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Review paper

CRISPR/Cas9 technology: towards a new generation of improved CAR-T cells for anticancer therapies

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Abstract

Chimeric antigen receptor (CAR)-modified T cells have raised among other immunotherapies for cancer treatment, being implemented against B-cell malignancies. Despite the promising outcomes of this innovative technology, CAR-T cells are not exempt from limitations that must yet to be overcome in order to provide reliable and more efficient treatments against other types of cancer. The purpose of this review is to shed light on the field of CAR-T cell gene editing for therapy universalization and further enhancement of antitumor function. Several studies have proven that the disruption of certain key genes is essential to boost immunosuppressive resistance, prevention of fratricide, and clinical safety. Due to its unparalleled simplicity, feasibility to edit multiple gene targets simultaneously, and affordability, CRISPR/CRISPR-associated protein 9 system has been proposed in different clinical trials for such CAR-T cell improvement. The combination of such powerful technologies is expected to provide a new generation of CAR-T cell-based immunotherapies for clinical application.

Key words: CAR-T cell; CRISPR/Cas9; gene editing; immunotherapy; cancer; clinical application

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Introduction

Cancer is widely known as one of the harshest diseases of our time due to its heterogeneity and aggressiveness. Mutations of diverse nature lead to an uncontrolled cell division that disrupts the normal functioning of the surrounding tissue and compromises patient's health in a very severe manner. The illness can be triggered by many different physical, chemical and biological agents as well as genetic predisposition. As a result, high incidence is found in a wide range of different social groups, and millions of deaths are reported annually (9.6 million in 2018) [1]. According to the World Health Organization (WHO), cancer mortality is expected to increase in the following decades (up to 13 million in 2030) [1].

Because of these devastating statistics, important efforts are being made in the development of anticancer strategies. Promising research projects are conducted in the field of immunotherapy, a therapeutic approach based on the boost of the patient's immune system to selectively target and kill tumour cells [2]. The proper modulation of the immune system brings considerable benefits in cancer treatment due to its potential ability to induce longer and more sustained responses against tumours. Besides, immunotherapy can function as an adjuvant or neo-adjuvant therapy for conventional treatments that suppress the immune system [3]. Adoptive cell transfer (ACT) has emerged as one of the most relevant types of treatment among other immunotherapy strategies [4, 5]. Following this approach, immune cells are isolated either from the patient or an adequate donor and are infused into the patient after being subject to anticancer stimulation or gene modification [4]. One of the most powerful ACT involves the use of chimeric antigen receptor (CAR)-modified T cells. This approach has been approved by the US Food and Drug Administration (FDA) in late 2017 for the treatment of relapsed or refractory B-lineage acute lymphoblastic leukaemia (ALL) and lymphomas, with high complete molecular response rates (CR) and negative minimal residual disease [6, 7].

CAR-T cell therapy for cancer treatment

CAR-T cell immunotherapy is based on using genetically modified T cells to precisely target and kill cancerous cells. Despite there are other strategies involving immune cells such as NK cells, it is worthy to take advantage of the main orchestrators of the immune system. Effector functions including immunomodulation, migration, clonal expansion, long immunological memory and cytotoxicity are found in T cells and are very valuable for antitumor therapies [8]. Nevertheless, native T cells are not able to overcome tumour expansion in most malignancies. This fact is due to the ability of cancer to evade the immune system and response with the so-called immune escape mechanisms. These are promoted by the increase of mutation rates and include immunosuppression and loss of immunogenicity and antigenicity by altered peptide processing mechanisms and human leukocyte antigen (HLA) loss [9, 10]. In order to surpass these escape mechanisms successfully, the implementation of an artificial CAR immunoreceptor provides a targeted strategy for surface-expressed antigens. The intracellular protein processing for peptide presentation and T-cell receptor (TCR)-based recognition by TCR and major histocompatibility complex (MHC) interaction (TCR-MHC) is simplified when expressing a transgene coding for the CAR receptor [10]. Instead, the tumour-associated antigen (TAA) is recognized directly by the CAR. This way, common tumour escape mechanisms such as HLA loss or altered peptide processing can be suitably bypassed [11]. The result is a swifter, alternative, robust, and sustainable activation of the immune response.

