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Review Paper

Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy

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Abstract

Chimeric antigen receptor(CAR) T-cell therapy has shown remarkable effects and promising prospects in patients with refractory or relapsed malignancies, pending further progress in the next-generation CAR T cells with more optimized structure, enhanced efficacy and reduced toxicities. The clustered regulatory interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) technology holds immense promise for advancing the field owing to its flexibility, simplicity, high efficiency and multiplexing in precise genome editing. Herein, we review the applications and explorations of CRISPR/Cas9 technology in constructing allogenic universal CAR T cells, disrupting inhibitory signaling to enhance potency and exploration of safer and more controllable novel CAR T cells.

Key words: CAR T cells; CRISPR; Cas9; cancer immunotherapy; genome editing

Introduction

Cancer immunotherapy is the fourth mainstream treatment after surgery, chemotherapy and radiotherapy. Adoptive T-cell immunotherapy, particularly chimeric antigen receptor (CAR) T cell therapy, has revolutionized cancer therapy especially after the FDA approval of Kymriah and Yescarta (CD19-directed CAR T cells in B-cell leukemia and lymphoma) [1–3]. CARs are synthetic receptors typically containing an antibody-derived target-binding extracellular domain, a hinge region, a transmembrane domain and an intracellular signaling moiety capable of activating T cells [4,5]. T cells programmed with CARs can specifically recognize and kill antigen-expressing cells without the restriction of major histocompatibility complex (MHC). Clinical data has demonstrated that CAR T-cell therapy can induce durable complete remissions (CRs) in patients with a variety of hematologic and solid cancers, especially in relapsed/refractory acute lymphoblastic leukemia (ALL) and multiple myeloma with striking response rates of 80–100% [6–8]. Despite of promising efficacy of CAR T-cell therapy, there are several challenges awaiting for solutions, such as insufficient quantity and poor quality of autologous T cells, CAR T cell exhaustion and tumor suppressive microenvironments, potential self-killing and uncontrollable proliferation.

Optimization of the CAR T designs is supposed as one of the main tracks to tackle these limitations. The first generation of CAR T cells with only CD3 zeta intracellular chain was found to have modest proliferative and cytotoxic capacity [9–12]. The second generation of CARs contains a single costimulatory domain (CD28 or 4-1BB), proven to attain an improved efficacy and *in vivo* survival, whereas the third generation has two or more costimulatory domains (CD28, 4-1BB, ICOS or OX40), not superior to the second generation [13–15]. More functional elements are considered to be added to the next generation of CARs, like interleukins genes to increase potency, chemokine receptors genes to improve T-cell trafficking and on–off switches or suicide genes to enhance safety and controllability [16–18]. The structures and features of every generation of CAR-T are shown in Table 1.

The development of genomic editing technologies opens a window to accelerate the fourth generation of CAR T cells.

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Table 1. Structure and features of every generation of CAR-T

Structure of CAR		First-generation CAR	Second-generation CAR	Third-generation CAR	Next-generation CAR	Universal CAR
Similarities	An extracellular antigen-recognition region consisting of an scFv A flexible hinge region derived from a CD8 molecule or CD28 or Fc region of an antibody A transmembrane derived from CD8 or CD28					
Differences	Intracellular domain	only CD3ζ	CD3ζ One costimulatory molecule: CD28 or 4-1BB	CD3ζ $\geq$ 2 Costimulatory molecules: CD28, 4-1BB, ICOS or OX40	CD3ζ One costimulatory molecule: CD28 or 4-1BB Functional elements: interleukins, chemokine receptors, on-off-switches or suicide genes etc.	CD3ζ One costimulatory molecule: CD28 or 4-1BB Knock-out of TCRs or/and HLAs
	In vivo Persistence	Low (days to 2 months)	Imporved (3 months to years)	Not superior to 2nd-generation	Exploration	Exploration
	Antitumor Effects	Low (ORR 0–40%)	Imporved (ORR depending on the tumor type)	Not superior to second-generation	Exploration	Exploration

There are currently three major genomic editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) [19–21]. Although ZFNs and TALENs have been applied to engineer T cells in clinical trials, the recognition of targetable DNA sequences is based on complicated protein conformation, a pair of Zn-finger binding domains or a pair of TALE DNA binding domains, accompanying with complex designs and relatively low gene-editing efficiencies [22,23]. CRISPR/Cas9, directed by a small guide RNA (sgRNA) to the target site, has become the most popular and developed of these tools due to its simplicity, flexibility, high efficiency and multiplexable genome editing capabilities [24–26]. A sgRNA-guided Cas9 nuclease induces a DNA double-stranded break at targeted genomic locations, subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ, an error prone repair pathway, can result in insertions or deletions of small nucleotide sequences and HDR can knock-in relatively large gene segments in the presence of a homology repair template at the site of interest [27–29]. Therefore, the combination with CRISPR/Cas9 technology will further expand the landscape of T-cell engineering. Besides knock-in of functional genes, such as interleukins and suicide genes, to product next-generation CAR T cells, other strategies comprises knock-out of endogenous genes, such as TCRs and MHCs, to develop 'off-the-shelf' universal CAR T cells [30], disruption of inhibitory receptors (such as PD-1 and TGF beta receptor) to ameliorate suppressive microenvironments [31,32], integration of the CAR cassette into the specific gene locus(such as TRAC and TET2) to improve efficiency and safety [33,34], deletions of target genes to avoid self-killing of CAR T cells [35]. CRISPR/Cas9 technology is unveiling a new era for CAR T-cell therapy. All gene-edited CAR T cells discussed here are shown in Table 2.

