nanoparticles—have been utilized in new immunotherapy strategies to further enhance therapeutic efficacy. Figure was created by the authors with BioRender.com.

2. Gene editing technologies used in T cells

The advent of gene editing has enabled more specific genetic manipulation to optimize T cell engineering and function, further advancing the scope of cellular therapies [19]. Many gene editing strategies have been explored for T cell engineering, including transposons, designer nucleases like zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 [24]. These platforms present various advantages and disadvantages in their editing specificity and efficiency, and ability to be delivered to T cells.

2.1. Transposons

Transposons (Fig. 2a) are units of DNA that can change their position within the genome and are used in non-viral cellular engineering [25]. The DNA transposon system involves a transposase that binds to terminal inverted repeats (TIRs) and mobilizes DNA flanked by these TIRs [26]. Since the *Sleeping Beauty* (SB) DNA transposon is capable of transposition in human cells, it has been used in several early clinical trials exploring CAR T cell therapy [27,28]. The SB platform can produce stable transgene expression with low genotoxicity and minimal disruption to other essential genes [29,30]. Compared to viral

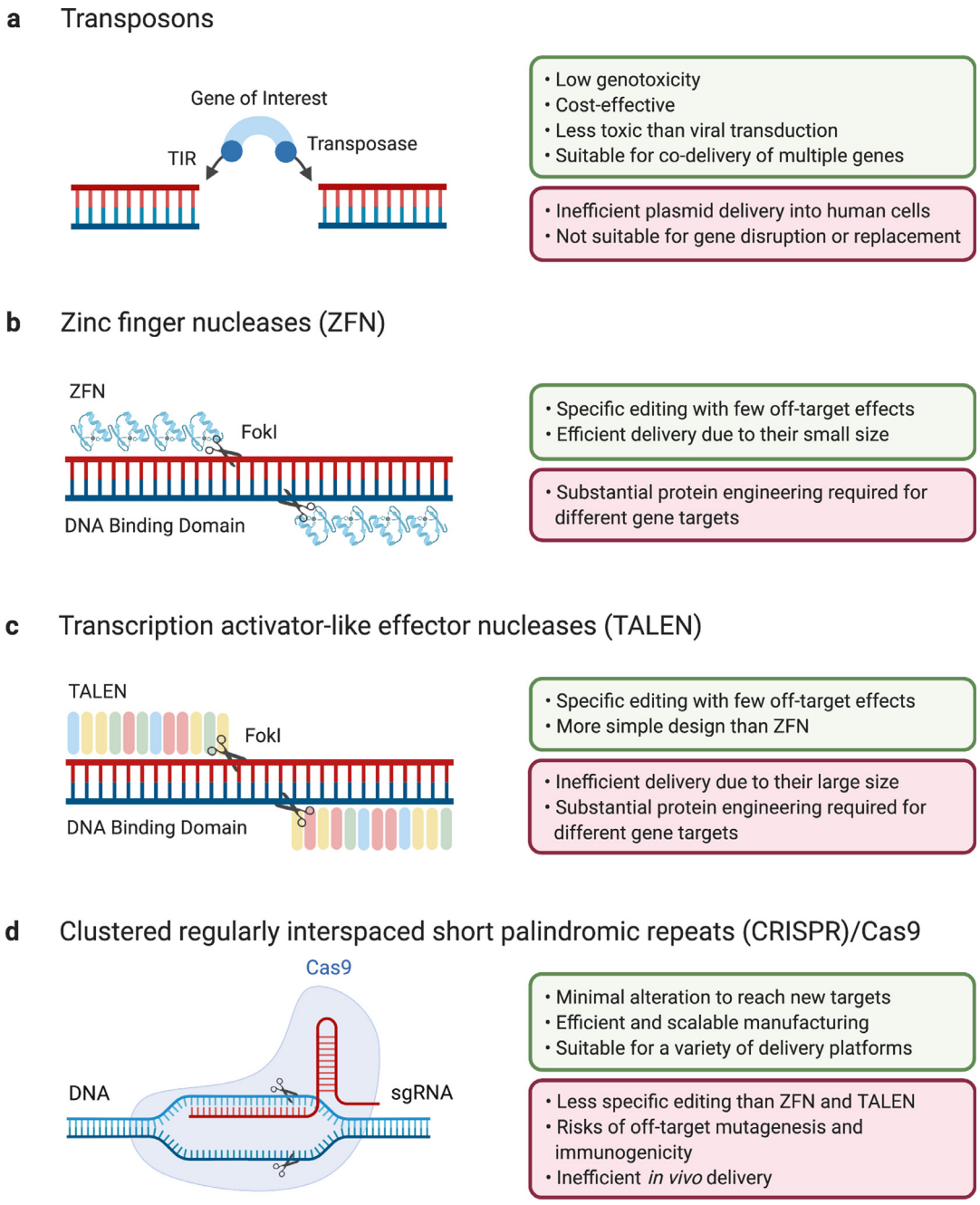

Fig. 2. Comparison of SB, ZFN, TALEN, and CRISPR/Cas9 platforms. Advantages (green) and disadvantages (red) of the (a) *Sleeping Beauty* (SB) transposon, (b) ZFN, (c) TALEN, and (d) CRISPR/Cas9 platforms in their editing specificity and efficiency, and ability to be delivered to T cells. Figure was created by the authors with BioRender.com.

systems, transposons are more cost-effective, less toxic, and can facilitate co-delivery of multiple genes [17,31,32]. Although the SB platform has promising applications in cancer immunotherapy, its use has been limited by low efficiency of plasmid DNA delivery into primary human cells [33,34]. Furthermore, this platform can only introduce a transgene into a cell while gene editing technologies like ZFN, TALEN, and CRISPR/Cas9 can disrupt or replace a specific gene, making them more versatile for T cell engineering [35].