CAR-T cell therapy limitations and strategies to overcome current boundaries

Although promising, CAR-T cell therapies are not exempt from side effects and still need further development to overcome their own limitations. For that purpose, many different genes are targeted with gene-editing technologies (Table 1).

On-target off-tumour effect

Problems such as targeting healthy cells and committing fratricide (on-target off-tumour effect) can be prevented by a rational selection of the CAR [12]. In T-cell-derived malignancies, it is challenging to identify suitable overexpressed TAA, as most TAA are shared between malignant T cells and CAR-T cells [13]. To avoid self-destruction, several groups have disrupted the expression of T-cell antigens of broad expression in CAR-T cells. The clusters of differentiation 3, 5 and 7 (CD3, CD5, and CD7) were successfully targeted by gene-editing technologies including transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated RNA-guided endonuclease Cas9 (CRISPR/Cas9). The resulted CAR-T cells exhibited remarkable antitumor efficacy with mitigated fratricide [14–16]. Pinz et al. generated CD4-CAR-T cells to treat peripheral T-cell lymphomas and reported significant fratricide with enrichment of $CD4^+$ $CD8^+$ CD4-CAR-T cells [17]. To maintain a constant ratio of CD4/CD8 CAR-T cells, it would be worthy to explore the effects of a CD4 knockout. Additionally, to overcome the potential fratricide effect of anti-CD319 CAR-T cells for multiple myeloma treatment, Galetto et al. inactivated CD319 (ubiquitously expressed in activated T cells) taking advantage of TALENs technology, achieving the prevention of the loss of CD319-positive CAR-T cells during T-cell expansion [18].

Toxicity and iatrogenic effects

Toxicities broadly reported in CAR-T cell treatments include cytokine release syndrome (CRS) and neurotoxicity (NT). CRS is caused by a cytokine storm generated by the release of pro-inflammatory cytokines (mainly IL-6, IL-2, IFN-γ and TNF-α) and is inherent to the potency of the treatment [19, 20]. Its severity comprises from mild to life-threatening iatrogenic effects, including severe symptoms such as cardiovascular dysfunction and hepatic failure [21]. Even so, it has been shown that the IL-6 receptor antibody tocilizumab can reduce or even terminate massive excess of T-cell stimulation by activated CAR-T cells almost immediately after administration [22, 23]. In the case of NT, the molecular mechanisms remain unclear, but both monocytes and macrophages seem to play a major role since they are the main producers of IL-6 during CAR-T cell therapies [24]. Many efforts are being made for implementing proper animal models to study the disorder [25]. Short treatments with systemic corticosteroids such as dexamethasone seem to improve patients' condition, while its prolonged use is usually avoided for the sake of efficacy and persistence of CAR-T cell treatment [21].

Another way to ameliorate these symptoms and improve the potency of the treatment can be achieved by antibody-induced

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Table 1. Strategies to genetically modify and improve T-cell and CAR-T cell-based immunotherapies