Production of allogeneic universal CAR T cells

Although currently widespread-used autologous CAR T cells have shown promising results in cancer therapy, limitations exist. Almost 10–15% of enrolled patients were unable to receive infusions of CAR T cells because of poor quality and insufficient quantities of autologous T cells unavailable for manufacturing or rapid disease progression and even death before successful production of certain amount of CAR T cells [1–3]. A UPenn team recently reported a patient relapsing after infusion of anti-CD19 CAR T cells with CD19-negative leukemia that aberrantly expressed the anti-CD19 CAR because the CAR gene was unintentionally introduced into a single leukemic B cell during T-cell manufacturing [36]. The development of universal 'off-the-shelf' CAR T cells from healthy donors can circumvent the constraints and potentially be the mainstream direction in the future. The major barriers of such universal CAR T cell products are graft-versus-host disease (GVHD) and rejection of the infused allogeneic T cells. Endogenous $\alpha\beta$ T cell receptors (TCRs) on adoptively transferred donor lymphocytes can recognize alloantigens in human leukocyte antigen (HLA) mismatched recipients resulting in GVHD; conversely, recognition of foreign HLA molecules on donor T cells may lead to rejection.

ZFNs and TALENs were successfully used to knock-out TCRα constant (TRAC) and TCRβ constant (TRBC) to generate TCR-negative CAR T cells to prevent GVHD without compromising CAR-mediated cytotoxicity [37,38]. Previous researches demonstrated that genetic knock-out of either *TRAC* or *TRBC* loci was sufficient to eliminate expression of $\alpha\beta$TCR on the T cell surface [39]. The Cellectis firstly reported the generation of TALEN-edited allogeneic universal anti-CD19 CAR T(UCART19) cells in which *TRAC* and *CD52* genes were knocked out [40]. CD52 disruption in the CAR T cells allowed effective targeted depletion of patients' autologous T cells using an anti-CD52 antibody (alemtuzumab). The first-in-man application of the products was two infants with high-risk CD19-positive ALL who achieved molecular remission after receiving the infusion of UCART19 cells and attained successful bridge-to-transplantation [41,42]. The remarkable results led to two clinical trials of UCART19 cells: CALM trial in adults and PALL trial in pediatric patients (NCT02746952 and NCT02808442). Pooled data of 20 patients showed acceptable and manageable safety with 15% (3/20) of

Table 2. Overview of the application of genomic editing technologies in CAR-T cells

Target of CAR	Delivery of CAR	Target locus	Gene-editing method	Delivery	Editing efficiency	Reference
CD19	SB electroporation	TRAC and TRBC	ZFNs	mRNA electrotransfer	15–37%	[33]
CD19	Lentivirus	TRAC and CD52	TALEN	mRNA electrotransfer	10–60%	[36]
CD19	AAV vector	TRAC (insert CAR to TRAC)	CRISPR/Cas9	Electroporation	~70%	[29]
CD19	SB electroporation	HLA-A	ZFNs	Nucleofection	40.70%	[40]
CD19	Lentivirus	B2M and TRAC	CRISPR/Cas9	RNA electroporation	52.55–65.21%	[26]
CD19		TRAC, B2M and PD-1			37.05–60.97%	
CD19	Lentivirus	B2M and TRAC	CRISPR/Cas9 (incorporating multiple gRNA cassettes in a single CAR vector)	Lentiviral vector	71.3 ± 6.7%	[42]
		TRAC, B2M and Fas			55.10%	
		TRAC, B2M and PD-1,CTLA-4			40.10%	
CD19	Lentivirus	TCR and B2M	CRISPR/Cas9	RNA electroporation	79.90%	[43]
		TRBC, B2M and PD-1			/	
PSMA	Lentivirus	dnTGF-βRII	/	Lentiviral vector	53.20%	[55]
CD19	Retrovirus	IL-15 and an suicide gene	Inducible caspase-9	Retroviral vector	65%	[57]
CD19	Retrovirus	Safety switch	Inducible caspase-9	Retroviral vector	61% ± 5%	[58]
CD19	Lentivirus	GM-CSF	CRISPR/Cas9	Lentiviral vector	82.20%	[61]
CD33	Lentivirus	CD33 in HSCs	CRISPR/Cas9	Electroporation	40–90%	[31, 64, 65]
CD7	Gammaretrovirus	CD7 in CAR T cells	CRISPR/Cas9	Electroporation	>80%	[66]