2.2. Designer nucleases

While numerous designer nucleases have been developed for gene editing, ZFN (Fig. 2b) and TALEN are most frequently used in T cell engineering [36,37]. Multiple zinc finger domains can be cloned in tandem to generate a "designer" enzyme that recognizes a specific DNA sequence [38]. Since the FokI endonuclease components function as a dimer, a pair of ZFN is required to bind at the target sites and cleave DNA [35]. Following this double-stranded cut, endogenous non-homologous end joining (NHEJ) or homologous recombination (HR) repair mechanisms are recruited to the break [24]. NHEJ can result in small insertions or deletions (indels) while HR is used for gene replacement [39]. The specificity of ZFN-mediated gene editing depends on the amino acid sequence of the fingers, number of fingers, and interaction of the nuclease domain [19]. As such, multiple linked zinc fingers can create highly specific recognition sites with minimal off-target effects [40–42]. In addition, the small size of ZFN enable efficient delivery in T cells [43]. The ZFN platform has been

investigated in cancer immunotherapy clinical trials, but its main application in T cells is to target CCR5 and CXCR4, the co-receptors for HIV entry into T cells [44–48]. While this technology is specific and effective, it is less efficient than CRISPR/Cas9 because it requires proteins to be specifically engineered for each target in the genome [24].

TALEN (Fig. 2c) is similar to ZFN in that it consists of an engineered designer nuclease. TALEN is composed of a non-specific DNA cleavage domain and a sequence-specific DNA-binding domain, which contains a highly conserved repeat sequence from transcription activator-like effector (TALE) [19]. Two TALEN modules are required to bind to the target site, and a FokI nuclease is fused to the DNA-binding domains to cleave DNA [49]. Similar to ZFN, the specificity of TALEN-mediated gene editing depends on the number and order of tandem repeats in a TALE [50]. However, TALEN is more difficult to deliver than ZFN due to the large size and repetitive nature of its functional components [51]. In clinical trials, the TALEN platform has been used to develop universal allogeneic T cells for cancer therapy, but its broad use has also been limited by the substantial protein engineering required to transition between different gene targets [52,53].

2.3. CRISPR/Cas9

In contrast to ZFN and TALEN, CRISPR/Cas9 (Fig. 2d) requires minimal alteration to reach new target sites and has been favoured for T cell engineering in recent clinical trials [53,54]. The CRISPR/Cas9 system is comprised of a single-stranded guide RNA (sgRNA) and a Cas9 endonuclease [55]. The sgRNA complements and binds the target DNA site while also binding the Cas9 protein that cleaves DNA [56]. CRISPR/Cas9 can enable genomic modifications through NHEJ or high-fidelity HR but is less specific than ZFN and TALEN, as it can tolerate multiple consecutive mismatches in the DNA target sequence [57]. There are concerns that CRISPR/Cas9 gene editing could promote tumour malignancy due to off-target mutagenesis or cause immunogenicity from anti-Cas9 responses [58,59]. However, CRISPR/Cas9 offers the potential for simultaneous multiple loci editing. While this strategy can be more complex to implement, it is more efficient and scalable overall [60]. Furthermore, CRISPR/Cas9 can be delivered in a variety of formats, including plasmid DNA encoding both the guide RNA (gRNA) and Cas9, messenger RNA (mRNA) for Cas9 translation with a separate gRNA, and ribonucleoprotein complexes (RNPs) of Cas9 protein and gRNA [53,61]. This versatility has enabled the development of various strategies, but *in vivo* delivery remains challenging because multiple components of the editing system must be delivered to the same cell [53].

3. Delivery methods to T cells

Several delivery platforms have been explored in T cells, and offer distinct advantages and disadvantages that have impacted their application in T cell therapies. In addition to viral transduction, non-viral delivery strategies—such as electroporation, cell squeezing, and nanoparticles—have been recently explored to improve the safety and efficacy of T cell therapies.

3.1. Viral

Viral transduction (Fig. 3a) has been used to achieve efficient delivery of the CAR transgene in traditional CAR T cell engineering [62]. Gamma-retroviruses and lentiviruses can integrate into the host genome to enable stable gene expression, while adenoviruses and adeno-associated viruses can induce transient expression [26]. Gamma-retroviruses and lentiviruses are most often used in manufacturing CAR T cells due to their high transduction efficiencies [63,64]. Similarly, lentiviruses have been used to deliver ZFN and CRISPR/Cas9 *ex vivo* to disrupt specific genes for improved T cell functionality [60,65,66]. In this case, the sgRNA component of the CRISPR/Cas9 system is generally delivered via lentiviral vectors for stable expression, while mRNA is delivered via electroporation for transient expression [60,67].

While viral delivery is highly effective at inducing gene expression, this method poses potential risks of genotoxicity and insertional mutagenesis caused by random insertion of transgenes into chromosomes [17,53]. While the exact causes remain unclear, a few patients have experienced fatal immune responses or developed cancer following viral gene therapy [68–71]. However, these random insertions can be therapeutically effective if gene disruption results in improved T cell activity [72]. The intrinsic toxicity and immunogenicity of viruses significantly hinder their applications *in vivo*, such as for cancer treatment, where repeated drug dosing is often required [73,74]. Furthermore, the small cargo capacity of viral systems inhibits co-delivery of multiple genes, which may be necessary for the development of advanced T cell therapies [18]. These limitations have motivated the exploration of non-viral delivery methods, including electroporation, cell squeezing, and nanoparticles.

3.2. Electroporation

Electroporation (Fig. 3b) uses pulsed high-voltage electrical currents to transiently create small pores in the cell membrane, allowing nanometre-sized cargo to enter the cell [75]. Electroporation can be used to deliver mRNA or plasmid DNA, enabling gene replacement or disruption [76,77]. This method can be as efficient as viral transduction and offers distinct advantages including a larger cargo capacity to facilitate the delivery of multiple genes or nucleic acids [78,79]. Electroporation has been used to deliver to muscle and skin cells *in vivo* and is currently being evaluated in phase I clinical trials for a DNA vaccine against SARS-CoV-2 [80,81]. However, it is not suitable for delivery to T cells *in vivo* due to its limited penetration depth and localized administration [82]. While used in *ex vivo* applications, the high voltage required for electroporation poses risks of cytotoxicity and loss of cytoplasmic content, which can adversely affect expression profiles [83–85]. In addition, electroporation may face scalability challenges because most commercial machines are designed for research and development rather than large-scale manufacturing [86]. Nucleofection is an advanced electroporation technique that can deliver cargo to the nucleus of a cell without breaking down the nuclear envelope, but it faces many of the same obstacles as electroporation regarding *in vivo* use. Although both electroporation and nucleofection face these limitations, they are still promising approaches for *ex vivo* gene editing in T cells [87].