Target (s)	Type of molecule (s)	Gene-editing technology	Purpose	Type of cancer	Results, improvements and limitations	Ref.
B2M, *Fas*,*TRAC*	Antigen presentation molecule, cell death-/survival-related molecule, antigen receptor molecule	CRISPR/Cas9	Prevention of HvG, apoptosis and GvHD	B-cell malignancies	• Enhancement of killing, cytokine secretion and survival • Fratricide resistance and prevention of GvHD	28
B2M, *CTLA4*, *PD1*, *TRAC*	Antigen presentation molecule, immune checkpoint, antigen receptor molecule	CRISPR/Cas9	Prevention of HvG, immunosuppre-sion and GvHD	B-cell malignancies	• Reduced alloreactivity and prevention of GvHD • Enhancement of antitumor activity • Limited efficiency in multiplex gene editing	28
B2M, *PD1*, *TRAC*, *TRBC*	Antigen presentation molecule, immune checkpoint, antigen receptor molecule	CRISPR/Cas9	Prevention of HvG, immunosuppre-sion and GvHD	B-cell malignancies	• Reduced alloreactivity and prevention of GvHD • Enhancement of antitumor activity	32
CD5	Immunoreceptor	CRISPR/Cas9	Prevention of fratricide	$CD5^+$ T-cell malignancies	• Enhancement of antitumor activity • Fratricide resistance • CAR upregulation	15
CD7, *TRAC*	Immunoreceptor, antigen receptor molecule	CRISPR/Cas9	Prevention of fratricide and GvHD	$CD7^+$ T-cell malignancies	• Robust killing performance maintained • Fratricide resistance and prevention of GvHD • Reduction of CAR-T cell viability	14
CD52, *TRAC*	Immunoreceptor	TALENs	Prevention of HvG and GvHD	$CD19^+$ lymphomas	• Prevention of GvHD and HvG • Cytotoxic activity maintained • Low editing efficiency (40%)	29
CD52, *TRAC*	Immunoreceptor, antigen receptor molecule	TALENs	Prevention of HvG and GvHD	Acute lymphoblastic leukaemia	• Molecular remission of the disease within 28 days • Residual GvHD in the skin and bone marrow	31
CD319, *TRAC*	Immunoreceptor, antigen receptor molecule	TALENs	Prevention of fratricide and GvHD	Multiple myeloma	• Enhancement of cytotoxicity • Fratricide resistance	18
CTLA4	Immune checkpoint	CRISPR/Cas9	Prevention of immunosuppres-sion	Human colonic adenocarcinoma	• Powerful immune activation with TNF-α and IFN-γ upregulation	44
DGK	Metabolic enzyme	CRISPR/Cas9	Prevention of immunosuppres-sion	Glioblastoma	• Resistance against TGF-β immunosupression • Enhancement of proliferation and functionality	69
GM-CSF	Inflammatory cytokine	CRISPR/Cas9	Prevention of loss of antitumor function	Acute lymphoblastic leukaemia	• Higher leukaemic regression and increased CAR-T cell survival	26
LAG3	Immune checkpoint	CRISPR/Cas9	Prevention of immunosuppres-sion	B-cell malignancies	• CAR-T cell phenotype and antitumor activity maintained • No improvement in resistance to exhaustion	58
NR4A	Transcription factor	Retrovirus	Prevention of immunosuppres-sion	Melanoma	• Prolonged survival • Enhancement of immune activation and antitumor activity	61
PD1	Immune checkpoint	CRISPR/Cas9	Prevention of immunosuppres-sion	Multiple myeloma	• Enhancement of antitumor activity and cytokine secretion	39
PD1	Immune checkpoint	CRISPR/Cas9	Prevention of immunosuppres-sion	Hepatocellular carcinoma	• Enhancement of antitumor activity • Improvement of persistence and infiltration	40
TGFβR2	Cellular receptor	CRISPR/Cas9	Prevention of immunosupression	Multiple myeloma	• Insensitivity to TGF-β	65
TRAC	Antigen receptor molecule	TALENs	Prevention of fratricide	Acute lymphoblastic leukaemia	• Potent antileukaemic effects	16

(Continued.)

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Table 1. Strategies to genetically modify and improve T-cell and CAR-T cell-based immunotherapies

Target (s)	Type of molecule (s)	Gene-editing technology	Purpose	Type of cancer	Results, improvements and limitations	Ref.
					• Fratricide resistance • Poor cell yields after conventional LV transduction followed by TRAC knockout	

B2M, β-2-microglobulin; CAR, chimeric antigen receptor; Cas9, CRISPR-associated protein 9; CD, cluster of differentiation; CRISPR, clustered regularly interspaced short palindromic repeats; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DGKα/ζ diacylglycerol kinase α/ζ subunit; GM-CSF, granulocyte-macrophage colony-stimulating factor; GvHD, graft-versus-host disease; HvG, host-versus-graft effect; IFN-γ, interferon γ; LAG3, lymphocyte activation gene 3; LV, lentivirus; NR4A, nuclear receptor 4A; PD1, programmed cell death protein 1; TALENs, transcription activator-like effector nucleases; TGFβ, transforming growth factor β; TGFβR2, TGFβ receptor 2; TNF-α, tumour necrosis factor α; TRAC/TRBC, T-cell receptor α/β constant subunit.