severe cytokine release syndrome (CRS) and 10% (2/20) of G1 cutaneous acute GVHD as well as promising efficacy with 88% (14/16) of CR or CR with incomplete blood count recovery (CRi) and 86% (12/14) of minimal residual disease-negative [43]. A MSKCC group showed that directing a CD19-specific CAR to the TRAC locus using CRISPR/Cas9 technology not only minimized the risks of insertional oncogenesis and TCR-induced GVHD, but also enhanced T-cell potency and delayed T-cell exhaustion [33].

ZFNs were also used to target the HLA-A locus to permanently and completely eliminate HLA-I expression in primary and genetically modified human T cells used in clinical trials to evade rejection [44]. In addition, elimination of HLA heavy chains or beta-2-microglobulin (*B2M*), the non-polymorphic subunit of HLA-I complex, would prevent rapid rejection of allogeneic cells [45]. However, ideal universal CAR T cells should be silenced both TCR and HLA to avoid GVHD and rejection without reducing persistence and cytotoxicity *in vivo*. CRISPR/Cas9 has an obvious advantage in simultaneously multiplex and highly efficient genomic editing compared with ZFNs and TALENs. CRISPR/Cas9 was readily applicable to generate double-knockout (*B2M* and *TRAC*, DKO) UCART19 cells with as similar safety and efficacy as wild-type anti-CD19 CAR T cells in preclinical studies [30]. One-shot CRISPR protocol for multiplex genome editing by incorporating multiple gRNA cassettes into a single CAR lentiviral vector was developed to generate DKO UCART19 cells [46]. By combining the lentiviral delivery of CAR with CRISPR RNA electroporation to co-introduce RNA encoding the Cas9 and gRNAs targeting endogenous TCR and B2M concurrently, an improved editing efficiency(~80%) was acquired to construct the DKO UCART19 cells that showed as potent antitumor activities as non–gene-edited CAR T cells both *in vitro* and in animal models [47]. However, the issue of whether such HLA-I negative CAR T cells will be the target of NK cells should be considered. Administering an anti-NK cell depletion antibody or engineering T cells with HLA-E are potential solutions to circumvent NK-mediated rejection [44, 48].

Recent advances in gene-editing technology, especially CRISPR/Cas9, allow for the production of universal CAR T cells starting from healthy donor in which the manufacture and quality of T cells can be preselected as well as GVHD and rejection can be avoided. The CRISPR/Cas9-modified universal CAR T cells need to be further tested for the safety and efficacy in clinical studies and there are currently six relevant ongoing clinical trials (Table 3).

Disruption of inhibitory signaling molecules

The function of T cells was proven to play a significantly important role in the therapeutic effect of CAR T cells [49]. However, T cells are exposed to persistent antigen in patients with malignant tumors, resulting in T-cell exhaustion [50]. Exhausted T cells lose robust effector functions and express multiple inhibitory receptors, such as programmed cell death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), domain-containing protein-3 (TIM-3) and lymphocyte-activated gene-3 (LAG-3), which inhibit T-cell proliferation and cytokine production leading to immune escape [51]. The inhibitory pathways also contribute to suppressive tumor microenvironment, a major barrier of CAR T-cell therapy in solid tumors. Immune checkpoint inhibitors, anti-PD-1/PD-L1 and anti-CTLA-4 antibodies, have shown promising clinical results and been approved by the FDA [52]. Thus, disruption of multiple inhibitory factors is expected to improve the potency of CAR T cells. Recent studies suggested that anti-CD19 CAR T cells with CRISPR-mediated triple-knockout of the TRAC/TRBC, B2M and PD-1 genes displayed stronger

Table 3. Current clinical trials about universal CAR T cells

CAR target	Gene-editing technology	Locus of knock-out	Diseases	Phase	R&D Unit	Study location	NCT ID
CD19	TALENs	TCR and CD52	B-ALL	1	Servier Group company	America, Europe	NCT02808442 NCT02735083 NCT02746952
CD19	CRISPR/Cas9	TRAC and HLA-I	ALL and NHL	1/2	Shanghai Bioray Laboratory Inc.	China	NCT03229876
CD19	CRISPR/Cas9	TRAC and B2M	B cell leukemia and lymphoma	1/2	Chinese PLA General Hospital	China	NCT03166878
CD19	CRISPR/Cas9	TCR and CD52	RR DLBCL	1	Nanjing Bioheng Biotech Co., Ltd	China	NCT04026100
CD123	TALENs	TCR and CD52	RR and Newly Diagnosed High-risk AML	1	Cellectis S.A.	Europe	NCT04106076 NCT03190278
CD123	TALENs	TCR and CD52	RR BPDCN	1	Cellectis S.A.	America	NCT03203369
BCMA	CRISPR/Cas9	TRAC and HLA-I	Multiple Myeloma	1/2	Shanghai Bioray Laboratory Inc.	China	NCT03752541