3.3. Cell squeezing

Cell squeezing (Fig. 3c) is a microfluidic delivery method that relies on mechanical membrane disruption *ex vivo*, which has a minimal effect on transcriptional responses and does not modulate T cell activity [85]. Cell squeezing has been used to deliver various compounds, including DNA, RNA, and proteins, to embryonic stem cells and immune cells [88]. Cell squeezing has been successfully used to deliver dextran molecules to murine T cells, indicating that it could be used in human T cells in the future [88]. Although cell squeezing has the potential to reduce the risks associated with electroporation, it requires isolating the cells for delivery, which limits its use to *ex vivo* engineering applications [85].

3.4. Nanoparticles

Nanoparticles (NPs) (Fig. 3d) are emerging delivery systems for gene editing with various advantages over viral, electrical, and mechanical-based delivery strategies [89]. Many types of NPs have

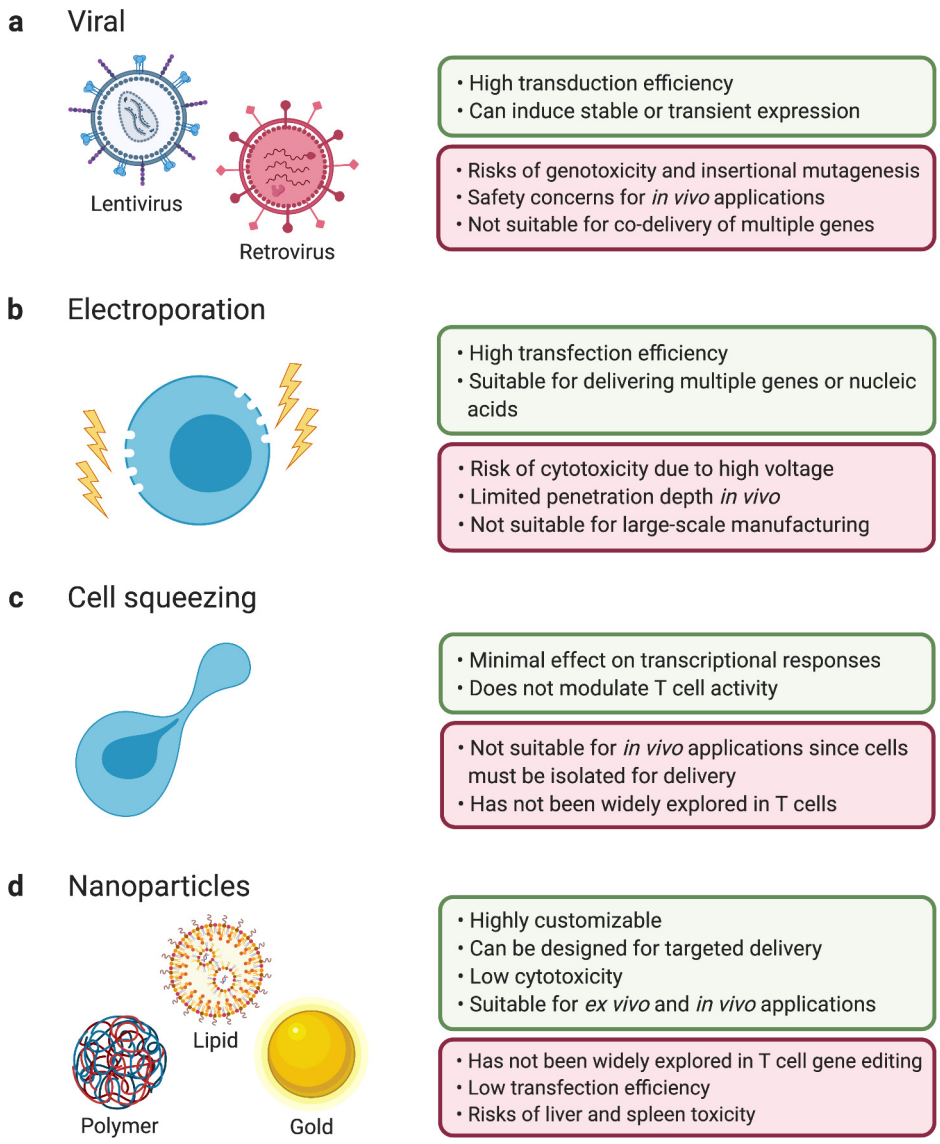

Fig. 3. Comparison of viral, electroporation, cell squeezing, and nanoparticle delivery. Advantages (green) and disadvantages (red) of (a) viral, (b) electroporation, (c) cell squeezing, and (d) nanoparticle delivery systems regarding their efficacy and safety. Figure was created by the authors with BioRender.com.

been used for delivery to T cells, including those composed of lipid, polymer, or gold materials [87]. Given the variety of platforms available, NPs are highly customizable and can deliver many different cargos, including DNA, mRNA, siRNA, miRNA, and even combinations of these nucleic acids [61,90]. Moreover, these platforms can be designed for targeted delivery by using selective surface modifications or controlled cargo release in response to T cell receptor activation [21]. NPs are less cytotoxic than viral or electroporation methods, resulting in higher viability and subsequent expansion capability of the engineered cell population [91,92]. NP delivery also offers a manufacturing advantage over cell squeezing because it does not require specific cell handling and can be easily implemented into established protocols for generating therapeutic cells [93]. Furthermore, NP platforms can be used to deliver cargos both *ex vivo* and *in vivo* due to the stability offered by particle encapsulation, but this has yet to be thoroughly explored in T cells [87,94].

