INTRODUCTION

1.1 Cell immunotherapy and the challenges it faces

With precise targeting and impressive efficacy, CAR-T (Chimeric Antigen Receptor Engineered T cell) and TCR-T (T Cell Receptor Engineered T cell) cell therapies have become powerful and innovative therapeutic modalities for cancer patients. CARs are recombinant receptors that redirect the T-cell activity towards target cells expressing specific surface antigen, independent of the classic peptide/MHC-TCR recognition patterns. While TCR-T cells are directed to recognize tumor-specific peptide epitopes-generated from inside the cells with the dependence on MHC molecules.

The first-generation of CAR consists of the binding moiety from a monoclonal antibody fused to the constant regions of a TCR, and later this design was modified to use a single-chain Fv fragment (scFv) of an antibody linked with CD3 zeta or FcγRIIIA γ signaling chain (Figure 1). Such engineered T cells specifically lysed target cells and produced cytokines. However, clinical studies showed its limited antitumor efficacies (Table 1), probably owing to the short persistence of CAR-T cells in vivo. The
second-generation of CAR (Figure 1) provided a costimulatory signal in combination with the primary activation signal.11,12 These CAR-T cells have a higher level of cytokine production, improved persistence in vivo and potent clinical activities (Table 1),13,14 enabling FDA’s first two approvals of CAR-T therapies in 2017. The third-generation of CAR contained two costimulatory domains combined with an activation domain (Figure 1), which suggested an enhancement of antitumor response compared to the second-generation CAR-T cells (Table 1).15,16 A clinical trial comparing the antitumor efficacy between the second- and the third-generation CAR-T cells is currently underway (NCT01853631). The fourth generation of CAR-T cells (Table 1), engineered based on the backbone of the second-generation CAR, were equipped with an inducible expression cassette to produce a transgenic cytokine, for example, IL-12, IL-18, upon the engagement of CAR to the specific tumor target (Figure 1).17

CAR-T and TCR-T cell therapies are showing promising results for cancer treatment, especially for targeting the B-cell lineage-restricted CD19 molecule expressed on B-cell leukemias and lymphomas with CD19-specific CAR-T cells.13,18-20 However, challenges including poor persistence, long manufacturing time, and limited infiltration of engineered T cells into immunosuppressive environment, still remain to be addressed. Through disrupting TCR and HLA genes, knocking out checkpoint inhibitory molecules, etc, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-Associated protein) technology holds enormous promise to enhance T-cell functionality and improve drug efficacy.

1.2 | CRISPR-Cas9 genome editing system: a genetic tool to enhance T-cell functionality

Cas9 functions as a RNA-dependent endonuclease and can be directed to the DNA target sites under the limitation of the protospacer adjacent motif (PAM), guided by a chimeric single-guide RNA (sgRNA).21 Cas9/sgRNA system could target any DNA sequence of interest by changing sgRNA guide sequence, and cut DNA to cause double-strand breaks (DSBs).22 These DSBs are repaired by error-prone nonhomologous end joining (NHEJ) or precise homology-directed repair (HDR) pathways. NHEJ leads to insertions or deletions (indel) of target gene and makes gene knock-out possible. HDR uses assisted recombination of DNA donor templates to reconstruct cleaved DNA with precise repair, which could be used to knock-in desired DNA.

Compared to other genome editing strategies such as transcription activator-like effector nucleases (TALENs), and
zinc-finger nucleases (ZFNs), CRISPR/Cas9 genome editing is more rapid, cost-effective, and it has been applied widely in plants and animals with its easier feasibility. Differences among the three types of genome editing systems have been discussed in numbers of review papers.23-26

2 | APPLICATIONS OF CRISPR/CAS9 TECHNOLOGY IN T-CELL THERAPY

Although commercial products of CAR-T have been successfully launched,20,27 including Kymriah and Yescarta from Novatis and Gilead/Kite, respectively, there is still much room to improve the existing T-cell therapy. We summarized the recent progress about how CRISPR/Cas9 system could be harnessed to produce advanced CAR-T cell products, with lower cost, reduced risk of causing malignancies, improved antitumor activities and efficacies (Table 2).

2.1 | Generation of off-the-shelf CAR-T cells

Current CAR-T therapy mostly focused on autologous T cells owing to the limitation of intrinsic MHC restriction. To shorten the manufacture cycle and lower the cost of CAR-T cell products, the concept of off-the-shelf CAR-T cells was emerged (Figure 2). Endogenous TCR on allogeneic T cells were eliminated by ZFN and TALEN to avoid graft-vs-host disease (GVHD), and HLA molecules were disrupted to prevent a rejection from recipient's immune system.28-30 It has been reported that universal CAR-T cells with TCRα chain and CD52 gene disrupted by TALEN were infused to two infants with relapsed refractory CD19+ ALL,31 demonstrating the therapeutic potential of gene-editing technology.