NHL, Non-Hodgkin lymphoma; RR, refractory or relapsed; DLBCL, diffuse large B-cell lymphoma; BPDCN, blastic plasmacytoid dendritic cell neoplasm.

antitumor functions in contrast to DKO UCART19 cells *in vitro* and in animal models [30,47]. High-fidelity Cas9s with the one-shot platform showed the feasibility of generating PD-1 and CTLA-4 dual inhibitory pathway-resistant DKO UCART19 cells by simultaneous disruption of quadruple genes [46].

The Fas receptor is a member of the tumor necrosis factor α (TNF-α) family of death receptors that mediate cell death [53]. Researches demonstrated that CAR T cell activity was attenuated due to cell Fas-FasL-dependent activation-induced cell death (AICD) [54]. Ren *et al.* [46] also utilized CRISPR/Cas9 technology to generate Fas-resistant universal CAR T cells that observed elevation of AICD resistance and prolonged survival.

Transforming growth factor-β (TGF-β) represses effector T-cell activities through binding the TGF-β receptors (TGFBRI and TGFBRII) to induce heterodimerization of the respective receptors and phosphorylation of the major TGF-β signal mediators SMAD2 and SMAD3, resulting in reduced cytokine production, cytotoxicity and amplification [55]. TGF-β also drives T-cell differentiation into regulatory T cells (Tregs) [56]. Thus, inhibiting TGF-β signaling, a potent immunosuppressive factor in a variety of solid tumors, has the potential to improve the immunosuppressive milieu. Previous studies demonstrated that TGF-β pathway could be blocked by using a dominant-negative TGFBRII (dnTGF-βRII), which lacked the intracellular domain necessary for downstream signaling [57]. Foster *et al.* [31] used a clinical grade retrovirus vector to construct dnTGF-βRII-expressing human antigen-specific cytotoxic T lymphocytes (CTLs) and found that TGF-β-resistant CTLs had a functional advantage over unmodified CTLs in the TGF-β-secreting lymphoma [31]. The clinical trial (NCT00368082) showed that TGF-β-resistant CTLs could safely expand and persist in patients with Hodgkin lymphoma without lymphodepleting chemotherapy and induced complete responses [58]. It was testified that adding dnTGF-βRII to PSMA-targeted human CAR T cells promoted T-cell proliferation and augmented prostate cancer eradication [59]. Chang *et al.* recently described a novel TGF-β CAR containing a scFv based on the sequences of TGF-β-neutralizing antibodies, demonstrating the ability to not only inhibit endogenous TGF-β signaling but also convert TGF-β into a stimulant of T-cell growth [60]. Above results support the potential value of the countermeasure of using CRISPR/Cas9 technology to generate TGF-β-resistant CAR T or UCART cells to improve potency of engineering T cells in solid tumors.

Exploration of safer and more controllable novel CAR T cells

Albeit unprecedented efficacy of CAR T-cell therapy, it is accompanied by serious and even life-threatening toxicities, including CRS, on-target/off-tumor toxicity, neurotoxicity, macrophage activation syndrome/ hemophagocytic lymphohistiocytosis and tumor lysis syndrome, which need to be paid more attention [61]. The most significant and common toxicity of CAR T-cell therapy is CRS, an inflammatory syndrome caused by multiple cytokines, including interferon γ, interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and granulocyte/macrophage colony-stimulating factor (GM-CSF), produced by the CAR T cells themselves and by other cells [62]. Tocilizumab, IL-6 receptor blockade, was approved by FDA for treatment of CAR T cell-induced severe or life-threatening CRS [63]. Thus, blocking relevant cytokines signaling is a hopeful strategy to ameliorate the dilemma and CRISPR/Cas9 can effectively knock-out related molecules. Sterner *et al.* [64]

described CRISPR/Cas9 mediated knock-out of GM-CSF and showed that GM-CSF-negative CAR T cells produced less GM-CSF without weakening antitumor activity *in vivo* compared to wild-type CAR T cells. Single or combined knock-out of other critical relevant cytokines in CAR T cells using CRISPR/Cas9 are needed to be further explored. Long-lasting B cell aplasia is a classical on-target/off-tumor toxicity of anti-CD19 and anti-CD20 CAR T-cell therapy [65, 66]. The insert of safety switches gene into CAR vector is a feasible method to terminate the effects without jeopardizing clinical responses. Diaconu *et al.* [67] demonstrated that the iC9 safety switch eliminated CD19-specific CAR T cells in a dose-dependent manner in a humanized mouse model, allowing either a selective containment of CAR T expansion in case of CRS or complete deletion on demand granting normal B-cell reconstitution.