Although NPs have been used to successfully deliver mRNA encoding CAR to T cells, as well as gene editing technologies to a variety of cell types, they have only recently been used for gene editing in T cells [95]. Lipid and polymer NPs have been used to deliver CRISPR/Cas9 *ex vivo* to T cells [96–99]. Other studies have used gold NPs to deliver small molecule drugs to T cells and CRISPR/Cas9 to other cell types, indicating the potential of this platform for further application in T cells [100,101]. Although NPs offer many advantages over other delivery strategies, they typically have lower transfection efficiencies, which has motivated the development of more advanced NPs aimed to overcome biological barriers to delivery [94,102,103]. NPs also tend to accumulate in the liver and spleen during clearance from the body, raising concerns about toxicity [104]. While these improved systems have yet to be thoroughly investigated for delivery to T cells, they hold immense potential to improve T cell immunotherapies.

4. Applications of genetically engineered T cells in cancer immunotherapy

Recent investigations (Table 1) demonstrate how the field has progressed as state-of-the-art gene editing and delivery technologies have been introduced in T cells. These investigations and later clinical trials (Table 2) have used gene editing strategies to develop T cells

Table 1
Investigations using gene editing in T cells for cancer immunotherapy.

Platform	Cancer	Target (Knockout)	Induced T cell Expression	Location of Delivery	Delivery Method (Cargo)
SB10	N/A	N/A	DsRed reporter gene	Ex vivo	Nucleofection (SB10/reporter plasmid or SB10, reporter plasmids)[29]
HSB5	Melanoma	N/A	P53, MART-1 TCRs	Ex vivo	Electroporation (TCR, HSB5 mRNA)[119]
SB11	Chronic lymphocytic leukaemia, mantle cell lymphoma, diffuse large B-cell lymphoma	TRAC, TRBC (ZFN)	CD19 CAR	Ex vivo	Nucleofection (CAR, SB plasmids, ZFN mRNA)[140]
	CD19+ B cell malignancies	N/A	CD19 CAR	In vitro, ex vivo	Electroporation (CAR, SB plasmids)[30]
	CD19+ B cell malignancies	HLA-A (ZFN)	CD19 CAR	In vitro, ex vivo	Nucleofection (CAR, SB plasmids, ZFN mRNA)[141]
	Chronic lymphocytic leukaemia	N/A	ROR1 CAR	Ex vivo	Electroporation (CAR, SB plasmids)[110]
	Melanoma	N/A	HERV-K CAR	Ex vivo	Electroporation (CAR, SB plasmids)[111]
	Melanoma, cholangiocarcinoma	N/A	AHNAK, ERBB2, ERBB2IP TCRs	Ex vivo	Nucleofection (TCR, SB plasmids)[120]
	Myelogenous leukaemia, acute lymphoblastic leukaemia	N/A	CD123 CAR (CIK cells)	Ex vivo	Nucleofection (CAR, SB plasmids)[113]
	Acute myelogenous leukaemia, acute lymphocytic leukaemia	N/A	CD13 CAR	Ex vivo	Electroporation (CAR, SB plasmids)[114]
	CD19+ leukaemia	N/A	CD19 CAR, mBIL15	Ex vivo	Electroporation (CAR, SB plasmids)[136]
SB100X	Haematological and certain non-haematological malignancies	N/A	WT1 TCR	In vitro, ex vivo	Lentivirus (CAR), nucleofection (TCR, SB plasmids)[31]
	CD19+ lymphoma	N/A	CD19 CAR	Ex vivo	Nucleofection (CAR, SB supercoiled DNA or CAR, SB plasmids)[17]
	Melanoma	TRAC, TRBC (miRNA)	TCR-engineered T cells	Ex vivo	Nucleofection (TCR minicircle DNA, SB mRNA, TCR-silencing miRNA)[121]
	Acute myeloid leukaemia	N/A	CD33 CAR (CIK cells)	Ex vivo	Nucleofection (CAR, SB plasmids)[115]
piggyBac	Leukaemia	N/A	CD19 CAR	Ex vivo, in vivo	CD3-targeted polymer nanoparticles (194-1BBz CAR/piggyBac plasmid, iPB7 plasmid), lentivirus (194-1BBz CAR)[95]
ZFN	Acute myeloid leukaemia	TRAC, TRBC	WT1 TCR	In vitro, ex vivo	Lentivirus (ZFN)[65]
	Chronic lymphocytic leukaemia, mantle cell lymphoma, diffuse large B cell lymphoma	TRAC, TRBC	CD19 CAR	Ex vivo	Nucleofection (CAR, SB plasmids, ZFN mRNA)[140]
	CD19+ B cell malignancies	HLA-A	CD19 CAR	In vitro, ex vivo	Nucleofection (CAR, SB plasmids, ZFN mRNA)[141]
	Metastatic melanoma	PD-1	N/A (TIL)	Ex vivo	Electroporation (ZFN mRNA)[125]
	N/A	CCR5, AAVS1	N/A	Ex vivo	Electroporation (ZFN mRNA), AAV6 (donor vector)[44]
TALEN	CD19+ lymphoma	CD52, TRAC, TRBC	CD19 CAR	Ex vivo	Electroporation (TALEN mRNA), lentivirus (CAR)[142]
	Acute lymphoblastic leukaemia	CD52, TRAC	CD19 CAR	Ex vivo	Electroporation (TALEN mRNA), lentivirus (CAR)[52]
	N/A	PD-1, TRAC	CD20 CAR	Ex vivo	Electroporation (TALEN mRNA), lentivirus (CAR)[132]
	Burkitt's lymphoma	GM-CSF	CD22 CAR	Ex vivo	Electroporation (TALEN mRNA), lentivirus (CAR)[130]
	Multiple myeloma	TRAC, CD52	BCMA CAR	Ex vivo	Electroporation (TALEN mRNA), lentivirus (CAR)[143]
	Burkitt's lymphoma	TRAC, IL2Ra, PD-1	CD22 CAR, IL-12P70	Ex vivo	Electroporation (TALEN mRNA), AAV6 (repair vector), lentivirus (CAR)[135]
megaTAL	B cell lymphoma	TRAC	CD19 CAR, TREX2, FOXO1, eGFP	In vitro, ex vivo	Polymer nanoparticles (TRAC-megaTAL, TREX2, FOXO1, eGFP mRNA), electroporation (eGFP mRNA), lentivirus (19-41BBζ CAR)[93]
TRC1-2 nuclease	B cell lymphoma	TRAC	CD19 CAR	Ex vivo	Electroporation (TCR1-2 nuclease mRNA), AAV6 (CAR donor vector)[22]
CRISPR/ Cas9	Epstein-Barr virus-associated gastric cancer	PD-1	N/A	Ex vivo	Nucleofection (sgRNA/Cas9 plasmid)[126]
	Acute lymphoblastic leukaemia	TRAC	CD19 CAR	Ex vivo	Electroporation (Cas9 mRNA, gRNA), AAV (1928z CAR repair vector)[134]
	Acute lymphoblastic leukaemia	TRAC, TRBC, B2M, Fas, PD1, CTLA-4	CD19 CAR	Ex vivo	Electroporation (Cas9 mRNA or protein), lentivirus (gRNA, CAR)[60]
	Colorectal carcinoma	CTLA-4	N/A (Cytotoxic T lymphocytes)	Ex vivo	Lentivirus (sgRNA, Cas9)[66]
	Erythroleukaemia, Burkitt's lymphoma	LAG-3	CD19 CAR	Ex vivo	Electroporation (Cas9 protein), nucleofection (sgRNA), lentivirus (CAR)[129]
	Acute lymphoblastic leukaemia, prostate carcinoma	TRAC, TRBC, PD-1, B2M, HLA class I genes	CD19 CAR, PSCA CAR	Ex vivo	Electroporation (Cas9 mRNA, gRNAs), lentivirus (CAR)[144]
	Acute lymphoblastic leukaemia, melanoma	TRBC	$\gamma\delta$ TCR	In vitro, ex vivo	Lentivirus (TCR, CRISPR/Cas9)[122]
	Burkitt's lymphoma	TRAC	CD19 CAR	Ex vivo	Electroporation (Cas9 mRNA), lentivirus (gRNA, CAR)[67]
	Glioblastoma	DGK	139 (EGFR VIII) CAR	Ex vivo	Nucleofection (RNPs), lentivirus (139 CAR)[124]
	Relapsed and refractory acute lymphoblastic leukaemia, non-Hodgkin's lymphoma	TRAC, CD7	CD7 CAR, CD19 CAR	In vitro, ex vivo	Electroporation (Cas9 mRNA, sgRNA), lentivirus (CAR)[138]
	Breast cancer (TNBC)	PD-1	Meso CAR	Ex vivo	Electroporation (RNPs), lentivirus (CAR)[127]
	Bladder cancer	CTLA-4	N/A (Peripheral blood CD8+ T cells)	Ex vivo	Electroporation (RNPs)[128]
	Hepatocellular carcinoma	PD-1	N/A	Ex vivo	Electroporation (liposomes encapsulating plasmid)[97]
	Glioblastoma	TRAC, B2M, PD-1	EGFR vIII CAR	Ex vivo	Electroporation (RNPs), AAV6 (CAR)[23]
	N/A	TRAC, RAB11A, CD4, TUBA1B, ACTB, FBL, CLTA	N/A	Ex vivo	Electroporation (polymer nanoparticle-stabilized RNPs)[99]
	Pancreatic carcinoma	TGFBR2	Meso CAR	Ex vivo	Nucleofection (RNPs), lentivirus (CAR)[131]
	Non-Hodgkin's lymphoma, other immune disorders	PTEN, PCSK9	N/A	In vivo	Lipid nanoparticles (Cas9 mRNA, sgRNA, RNPs)[98]
	N/A	TRAC	IL-15, mClover3, CAR, BiTE	Ex vivo	Nucleofection (RNPs, DNA for HDR)[133]