Compared to ZEN and TALEN technologies, CRISPR/Cas9 gene editing system holds greater promise due to its simplicity and high effectiveness to increase the efficacy of therapeutic agents or work as standalone therapeutics. TRAC and B2M genes have been knocked out simultaneously by CRISPR/Cas9 to generate universal CAR-T cells.32 Besides, to improve antitumor activity, multiplex genomic editing of CAR-T cells by CRISPR/Cas9 has been reported.33,34 This one-shot CRISPR system has shown to improve gene targeting efficiency and facilitate the manufacture of universal CAR-T cells deficient in CD3 and HLA-class I.33

Nevertheless, there is a potential issue when β2M and TCR gene loci are eliminated to prevent allo-rejection. The elimination of HLA-class I of T cell could increase the attack from NK cell due to its “missing self” phenotype,35 which should be taken into consideration for the future therapy.
To avoid oncogenic transformation and transcriptional silencing caused by random integration of CAR into genome by lentivirus infection, knocking in CAR at designed gene locus via homologous recombination (HR) has been achieved (Figure 2). Schumann et al. conducted a targeted nucleotide replacement in CXCR4 and PD-1 (PDCD1) gene loci by electroporating Cas9:sgRNA ribonucleoproteins (Cas9 RNPs) with homology-directed repair template oligonucleotides, establishing applications of Cas9 RNP technology for genome engineering in human T cells. Eyquem et al showed that human T cells were electroporated with Cas9 mRNA and sgRNA to specifically insert a CD19-specific CAR into TRAC locus, which resulted in not only uniform CAR expression but also enhanced T-cell potency. These results indicate site-specific knocking-in a CAR may provide a safer and potent T-cell product.

In addition to CAR knock-in, a TCR that recognizes NY-ESO-1 tumor antigen has also been knocked into endogenous TCR gene locus, giving rise to specific recognition of tumor antigens and productive antitumor cell responses. Such precise knock-in of CAR or TCR into a specific gene locus by CRISPR/Cas9 system leads to the enhancement of antitumor responses and brings additional clinical benefits to the patients engrafted with engineered T cells.
Inhibition of tumor growth. Interestingly, it has been reported that blockade of PD-1, LAG-3 (Lymphocyte Activation Gene 3) or CTLA-4 led to a compensatory upregulation of the other checkpoint pathways, which means combinatorial blockade strategies should be applied in practice. This conclusion is in accordance with Tanvetyanon’s review that combinatorial blockade of PD-1 and CTLA-4 may produce a higher antitumor response than PD-1 blockade alone in patients. The clinical potential of combined disruption of PD-1 and LAG-3 has also been explored in the CAR-T cell therapy.

Except knock-out of checkpoint molecules, an alternative way is to coexpress the PD-1-blocking scFv with CAR, which has improved the antitumor activities of CAR-T cells. CRISPR/Cas9 has also been used in TCR-T cells to disrupt PD-1 gene. The clinical trial with the strategy to disrupt endogenous TCR and PD-1 gene is ongoing (NCT03399448).

2.4 Generation of CAR-T cells expressing exogenous cytokines for efficacy improvement

In addition to TCR engagement (Signal 1) and costimulatory signaling (Signal 2), cytokines play essential roles in regulating T-cell function. Constitutive expression of IL-12 in CAR-T cells to destroy antigen-loss cancer cells, and improve antitumor efficiency has been reported. IL-15, which is functionally associated with T-cell memory, was coexpressed with anti-CD19 CAR to develop long-term persisting CAR-T cells. CRISPR/Cas9 has also been used in TCR-T cells to disrupt PD-1 gene. The clinical trial with the strategy to disrupt endogenous TCR and PD-1 gene is ongoing (NCT03399448).

3 DIFFERENT METHODS OF GENE DELIVERY SYSTEMS FOR CRISPR/CAS9-BASED CELL IMMUNOTHERAPY

An efficient gene delivery system is critical for the success of CAR and TCR cell therapies and the efficacy of gene editing. There are several major delivery systems (Table 3). Gammaretrovirus and lentivirus belong to the retroviral family and possess the intrinsic capability to integrate into the host genome, allowing long-term and stable transgene expression, with its capacity less than 4.8 kb (Table 3). Adenovirus has strong immunogenicity, with its capacity less than 4.8 kb (Table 3). AAV vectors have broad spectrum of target cell types and low immunogenicity, with its capacity less than 4.8 kb (Table 3).