There are two reported cases indicating the risks of unexpected situations in the manufacture of CAR T cells and potential carcinogenicity *in vivo*. One CD19-negative relapsed patient after CD19-targeted CAR T cell therapy was found that the CAR gene was unintentionally introduced into a single dominantly-proliferative leukemic B cell and its product bound in cis to the CD19 epitope on the surface of leukemic cells, masking it from recognition by CAR T cells [36]. Another case was a 78-year-old man with advanced relapsed/refractory chronic lymphocytic leukemia who obtained CR after the second infusion. Unexpectedly, 94% of CAR T cells at the peak of the response originated from a single clone in which lentiviral vector-mediated insertion of the CAR transgene disrupted the methylcytosine dioxygenase TET2 gene [34]. Therefore, there is a need to incorporate inducible safe switches or suicide genes into the CAR T cells, which can provide a means to eliminate the CAR T cells in case of unexpected toxicities. Hoyos *et al.* [68] generated a novel anti-CD19 CAR construct that incorporates the IL-15 gene and an inducible caspase-9(iC9)-based suicide gene and >95% of transgenic cells could be efficiently eliminated within 24 h upon pharmacologic activation of the suicide gene [68]. Adding inducible safe switches or suicide genes to generate more controllable and safer CAR T cells will be widespread-used by multiplexed CRISPR/Cas9 technology. There are three ongoing clinical trials (NCT02107963, NCT01822652 and NCT02439788) incorporating the iC9 construct into CAR T-cell products to provide a method to eliminate autologous CAR T cells in case of potential off-target toxicity.

Other applications

CAR T-cells therapy has an obvious barrier in acute myeloid leukemia (AML) because myeloid-directed immunotherapy will eradicate normal as well as malignant cells, leading to bone marrow failure, as has been shown in several preclinical studies of CD33 or CD123 directed CAR T cell therapy [69, 70]. Several groups developed a novel approach to circumvent the problem with potent anti-CD33 CAR T cells followed by infusions of CRISPR/Cas9-modified CD33-konckout normal hematopoietic stem cells (HSCs), thus allowing persistent antigen-specific cytotoxicity along with reconstitution of effective hematopoiesis [35,71,72]. Extending the success of CAR T cells to T-cell malignancies is also problematic because most target antigens are expressed on both normal and malignant cells, resulting in CAR T-cell fratricide. CD7 is a transmembrane protein highly expressed in T-cell acute leukemia (T-ALL) and largely confined to T cells and natural killer cells. Studies showed that CD7-specific CAR T cell impaired expansion due to self-killing of the CAR T cells. Diogo *et al.* [73] explored that targeted disruption of the CD7 gene using CRISPR/Cas9 prior to CAR expression minimized fratricide in T cells and allowed the expansion of the CD7-knock-out anti-CD7 CAR T cells with robust antitumor activity for preclinical and potential clinical application. Hence, the CRISPR/Cas9 system can be applied to disrupt the targeted antigens to avoid self-killing of the CAR T cells and broaden the therapeutic index.

Conclusion and outlooks

The unprecedented responses of CAR T cells in advanced malignancies promote the rapid growth of the therapeutic approach and the development of the smarter and commercialized CAR T cells is an inevitable mainstream trend, such as a split, universal and programmable CAR system to prevent relapse, mitigate overactivation and enhance specificity [74]. CRISPR/Cas9 genomic editing technology holds promising explorations and applications to create the next-generation CAR T-cell products, including universal CAR T cells by disrupting endogenous TCR and HLA, more potent CAR T cells by ablating inhibitory modulators, more controllable CAR T cells by adding inducible safe switches or suicide genes and novel CAR T cells by knock-out of the targeted antigens to avoid self-killing.

However, the gene-editing specificity and efficiency of CRISPR/Cas9 technology are of significant importance in therapeutic application. The first concern of CRISPR/Cas9 gene editing is off-target effects, which introduce random mutations, hence activating oncogenes or impacting tumor-suppressor genes to unintentional deleterious consequences [75]. Multiple strategies, such as careful selection of the target site, optimized sgRNA design and Cas9 activity, prior off-target detection assays, have been attempted to minimize the safe risks of off-target effects [76–78]. Attempts to increase HR frequencies using HR enhancers or NHEJ inhibitors are currently ongoing and may further promote precise gene engineering [79, 80]. Another challenge for therapeutic gene editing is efficient and nontoxic delivery into CAR T cells. There are three main methods to deliver CRISPR/Cas9 system, including a DNA plasmid-based system, an all-RNA-based system and a Cas9 ribonucleoprotein complex as delivery [81]. Viral vectors with high efficiency and potential hazards, such as mutagenesis, immunogenicity and off-target effects, are widely applied for donor DNA delivery and electroporation has emerged as new method to deliver CRISPR/Cas9 elements with safety, simplicity and flexibility [82]. Viral and non-viral vectors have specific merits and beneficial combinations of different delivery means are being explored to ensure efficiency and safety [33]. As technical progresses to reduce off-target effects and improve delivery efficiency, CRISPR/Cas9 technology provides an extraordinary potential to construct novel CAR T cells and streamlines the burgeoning realm of immunotherapy.