Table 2
Clinical trials using gene editing in T cells for cancer immunotherapy.

Platform	Cancer	Target (Knockout)	Induced T cell Expression	Delivery Method (Cargo)	Phase	Trial number
SB11	B cell lymphoma	N/A	CD19 CAR	Nucleofection (SB plasmid, CAR)	I	NCT00968760
	Acute lymphoblastic leukaemia, acute biphenotypic leukaemia, non-Hodgkin's lymphoma, small lymphocytic lymphoma, chronic lymphocytic leukaemia	N/A	CD19 CAR	Nucleofection (SB plasmid, CAR)	I	NCT01497184
	B-lineage lymphoid malignancies	N/A	CD19 CAR	Nucleofection (SB plasmid, CAR)	I	NCT01362452
	Chronic lymphocytic leukaemia	N/A	CD19 CAR	Nucleofection (SB plasmid, CAR)	I	NCT01653717
TALEN	Acute lymphoblastic leukaemia	CD52, TRAC, TRBC	CD19 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT02746952
	Relapsed and refractory acute lymphoblastic leukaemia	CD52, TRAC, TRBC	CD19 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT02808442
	Relapsed and refractory multiple myeloma	CD52, PD-1	CS1 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT04142619
	Acute lymphoblastic leukaemia	CD52, PD-1	CD22 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT04150497
CRISPR/Cas9	Metastatic non-small cell lung cancer	PD-1	N/A	Nucleofection (plasmid)	I	NCT02793856
	Epstein-Barr virus-associated cancers	PD-1	N/A	Nucleofection (plasmid)	I/II	NCT03044743
	Advanced oesophageal cancer	PD-1	N/A	Electroporation (plasmid)	N/A	NCT03081715
	Relapsed and refractory CD19+ leukaemia and lymphoma	TRAC, TRBC, B2M	CD19 CAR	Electroporation (mRNA), lentivirus (CAR)	I/II	NCT03166878
	Relapsed and refractory acute myeloid leukaemia	TRAC	CD123 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT03190278
	Advanced refractory myeloma, metastatic sarcoma	TRAC, TRBC, PD-1	N/A	Electroporation (RNPs), lentivirus (TCR)	I	NCT03399448
	Mesothelin-positive multiple solid tumours	PD-1, TRAC	Mesothelin-directed CAR	Lentivirus (DNA, CAR)	I	NCT03545815
	Mesothelin-positive multiple solid tumours	PD-1, TRAC	Mesothelin-directed CAR	Lentivirus (DNA, CAR)	I	NCT03747965
	Refractory B cell malignancies	Unknown	CD19 CAR	Electroporation (mRNA)	I/II	NCT04035434
	Refractory B cell malignancies	HPK1	CD19 CAR	Electroporation (mRNA), lentivirus (CAR)	I	NCT04037566
	Metastatic gastrointestinal epithelial cancer	CISH	N/A (TIL)	Electroporation (mRNA)	I/II	NCT03538613
	T cell leukaemia and lymphoma	CD28	CD7 CAR	Undefined	I	NCT03690011

with improved responses to antigens, enhanced antitumour activity, and optimized functionality for universal CAR T cell therapies.