3.2 Transposon

Besides the viral vector delivery systems described above, Transposon has emerged as a new potential delivery tool for transferring genes of interest (Figure 3B). The vector system of DNA transposon comprises a transposon containing a gene of interest flanked by terminal inverted repeats (TIRs), and a transposase that binds to TIRs. These nonviral vector integration systems, such as PiggyBac (PB) and Sleeping Beauty (SB), also showed advantages for gene delivery (Table 3).
| Table 3 | Different methods of delivery systems for CRISPR/Cas9-based cell immunotherapy |
|---------|--------------------------------------------------------------------------------|
| **Forms of payload** | **Capacity** | **Advantages** |
| Viral delivery system | Infection | AdV | 7.5-37 kb | 1) Broad infectivity; 2) High titers; 3) Large cloning capacity; 4) Transient expression without integration |
| | | AAV | 5 kb | 1) Relatively broad host spectrum; 2) Low immunogenicity; 3) Transient gene expression |
| | | Gammaretroviral & lentiviral vectors | 8-9 kb | 1) Stable gene expression; 2) Lentivirus infects dividing and nondividing cells |
| Nonviral delivery system | Electroporation | Minigene/minicircle (Transposon) | More than 100 kb | 1) Lower immunogenicity; 2) Efficient stable genome modification; 3) Reduced cost |
| | | DNA | More than 100 kb | 1) Easy to operate; 2) Lower cost; 3) Scalable manufacturing |
| | | mRNA | Flexible | 1) Higher efficiency; 2) Rapid expression; 3) Reduce off-target effect |
| | | RNP | Flexible | 1) Lower off-target; 2) Lower cellular toxicity |
| Nano-carrier | RNA, DNA, or protein | Flexible | 1) Flexible payload sizes and formats; 2) Low immunogenicity; 3) Transient or stable gene expression |
| Squeeze | Different bioactive materials, including small molecules, polysaccharides, siRNA, proteins, carbon nanotubes and quantum dots | Flexible | 1) Diverse range of payloads; 2) Higher efficiency; 3) Unchanged expression profiles; 4) High throughput; 5) Improved safety |
| **Disadvantages** | **Reference** | **Clinical trials** |
| 1) High immunogenicity | 55-57 | None |
| 1) Limited cargo size; 2) Difficult to produce pure viral stock | 58-59 | Projected by Editas Medicine in LCA10 patients |
| 1) Genotoxicity; 2) Random integration | 60-62 | CAR-T (gammaretroviral): NCT01583686, NCT02744287 (lentiviral): NCT02935543, NCT03274219, NCT03166878 |
| 1) Cellular toxicity (Transposon); 2) Random integration | 63-65 | NCT03389035, NCT00968760, NCT01497184, NCT01362452, NCT01653717, NCT02529813 |
| 1) Reduced cell viability; 2) Spontaneous vector integration | 28,29,37,40 | CRISPR: NCT03057912, NCT03164135, NCT0365678, NCT03545815, NCT03081715, NCT03598967, NCT03690011, NCT02863913, NCT02867345, NCT02867332, NCT02793856, NCT03044743 |
| 1) Medium cost; 2) Fast degradation | 66-70 | NCT03399448, NCT03166878 |
| 1) High cost; 2) Fast degradation | None for CRISPR/Cas9 |
| Requires special expertise | 51-53,71 | NCT02340156, NCT03020017, NCT01455389 |

AdV, adenovirus; AAV, adenovirus associated virus; RNP, ribonucleoprotein.
LCA10, TYPE 10 leber's congenital amaurosis; CAR, chimeric antigen receptor; CRISPR, clustered, regularly interspaced, short palindromic repeats.
Transposon system was exploited in clinical trials for cancer immunotherapy. Human T cells genetically modified by the SB transposon/transposase system to express a CD19-specific CAR were evaluated in 2016. The clinical results demonstrated that these SB platform-engineered CAR-T cells were safe, and further supported the clinical development of this nonviral gene therapy approach. Clinical trials at MD Anderson Cancer (Table 3) have shown that these transposon-engineered CAR-T products work robustly and feasibly.

### 3.3 | Electroporation

Cas9 mRNAs or proteins were electroporated into CAR-T cells (Figure 3C) to knock-out specific genes like TCR, CTLA-4, and PD-1 gene. Ren et al accomplished a versatile system for rapidly generating multiplex genome-edited CAR-T cells, by lentiviral infection of one-shot CAR vector with multiple sgRNAs and electroporation of Cas9 mRNA. Other labs developed a protocol for combined Cas9 RNP-mediated gene editing and lentiviral transduction to generate PD-1 deficient anti-CD19 CAR T cells. Hu et al explored a simplified protocol for generating PD-1 deficient CD133-specific CAR T cells, by nucleofecting plasmids of CRISPR/Cas9 system to disrupt PD-1 gene and piggyBac transposon system for CAR gene expression. This convenient method avoids manufacturing of RNAs or proteins and reduces the processing of T-cell modification.