Key Points

- CAR T-cell therapy has shown promising responses in both hematologic and solid cancers. However, there are some limitations awaiting for solutions, such as insufficient quantity and poor quality of autologous T cells, CAR T cell exhaustion and tumor suppressive microenvironments, potential self-killing and uncontrollable proliferation.

- Genomic editing technologies, especially CRISPR/Cas9 with flexibility, simplicity, high efficiency and multiplexing open a window to develop next-generation CAR T cells.
- CRISPR/Cas9 genomic editing technology holds promising explorations and applications to create next-generation CAR T cell products, including universal CAR T cells by disrupting endogenous TCR or HLA, more potent CAR T cells by ablating of inhibitory modulators and more controllable CAR T cells by adding inducible safe switches or suicide genes.
- CRISPR/Cas9 technology is unveiling a new era for CAR T cell therapy.

Conflict of interest

The authors declare that they have no conflict of interests.

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References

1. Neelapu SS, Locke FL, Bartlett NL, *et al*. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med* 2017;**377**(26):2531–44.
2. Maude SL, Laetsch TW, Buechner J, *et al*. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med* 2018;**378**(5):439–48.
3. Schuster SJ, Bishop MR, Tam CS, *et al*. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med* 2019;**380**(1):45–56.
4. Guedan S, Calderon H, Posey AD, Jr, *et al*. Engineering and design of chimeric antigen receptors. *Mol Ther Methods Clin Dev* 2019;**12**:145–56.
5. Kulemzin SV, Kuznetsova VV, Mamonkin M, *et al*. Engineering chimeric antigen receptors. *Acta Nat* 2017;**9**(1):6–14.
6. Fry TJ, Shah NN, Orentas RJ, *et al*. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med* 2018;**24**(1):20.
7. Adaniya SPS, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: a review of current data and potential clinical applications. *Am J Hematol* 2019;**94**:S28–33.
8. Raje N, Berdeja J, Lin Y, *et al*. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med* 2019;**380**(18):1726–37.
9. Sadelain M, Brentjens R, Riviere I. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 2009;**21**(2):215–23.
10. Brocker T, Karjalainen K. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. *J Exp Med* 1995;**181**(5):1653–9.
11. Kershaw MH, Westwood JA, Parker LL, *et al*. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 2006;**12**(20):6106–15.
12. Till BG, Jensen MC, Wang JJ, *et al*. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 2008;**112**(6):2261–71.
13. Salter AI, Ivey RG, Kennedy JJ, *et al*. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Sci Signal* 2018;**11**(544). doi: 10.1126/scisignal.aat6753.
14. Till BG, Jensen MC, Wang JJ, *et al*. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood* 2012;**119**(17):3940–50.
15. Ramello MC, Benzaid I, Kuenzi BM, *et al*. An immunoproteomic approach to characterize the CAR interactome and signalosome. *Sci Signal* 2019;**12**(568). doi: 10.1126/scisignal.aap9777.
16. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. *Expert Opin Biol Ther* 2015;**15**(8):1145–54.
17. Moon EK, Carpenito C, Sun J, *et al*. Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. *Clin Cancer Res* 2011;**17**(14):4719–30.
18. Guedan S, Calderon H, Posey AD, *et al*. Engineering and design of chimeric antigen receptors. *Mol Therapy-Methods Clin Dev* 2019;**12**:145–56.
19. Urnov FD, Rebar EJ, Holmes MC, *et al*. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010;**11**(9):636–46.
20. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 2014;**32**(4):347–55.
21. Miller JC, Tan S, Qiao G, *et al*. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* 2011;**29**(2):143–8.
22. Carroll D. Progress and prospects: zinc-finger nucleases as gene therapy agents. *Gene Ther* 2008;**15**(22):1463–8.
23. Moscou MJ, Bogdanove AJA. Simple cipher governs DNA recognition by TAL effectors. *Science* 2009;**326**(5959):1501–1.
24. Cong L, Ran FA, Cox D, *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013;**339**(6121):819–23.
25. Haurwitz RE, Jinek M, Wiedenheft B, *et al*. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science* 2010;**329**(5997):1355–8.
26. Mali P, Yang LH, Esvelt KM, *et al*. RNA-guided human genome engineering via Cas9. *Science* 2013;**339**(6121):823–6.
27. Ehrke-Schulz E, Schiwon M, Hagedorn C, *et al*. Establishment of the CRISPR/Cas9 system for targeted gene disruption and gene tagging. *Methods Mol Biol* 2017;**1654**:165–76.
28. Zhang JP, Li XL, Li GH, *et al*. Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. *Genome Biol* 2017;**18**(1):35.
29. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, *et al*. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* 2016;**540**(7631):144.
30. Liu XJ, Zhang YP, Cheng C, *et al*. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Res* 2017;**27**(1):154–7.
31. Foster AE, Dotti G, Lu A, *et al*. Antitumor activity of EBV-specific T lymphocytes transduced with a dominant negative TGF-beta receptor. *J Immunother* 2008;**31**(5):500–5.
32. Chong EA, Melenhorst JJ, Lacey SF, *et al*. PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. *Blood* 2017;**129**(8):1039–41.