4.1. Engineering T cells to target specific antigens

In a normal immune response, individual T cells express distinct TCRs that can recognize an antigen in the context of the major histocompatibility complex (MHC) to activate and proliferate [105]. Genetic engineering can be used to enhance the cancer-targeting ability of primary T cells via the incorporation of exogenous receptors [15]. Specifically, T cells have been engineered to express CARs or TCRs with specificity for a tumour-associated antigen to enhance their therapeutic response. CARs are synthetic transmembrane receptors that combine an extracellular antigen recognition domain with the intracellular co-stimulatory domains from CD28 or 4-1BB, whereas engineered TCRs are affinity-enhanced synthetic receptors with the same structure as the native TCR [24]. The 1st generation CAR initially combined the extracellular antigen recognition domain as a single-chain variable fragment to the intracellular signalling domain from CD3ζ [6]. This CAR was later improved by adding the co-stimulatory endodomain from either CD28 or 4-1BB to CD3ζ to the intracellular side [6]. This 2nd generation CAR has formed the basis for current clinically approved CAR T cell therapies. The 3rd generation CAR further improved antitumour activity with the addition of both CD28 and 4-1BB co-stimulatory domains to CD3ζ [6]. Both CARs and TCRs have been widely explored in T cell engineering applications [24,106].

In contrast to transgenic TCRs, CARs can respond to surface antigens independent of MHC, expanding the target space for T cell therapy [15,107]. Many investigations have focused on generating CD19-specific CAR T cells to treat relapsed or refractory B cell lymphoid malignancies [108]. Clinically approved CAR T cell therapies have relied on retroviral or lentiviral transduction of the CAR transgene [1–4,109]. Recently, other delivery technologies have also generated CAR T cells targeting a variety of antigens. Maiti et al. delivered SB plasmid DNA via electroporation to genetically modify T cells to express CD19-specific CARs [30]. This provided the groundwork for future phase I clinical trials, in which SB was used to modify patient- and donor-derived T cells to express 2nd generation CD19-specific CARs [27,28]. Patients with advanced non-Hodgkin lymphoma (NHL) and ALL underwent haematopoietic stem cell transplantation (HSCT) and infusion of either autologous or allogeneic CAR T cells for adjuvant therapy [28]. The infusion of CAR T cells showed no acute or latent toxicities and did not exacerbate graft-versus-host disease (GVHD) [28]. NP delivery platforms have also been used to produce antigen-specific CAR expression in T cells. Smith et al. delivered SB plasmid DNA via polymeric NPs to generate CD19-specific CAR T cells that effectively targeted tumour cells to induce long-term disease remission [95].

Although CD19-specific CAR T cell therapies have been successful in treating B cell malignancies, they can lead to loss of normal $CD19^+$ B cells, humoral immunity, and potentially the development of $CD19^-$ B cell cancers [110]. As a result, other studies have explored CARs for antigens expressed solely on tumour cells to avoid off-target toxicity [110,111]. Unlike CD19, the receptor tyrosine kinase-like orphan receptor 1 (ROR1) is expressed on B cell malignancies and solid tumours, but not normal cells [110]. Thus, Deniger et al. delivered two SB transposons via nucleofection to produce 2nd generation ROR1-specific CAR T cells [110]. Similarly, Krishnamurthy et al. engineered CAR T cells with the SB platform to target human endogenous retrovirus K (HERV-K), which is upregulated on melanoma cancers [111]. Adusumilli et al. engineered CAR T cells to target mesothelin (MSLN), which is highly expressed in malignant pleural mesothelioma and metastatic lung and breast cancers [112]. Other investigations have extended this approach by using the SB platform to generate interleukin-3 receptor α-chain (CD123)-specific CAR T cells and CD33-specific CAR cytokine-induced killer (CIK) cells to treat other haematological malignancies, such as acute myelogenous leukaemia (AML) [113–115]. While the initial success of CAR T cell therapy has expanded its use into clinical trials, it can lead to the development of cytokine release syndrome (CRS) [116]. This systemic inflammatory syndrome is caused by activated T cell proliferation with rapid release of inflammatory cytokines, which can be toxic to the patient [116].

Engineered TCRs have also been applied to target tumour-associated antigens and have various advantages over CARs including

decreased CRS and the ability to recognize a larger array of potential antigens [117,118]. Peng et al. delivered the SB platform via electroporation to introduce TCRs targeting p53 and MART-1 in peripheral blood lymphocytes [119]. The modified lymphocytes had comparable transgene expression and phenotypic function to those transduced with retroviruses [119]. Field et al. also compared lentiviral delivery and SB nucleofection by generating murine-human chimeric TCR-engineered T cells to target Wilms' tumour 1 (WT1) [31]. The SB platform had a slightly lower transfection efficiency than lentiviral integration, but SB-modified cells could be readily expanded and had more randomly distributed integration sites, reducing the chance of oncogenesis by disruption of an actively transcribed gene [31]. Deniger et al. delivered the SB platform via electroporation to engineer mutation-specific TCRs unique to each patient's tumour, creating a personalized T cell therapy to produce improved patient outcomes [120]. Despite their advantages, engineered TCRs have yet to reach the same level of clinical application as CARs due to their lower antitumour activity and higher risk of off-target reactivity [105].