The key benefit of using nanoparticle over electroporation is high viability and expansion capability of manipulated cells, allowing its broader applications.

### 3.5 | Microfluidics-based CellSqueeze

Although gene delivery by electroporation has been widely used, it was found by genome-wide approach that electroporation treatment may disrupt the expression profiles of key functional transcripts and lead to the perturbation of cytokine secretion. A microfluidic delivery system called cell squeezing was recently used for compound delivery. Its mechanism of action is based on mechanical membrane disruption (Figure 3E), which has minimal effects on transcriptional responses and will not modulate T-cell activity. The CellSqueeze technology from SQZ Biotechnologies Co. (Table 3) is able to introduce a wide range of compounds into varieties of cell types like immune cells, embryonic stem cells etc.

Compared with other delivery systems described above, CellSqueeze technology could achieve high delivery efficacy without adversely affecting cell viability and expression profiles.

### 4 | CHALLENGES AND THE FUTURE DIRECTIONS OF THE APPLICATION OF CRISPR/CAS9 TECHNOLOGY

#### 4.1 | An increased risk of tumor malignancy

CRISPR-Cas9 technology has made gene editing simpler and faster than ever. However, a study points out this popular gene-editing tool could inadvertently cause cancer. It found that Cas9 RNP delivery triggers a p53-dependent DNA damage response that suppresses gene correction.

#### 4.2 | Failure of genome editing caused by the immunogenicity elicited from anti-Cas9 responses

A recent study showed that the most widely used forms of CRISPR could be an immunogen in humans. Instead of modifying the genome while used therapeutically, the editing tool could trigger an adaptive immunity to Cas9 proteins, raising considerable concerns for the future CRISPR clinical trials.

#### 4.3 | An increased risk of off-target mutagenesis

The off-target mutation, which may cause genomic instability and disrupt the functionality of other normal genes, is still
A. Suspension of cells and target materials

B. Cell deformation (squeezing)

C. Temporary disruption of the cell membrane

D. Target material enters the cell

E. Membrane recovers

Transposase binding

“Cut and Paste”

Genomic integration

Virus delivery

Nanocarrier

mRNA

Nanocarrier: DNA, RNA, or protein

T cell

Electroporation:

Plasmid, RNP, Chem-RNA, RNA

Target gene

Transposase

TIR

TIR

Target gene

Cas9

PAM

NGG

NCC

PAM

Cas9

sgRNA

Cas9

TIR

TIR

Target gene

CD28/4-1BB

TM

CD8

T cell

E. Membrane recovers

A. Suspension of cells and target materials

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Transposase binding

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Electroporation:

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Target gene

Transposase

TIR

TIR

Target gene

Cas9

PAM

NGG

NCC

PAM

Cas9

sgRNA

Cas9

TIR

TIR

Target gene

CD28/4-1BB

TM

CD8

T cell
the major concern of CRISPR/Cas9 system in biomedical and clinical application. Although the targeting specificity of Cas9 is believed to be tightly controlled by the guide sequence of sgRNA and the presence of PAM, potential off-target cleavage activity could still occur with even three to five base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence. Moreover, Cas9 stays in the cells for a period of time after treatment, which increases the incidence of DNA being cut in the wrong place.

4.4 | Inefficient delivery systems for CRISPR/Cas9

There are several delivery systems for CRISPR/Cas9 as described previously, but the delivery efficiency of each system is still not satisfying, especially for in vivo application. In addition, large DNA fragment knock-in and multiplex genome editing are generally hard to achieve.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

CRISPR/Cas9 has provided a simple, cheap, and fast way to manipulate genomes. CRISPR-edited CAR-T and TCR-T cells hold out great hope and potential for the next generation cancer immunotherapy, particularly for the treatment of solid tumors. Considering the safety issues related to CRISPR/Cas9 system, mutated Cas9 including Cas9 nicksense,80 truncated sgRNA,81 or other more accurate nucleases with longer PAM sequences are explored to reduce off-target effect. As to improve delivery efficiency of CRISPR, new delivery platforms like nanocarrier and CellSqueeze technology, have recently emerged. With rapid improvement in the field of gene therapy, CRISPR/Cas9 genome editing system is expected to have broader therapeutic applications in cancer immunotherapies.

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