33. Eyquem J, Mansilla-Soto J, Giavridis T, *et al*. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 2017;**543**(7643):113–7.
34. Fraietta JA, Nobles CL, Sammons MA, *et al*. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. *Nature* 2018;**558**(7709):307–12.
35. Kim MY, Kenderian SS, Schreeder D, *et al*. Genome editing using CRISPR-Cas9 to increase the therapeutic index of antigen-specific immunotherapy in acute myeloid Leukemia. *Mol Ther* 2016;**24**:S108–8.
36. Ruella M, Xu J, Barrett DM, *et al*. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. *Nat Med* 2018;**24**(10):1499–503.
37. Torikai H, Reik A, Liu PQ, *et al*. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* 2012;**119**(24):5697–705.
38. Berdien B, Mock U, Atanackovic D, *et al*. TALEN-mediated editing of endogenous T-cell receptors facilitates efficient reprogramming of T lymphocytes by lentiviral gene transfer. *Gene Ther* 2014;**21**(6):539–48.
39. Morgan NV, Goddard S, Cardno TS, *et al*. Mutation in the TCRalpha subunit constant gene (TRAC) leads to a human immunodeficiency disorder characterized by a lack of TCRalphabeta+ T cells. *J Clin Invest* 2011;**121**(2):695–702.
40. Poirot L, Philip B, Schiffer-Mannioui C, *et al*. Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. *Cancer Res* 2015;**75**(18):3853–64.
41. Qasim W, Zhan H, Samarasinghe S, *et al*. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med* 2017;**9**(374). doi: 10.1126/scitranslmed.aaj2013.
42. Qasim W, Amrolia PJ, Samarasinghe S, *et al*. First clinical application of Talen engineered universal CAR19 T cells in B-ALL. *Blood* 2015;**126**(23). doi: 10.1182/blood.V126.23.2046.2046.
43. Benjamin R, Graham C, Yallop D, *et al*. Preliminary data on safety, cellular kinetics and anti-leukemic activity of UCART19, an allogeneic anti-CD19 CAR T-cell product, in a pool of adult and Pediatric patients with high-risk CD19+relapsed/refractory B-cell acute lymphoblastic leukemia. *Blood* 2018;**132**. doi: 10.1182/blood-2018-99-111356.
44. Torikai H, Reik A, Soldner F, *et al*. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood* 2013;**122**(8):1341–9.
45. Riolobos L, Hirata RK, Turtle CJ, *et al*. HLA engineering of human pluripotent stem cells. *Mol Ther* 2013;**21**(6):1232–41.
46. Ren J, Zhang X, Liu X, *et al*. A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget* 2017;**8**(10):17002–11.
47. Ren JT, Liu XJ, Fang CY, *et al*. Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin Cancer Res* 2017;**23**(9):2255–66.
48. Gornalusse GG, Hirata RK, Funk SE, *et al*. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat Biotechnol* 2017;**35**(8):765–72.
49. Ritchie DS, Neeson PJ, Khot A, *et al*. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. *Mol Ther* 2013;**21**(11):2122–9.
50. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 2015;**15**(8):486–99.
51. Hoos A. Development of immuno-oncology drugs - from CTLA4 to PD1 to the next generations. *Nat Rev Drug Discov* 2016;**15**(4):235–47.
52. Hodi FS, O'Day SJ, McDermott DF, *et al*. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;**363**(8):711–23.
53. Waring P, Mullbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. *Immunol Cell Biol* 1999;**77**(4):312–7.
54. Kunkele A, Johnson AJ, Rolczynski LS, *et al*. Functional tuning of CARs reveals Signaling threshold above which CD8(+) CTL antitumor potency is attenuated due to cell Fas-FasL-dependent AICD. *Cancer Immunol Res* 2015;**3**(4):368–79.
55. Pickup M, Novitskiy S, Moses HL. The roles of TGFbeta in the tumour microenvironment. *Nat Rev Cancer* 2013;**13**(11):788–99.
56. Liu VC, Wong LY, Jang T, *et al*. Tumor evasion of the immune system by converting CD4(+) CD25(−) T cells into CD4(+) CD25(+) T regulatory cells: role of tumor-derived TGF-beta. *J Immunol* 2007;**178**(5):2883–92.
57. Ebner R, Chen RH, Shum L, *et al*. Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. *Science* 1993;**260**(5112):1344–8.
58. Bollard CM, Tripic T, Cruz CR, *et al*. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol* 2018;**36**(11):1128–39.
59. Kloss CC, Lee J, Zhang A, *et al*. Dominant-negative TGF-beta receptor enhances PSMA-targeted human CAR T cell proliferation and augments prostate cancer eradication. *Mol Ther* 2018;**26**(7):1855–66.
60. Chang ZL, Lorenzini MH, Chen X, *et al*. Rewiring T-cell responses to soluble factors with chimeric antigen receptors. *Nat Chem Biol* 2018;**14**(3):317–24.
61. Wang Z, Guo Y, Han W. Current status and perspectives of chimeric antigen receptor modified T cells for cancer treatment. *Protein Cell* 2017;**8**(12):896–925.
62. Brudno JN, Kochenderfer JN. Recent advances in CAR T-cell toxicity: mechanisms, manifestations and management. *Blood Rev* 2019;**34**:45–55.
63. Le RQ, Li L, Yuan WS, *et al*. FDA approval summary: Tocilizumab for treatment of chimeric antigen receptor T cell-induced severe or life-threatening cytokine release syndrome. *Oncologist* 2018;**23**(8):943–7.
64. Sterner RM, Cox MJ, Sakemura R, *et al*. Using CRISPR/Cas9 to knock out GM-CSF in CAR-T cells. *Jove-J Visual Exp* 2019;(149). doi: 10.3791/59629.
65. Zhang WY, Wang Y, Guo YL, *et al*. Treatment of CD20-directed chimeric antigen receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an early phase IIa trial report. *Signal Transduct Target Ther* 2016;**1**:.
66. Maude SL, Frey N, Shaw PA, *et al*. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* 2014;**371**(16):1507–17.
67. Diaconu I, Ballard B, Zhang M, *et al*. Inducible Caspase-9 selectively modulates the toxicities of CD19-specific chimeric antigen receptor-modified T cells. *Mol Ther* 2017;**25**(3):580–92.
68. Hoyos V, Savoldo B, Quintarelli C, *et al*. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide

gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia* 2010;**24**(6):1160–70.
69. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, *et al*. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. *Leukemia* 2014;**28**(8):1596–605.
70. Mardiros A, Dos Santos C, McDonald T, *et al*. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* 2013;**122**(18):3138–48.
71. Kim MY, Yu KR, Kenderian SS, *et al*. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid Leukemia. *Cell* 2018; **173**(6):1439.
72. Borot F, Wang H, Ma Y, *et al*. Gene-edited stem cells enable CD33-directed immune therapy for myeloid malignancies. *Proc Natl Acad Sci U S A* 2019;**116**(24):11978–87.
73. Gomes-Silva D, Srinivasan M, Sharma S, *et al*. CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies. *Blood* 2017;**130**(3):285–96.
74. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. *Cell* 2018;**173**(6):1426–38 e1411.
75. Peng R, Lin G, Li J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS J* 2016;**283**(7):1218–31.
76. Liu J, Zhou G, Zhang L, *et al*. Building potent chimeric antigen receptor T cells with CRISPR genome editing. *Front Immunol* 2019;**10**:456.
77. Jiang J, Zhang L, Zhou X, *et al*. Induction of site-specific chromosomal translocations in embryonic stem cells by CRISPR/Cas9. *Sci Rep* 2016;**6**:.
78. Torres R, Martin MC, Garcia A, *et al*. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. *Nat Commun* 2014;**5**:3964.
79. Maruyama T, Dougan SK, Truttmann MC, *et al*. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol* 2015;**33**(5):538–42.
80. Riesenberg S, Maricic T. Targeting repair pathways with small molecules increases precise genome editing in pluripotent stem cells. *Nat Commun* 2018;**9**.
81. Jensen TI, Axelgaard E, Bak RO. Therapeutic gene editing in haematological disorders with CRISPR/Cas9. *Br J Haematol* 2019;**185**(5):821–35.
82. Xu XJ, Wan T, Xin HH, *et al*. Delivery of CRISPR/Cas9 for therapeutic genome editing. *J Gene Med* 2019;**21**(7). doi: 10.1002/jgm.3107.