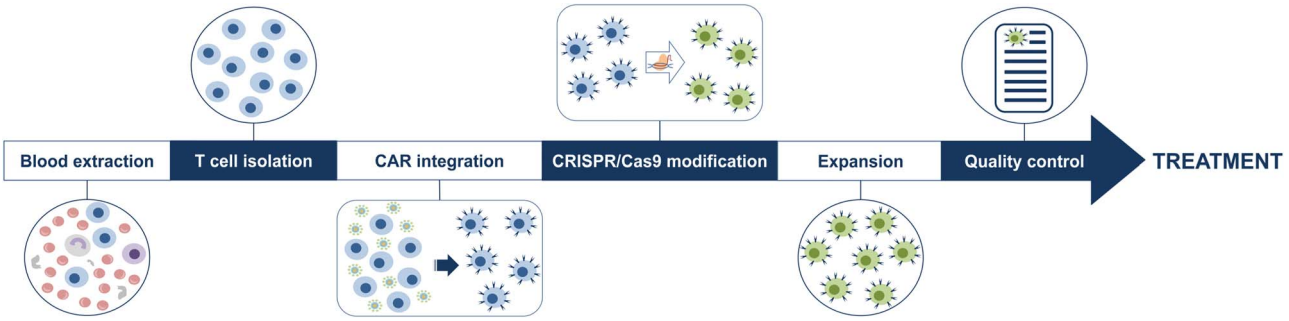


Figure 1. Schematic overview of CRISPR/Cas9-improved CAR-T cell production for tumour treatment. After blood extraction from patient or healthy donor, T cells are properly isolated. The integration of the CAR (usually achieved by lentiviral transduction) is performed. Resulting CAR-T cells are modified by CRISPR/Cas9 system for further improvement. CRISPR/Cas9-modified CAR-T cells are expanded and studied for treatment. Abbreviations: CAR, chimeric antigen receptor; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats.

Table 2. Registered clinical trials that incorporate CAR-T cell therapy and further genetic modifications by CRISPR/Cas9 to enhance effector function or to broaden the applicability of the cellular product

Cancer	Indication	Target antigen	Modification by CRISPR	Purpose	Status	Phase	Sponsor	ClinicalTrials.gov ID
B-cell malignancies	Relapsed/ refractory	CD19	CD19 CAR integration	• Cost reduction	Recruiting	1/2	CRISPR therapeutics AG	NCT04035434
B-cell malignancies	Relapsed/ refractory	CD19	Knockout of TCR and B2M	• Broaden applicability • Cost reduction	Recruiting	1/2	Chinese PLA General Hospital	NCT03166878
B-cell malignancies	Relapsed/ refractory	CD19/CD20/ CD22	Knockout of TCR and B2M	• Broaden applicability • Cost reduction	Recruiting	1/2	Chinese PLA General Hospital	NCT03398967
B-cell malignancies	Relapsed/ refractory	CD19	HPK1 knockout	• Enhance effector function	Recruiting	1/2	Xijing Hospital	NCT04037566
Solid tumours	Refractory	Mesothelin	PD-1 knockout	• Enhance effector function	Recruiting	1/2	Chinese PLA General Hospital	NCT03747965
Solid tumours	Refractory	Mesothelin	PD-1 and TCR knockout	• Enhance effector function • Broaden applicability • Cost reduction	Recruiting	1/2	Chinese PLA General Hospital	NCT03545815

B2M, β-beta 2-microglobulin; CD, cluster of differentiation; HPK1, haematopoietic progenitor kinase 1; PD-1, programmed cell death protein 1; TCR, T- cell receptor.

blockade of immune checkpoints and gene modification. Sterner et al. critically studied the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibition in CD19-CAR-T cells. *In vitro*, they tested the effects of lenzilumab in CD19-CAR-T cells that were cocultured with $CD19^+$ ALL cell line. The provided results showed that the antibody neutralized GM-CSF without inhibiting CAR-T cell proliferation. Subsequently, they performed GM-CSF knockout in CD19-CAR-T cells using CRISPR/Cas9 technology and reported that the potency of the treatment was enhanced since significantly higher leukemic regression and increased CAR-T cell survival were observed. Moreover, with the blockade of GM-CSF by lenzilumab, CRS and neuroinflammation were reduced after CAR-T cell therapy in xenograft models. Phase II clinical trials with lenzulimab in combination with CD19-CAR-T cells therapy are expected [26].