4.2. Enhancing antitumour activity of T cells

Recent investigations have used similar strategies to generate antigen-specific T cells while also utilizing gene editing to increase their antitumour activity. A variety of gene editing platforms have been used to replace the endogenous TCRs with a transgenic TCR to eliminate competition in signalling and promote T cell activation driven by the introduced receptor [65,121,122]. For example, Clauss et al. used SB transposon minicircle vectors encoding RNA and miRNA to express the engineered TCR and disrupt the endogenous TCR α (TRAC) and β (TRBC) chains, respectively [121]. The use of miRNA reduced mispairing with the endogenous TCRs, increased surface expression of the transgenic TCR, and enhanced antigen-specific T cell functionality [121]. Similarly, Provasi et al. used ZFN to disrupt the endogenous TCRs and stably express WT1-specific TCRs in T cells [65]. The engineered T cells showed improved recognition to antigen and sustained antitumour activity *in vivo* without off-target reactivity [65]. Legut et al. used CRISPR/Cas9 to simultaneously disrupt TRBC and transduce a cancer-specific TCR, which resulted in increased surface expression of the transgenic TCR and improved responses to antigen [122]. Moffett et al. described a "hit-and-run programming" of T cells and haematopoietic stem cells in which NPs are delivered to transiently express a megaTAL nuclease mRNA targeting TRAC [93]. The NPs did not affect virus-mediated gene transfer, so the same cells were then transduced with a lentiviral vector delivering a tumour-specific CAR [93]. NPs have also been used to inhibit TGFβ signalling and subsequently increase T cell antitumour activity. Schmid et al. used polymer NPs targeting $CD8^+$ T cells to deliver and release the TGFβ inhibitor SD-208 to mouse T cells *in vivo,* extending the survival of tumour-bearing mice [123]. This strategy also enabled delivery of TLR7/8 agonist to target PD-1, increasing the antitumour activity of $CD8^+$ T cells [123]. Similarly, Yang et al. delivered the TGFβ inhibitor SB525334 via gold NPs to T cells *in vivo* to enhance their cytokine polyfunctionality in a cancer vaccine model [101].

In addition to disrupting the endogenous TCRs, gene editing has been used to disrupt genes that contribute to T cell exhaustion and enhance antitumour activity. Various inhibitory signals can affect T cell signalling pathways and reduce the efficacy of T cell immunotherapies. Thus, investigations have focused on disrupting the immune checkpoint receptors, programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) [124]. Beane et al. used ZFN delivered via electroporation of mRNA to disrupt PD-1 in melanoma tumour infiltrating lymphocytes (TILs) [125]. The edited TILs showed improved *in vitro* effector function and a significantly increased polyfunctional cytokine profile [125]. In addition, studies have used CRISPR/Cas9 to disrupt PD-1 in T cells for increased antitumour activity in a variety of cancers, including gastric, breast, and liver cancer [97,126,127]. Lu et al. used CRISPR/Cas9 delivered via liposomes to disrupt PD-1, which generated T cells with high antitumour activity that could secrete the pro-inflammatory cytokine IFN-γ and kill HepG2 cells *in vitro* [97]. Similarly, CRISPR/Cas9 has been used to disrupt CTLA-4 in cytotoxic T lymphocytes (CTLs). Shi et al. delivered CRISPR/Cas9 via lentiviral vector to disrupt CTLA-4 in CTLs, enhancing their antitumour activity in a mouse xenograft model of colorectal carcinoma [66]. Zhang et al. delivered RNPs via electroporation to disrupt CTLA-4, generating CTLs with an enhanced immune response and increased cytotoxicity against bladder cancer cells *in vitro* [128]. Beyond PD-1 and CTLA-4, investigations have disrupted lymphocyte activation gene-3 (LAG-3) or diacylglycerol kinase 1 (DGK) to increase T cell activity, and granulocyte-macrophage colony-stimulating factor (GM-CSF) to prevent CRS [124,129,130]. CRISPR/Cas9-mediated knockout of endogenous TGF-β receptor II (TGFBR2) has also been recently demonstrated to increase T cell activity in solid tumours by reducing the Treg conversion that results in CAR T cell exhaustion [131].

Given the benefits of disrupting both the endogenous TCRs and immune checkpoint receptors, recent studies have combined these strategies. Gautron et al. used TALEN to disrupt both TRAC and PD-1, improving CAR T cell functionality [132]. In a recent phase I first-in-human clinical trial, CRISPR/Cas9 was used to remove the endogenous TCRs in T cells from three patients with refractory cancer, which reduced TCR mispairing and enhanced expression of the cancer-specific TCR transgene NY-ESO-1 [54]. In addition, disrupting PD-1 increased T cell antitumour activity [54]. The CRISPR-based system was able to achieve highly specific editing at the targeted loci without clinical toxicity, and the edited T cells effectively targeted tumour cells [54]. Introducing an engineered TCR while disrupting an immune checkpoint inhibitor resulted in persistent antitumour T cell activity.

Recent advances in non-viral gene editing strategies have also enabled efficient site-specific integration of CAR or other transgenes to further improve T cell functionality. By targeting the locus of the endogenous TCR, transgene introduction can simultaneously disrupt gene expression [133]. Eyquem et al. used CRISPR/Cas9 delivered via electroporation to direct a CD19-specific CAR into the TRAC locus. The CAR T cells had enhanced potency and delayed effector differentiation and exhaustion [134]. Similarly, Sachdeva et al. used TALEN delivered via electroporation to insert a CAR into TRAC, and interleukin-12 (IL-12P70) into either interleukin-2 receptor subunit alpha (IL2Rα) or PD-1 locus [135]. The edited CAR T cells secreted IL-12P70 in a tumour cell-dependent manner and had improved antitumour activity *in vitro* and *in vivo* [135]. Similarly, other investigations have introduced pro-inflammatory cytokines into T cells to promote a memory response and increase their antitumour activity. Hurton et al. used a SB plasmid-based system delivered via electroporation to co-express CAR with a membrane-bound chimeric interleukin-15 (mbIL15) [136]. This resulted in CAR T cells that were phenotypically similar to T memory stem cells, a rare T cell subset with potential for long-term persistence [136].