Graft-versus-host disease and host-versus-graft effect

Many other limitations can be smartly surpassed by gene editing as well. It is remarkable to state that CAR-T cell therapies share the same inconveniences as organ transplantations. Graft-versus-host disease (GvHD) and host-versus-graft effect (HvG)

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Table 3. Optimized strategies for gene editing in CAR-T cells

Engineered CAR-T cell	Target gene (s)	sgRNA design (s)	sgRNA:Cas9 delivery system	Transfection method	Gene-editing efficiency	Limitations	Ref.
CD19-CAR-T cells PSCA-CAR-T cells	*B2M* *PD1* *TRAC* *TRBC*	CGCGAGCACAGCTAAGGCCA GGCCAGGATGGTTCTTAGGT AGAGTCTCTCAGCTGGTACA GCAGTATCTGGAGTCATTGA	mRNA	Electroporation	50% NHEJ (multiplex) 70–90% NHEJ (single gene disruption)	Multiplex yielded lower gene-editing efficiencies	32
CD19-CAR-T cells	*LAG3*	GTTTCTGCAGCCGCTTTGGG	RNP	Electroporation	70% NHEJ (single gene disruption)	Gene-editing efficiency could be further optimized	58
GPC3-CAR-T cells	*PD1*	GTCTGGGCGGTGCTACAACT	RNP	Electroporation	85% NHEJ (single gene disruption)	-	40
139 CAR-T cells	*DGKα* *DGKζ*	CTCTCAAGCTGAGTGGGTCC ACGAGCACTCACCAGCATCC	RNP	Electroporation	60–70% NHEJ (single gene disruption)	Gene-editing efficiency could be further optimized	69
CD7-CAR-T cells CD19-CAR-T cells	*CD7* *TRAC*	ATCACGGAGGTCAATGTCTA GAGAATCAAAATCGGTGAAT	DNA plasmid	Electroporation	70 NHEJ (multiplex) 90% NHEJ (single gene disruption)	-	14
CD19-CAR-T cells PSCA-CAR-T cells	*B2M* *CTLA4* *Fas* *PD1* *TRAC* *TRBC*	CGCGAGCACAGCTAAGGCCA GCAAAGGTGAGTGAGACTTT GAGGGTCCAGATGCCCAGCA GGCCAGGATGGTTCTTAGGT AGAGTCTCTCAGCTGGTACA GCAGTATCTGGAGTCATTGA	LV-based one-shot system/ mRNA RNP mRNA	Electroporation	50% NHEJ (multiplex) and 90% NHEJ (single gene disruption) 82% NHEJ (single gene disruption) 76% NHEJ (single gene disruption)	Multiplex yielded lower gene-editing efficiencies Limited lentiviral packaging size resulted in reduced gene-editing efficiency	28

B2M, β-2-microglobulin; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DGKα/ζ diacylglycerol kinase $\alpha/\ \zeta$ subunit; DNA, deoxyribonucleic acid; GPC3, glypican 3; LAG3, lymphocyte activation gene 3; LV, lentivirus; mRNA, messenger ribonucleic acid; NHEJ, non-homologous end joining; PD1, programmed cell death protein 1; PSCA, prostate stem cell antigen; RNP, ribonucleoprotein; TRAC/TRBC, T-cell receptor α/β constant subunit.

are susceptible to occur, but can be prevented by knocking out genes such as *TRAC* and *B2M*. *TRAC* encodes for α constant subunit of TCR which recognizes the alloantigens presented by HLA and induces T-cell alloreactivity [27]. The second encodes for β-2-microglobulin (B2M), an essential component of HLA indispensable for alloantigen presentation. Incompatibilities between host and graft can be prevented by knocking out the expression of both subunits to generate universal CAR-T cells [28, 29]. To further prevent HvG, lympho-depleting drugs such as alemtuzumab (CD52 blocking antibody) are combined with the treatment. Not surprisingly, these agents exhibit toxicity against CAR-T cells and compromise the efficacy of the treatment. To cope with this issue, the proper knockout of CD52 in CAR-T cells has been shown feasible [29].