4.3. Generating universal CAR T cells

Although autologous CAR T therapies have had successful clinical outcomes, their widespread application has been limited by the complexity and cost of manufacturing patient-derived CAR T cells [137]. These therapies heavily rely on the ability to harvest sufficient autologous T cells from cancer patients [138]. Although donor-derived CAR T cells could overcome many of the immune defects associated with cancer treatment and simplify the manufacturing process, current CAR T cell therapies use autologous T cells to prevent GVHD [6]. Incompatibility between the major and/or minor histocompatibility antigens from the host and donor causes allogeneic T cells to be

rejected [139]. In order to create safe allogeneic CAR T cell therapies, this incompatibility must be avoided or adverse immunological interactions must be suppressed [139].

Recent investigations have applied gene editing to modify allogeneic T cells from healthy donors and generate CAR T cells that are universally accepted by other patients [26]. Specifically, disrupting the endogenous TCRs of allogeneic T cells prevented them from recognizing host antigens, which leads to GVHD [53]. In the first study to generate universal CAR T cells, Torikai et al. used ZFN to disrupt TRAC and TRBC in CD19-specific donor CAR T cells, which prevented GVHD without compromising CAR-dependent effector functions [140]. Allogeneic CAR T cells can also be targeted by host T cells in a host-versus-graft (HVG) effect. To prevent the host cells from killing newly introduced CAR T cells, human leukocyte antigen (HLA) expression on allogeneic T cells can be disrupted [6]. In addition to targeting TRAC and TRBC, Torikai et al. delivered ZFN via electroporation to disrupt HLA-A in CD19-specific CAR T cells and embryonic stem cells, which prevented an adverse immune response [141].

The HVG effect in allogeneic CAR T cell therapy can also be mitigated by using a lymphodepleting agent to suppress the host immune system [139]. However, these agents can be toxic to the introduced CAR T cells, so investigations have worked to generate universal CAR T cells with lymphodepletion resistance. Poirot et al. used TALEN to generate universal CAR T cells by disrupting the endogenous TCRs and CD52, a protein targeted by the chemotherapeutic agent alemtuzumab [142]. The CAR T cells did not elicit GVHD and targeted $CD19^+$ tumours, even in the presence of alemtuzumab [142]. Similarly, Qasim et al. used TALEN to disrupt the TRAC and CD52 loci in donor cells and generate universal CD19-specific CAR T cells [52]. Two infants with relapsed and refractory ALL, undergoing lymphodepleting chemotherapy and anti-CD52 serotherapy, were infused with these CAR T cells and achieved molecular remission within 28 days [52]. This strategy has also been used to develop universal CAR T cells that target different antigens. Sommer et al. used TALEN delivered via electroporation to generate universal B cell maturation antigen (BCMA)-specific CAR T cells with lymphodepletion resistance and reduced risk of GVHD [143]. The allogeneic BCMA-specific CAR T cells induced sustained antitumour responses and maintained their phenotype and potency after scale-up manufacturing [143].

Compared to designer nucleases, CRISPR/Cas9 multiplex gene editing offers a more efficient strategy for generating CAR T cell therapies [53,60]. Ren et al. used a one-shot CRISPR protocol that incorporated multiple gRNAs into a lentiviral vector to disrupt the endogenous TCR and HLA class I genes [60]. The universal CAR T cells were also designed to exhibit resistance to inhibitory pathways such as PD-1 and CTLA-4 [60]. To further develop this therapy, the same group used this protocol to simultaneously generate universal CAR T cells and disrupt immune checkpoint receptors that inhibit T cell activity [144]. Specifically, they used CRISPR/Cas9 delivered via electroporation to disrupt the endogenous TCR and PD-1, enhancing antitumour activity [144]. They also disrupted β-2 microglobulin (B2M) to suppress the HVG effect in allogeneic T cells [144]. CRISPR/Cas9 has also been used to prevent unintended CAR T cell fratricide caused by shared antigen expression between CAR T cells and malignant T cells [138]. Cooper et al. used CRISPR/Cas9 to develop fratricide-resistant universal CD7-specific CAR T cells that targeted T cell ALL *in vitro* and *in vivo* without inducing GVHD [138].

5. Conclusions and future directions

Gene editing technology has transformed adoptive T cell therapies by increasing their potential to address currently unmet clinical needs. Designer nuclease and CRISPR/Cas9 gene editing platforms can facilitate precise genetic modification to generate engineered T cells with improved responses to antigens, enhanced antitumour activity, and potential for use in allogeneic applications. T cell immunotherapies have shown success treating B cell malignancies, but there are still challenges in applying these therapies to other types of cancer. Recent CRISPR/Cas9-based genome-wide screens have discovered novel drug targets to further advance genetically engineered T cell therapies [145–147]. Identifying new gene targets to increase the efficacy of T cells in immunosuppressive tumour microenvironments could extend the use of these therapies to solid tumours. In addition, future investigations must confirm the efficacy and safety of gene editing technologies for *in vivo* applications. Novel delivery systems, such as cell squeezing and nanoparticles, should be further explored for delivering gene editing technologies to T cells to potentially improve efficiency and reduce cytotoxicity. Ongoing advances in gene editing strategies, identification of new drug targets, and implementation of novel delivery platforms could broaden the application of T cell immunotherapies to successfully treat other haematological malignancies and even solid tumour cancers.

Search strategy and selection criteria

Data for this review were identified by searches of PubMed, Google Scholar, ClinicalTrials.gov, and references from relevant articles. Search terms used include “gene editing”, “T cell”, “CAR T cell”, “engineered TCR”, “Sleeping Beauty”, “ZFN”, “TALEN”, and “CRISPR/Cas9”. Only articles published in English between 1988 and 2021 were included.

Contributors

E.S.A., M.M.B., and M.J.M. contributed to the conceptualization, review, and editing of the final manuscript. E.S.A. and M.M.B. contributed to the literature search and writing of the original draft. E.S.A. designed the figures.

Declaration of Competing Interest

E.S.A., M.M.B., and M.J.M. declare no conflicts of interest.

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