The use of allogeneic CAR-T cells has substantial benefits for many immunosuppressed patients whose autologous therapies are insufficient. Likewise, non-impaired CAR-T cells of regular fitness and excellent quality are guaranteed [30]. Different groups have generated universal CAR-T cells following this approach. Qasim et al. knocked out *CD52* and *TRAC* using TALENs in CD19-CAR-T cells for treating two B-ALL patients. Infused CAR-T cells promoted molecular remissions of the disease within 28 days and exhibited residual GvHD in the skin and marrow [31]. Other researchers have proceeded with *TRAC* and *B2M* knockouts along with other targets of interest using multiplex gene editing by CRISPR/Cas9. Reduced alloreactivity and prevention of GvHD were observed in animal models treated with $TCR^{-}/B2M^{-}$ CAR-T cells [32].

Exhaustion and immunosuppresion

Gene editing is also useful for improving CAR-T cell function in terms of efficacy. By disrupting the immune checkpoints, more powerful responses are expected. The checkpoints comprise molecules and pathways that present a key role in the maintenance of self-tolerance, indispensable to spare own cells and tissues from immune reactivity [33]. Tumour cells are known to sequester inhibitory checkpoints to suppress immune activity and escape. In T cells, the main inhibitory checkpoints are programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), two surface receptors that regulate T-cell stimulation in late and early immune responses, respectively [34]. PD1 regulates T-cell activation through binding to its ligands, PDL1 and PDL2. This PD1 binding reduces survival and inhibits T-cell proliferation, suppressing interleukin production and the inflammatory response [35]. In contrast, anti-PD1 and anti-PD1 ligand antibodies significantly improve immune antitumor activity in different types of malignancies, being approved by the FDA for the treatment of melanoma, kidney and non-small cell lung cancer among many others [36–38]. Also, diverse research groups have studied the functional benefit of PD1 knockout in CAR-T cells. It was formerly demonstrated that the PD1 knockout in cytotoxic T cells enhances the antitumor activity *in vitro* and *in vivo* against multiple myeloma [39]. Besides, PD1 disruption enhances antitumor activity of CAR-T cells against hepatocellular carcinoma *in vivo*, as well as improving the persistence and infiltration of CAR-T cells in the tumour [40].

Regarding the other inhibitory checkpoint, CTLA4 is a homolog of the CD28 costimulatory molecule that binds to APC's B7 receptors with higher affinity, but contrary to CD28:B7 interaction, CTLA4:B7 binding does not produce the costimulatory signal needed for T-cell activation. Furthermore, CTLA4:B7 interaction may actually produce inhibitory signals that counteract T-cell stimulation [41, 42]. CTLA4 blockade with specific antibodies such as ipilimumab can greatly enhance antitumor responses in melanoma with long-lasting tumour regression [43]. In addition, a recent study proved that the knockout of CTLA4

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correlates with greater antitumor activity in cytotoxic T cells *in vitro* as well as *in vivo* xenograft models, with a significant reduction of tumour burden in comparison to the control. Moreover, a twofold increase of TNF-α and IFN-γ levels was reported *in vitro*, revealing a powerful immune activation [44].

Despite their similarities, PD1 and CTLA4 display different signalling pathways, anatomic location, the timing of downregulation and ligand distribution [34]. Therefore, it is essential to target both to guarantee the effectiveness of CAR-T cell treatments. Ren et al. successfully achieved the generation of quadruple CAR-T cell knockouts involving *PD1*, *CTLA4*, *B2M* and *TRAC*, although the efficiency of gene editing was severely compromised [28]. The researchers also generated and tested *TRAC/B2M/Fas* knockout since cell death induced by Fas/FasL interaction is exhibited in T cells [45] and is predominant in CAR-T cells [46, 47]. A reduction of apoptosis was observed *in vitro* and *in vivo*, along with enhanced killing ability and cytokine secretion [28].

More immune checkpoints were successfully targeted to improve CAR-T cell treatment. Increased expression of lymphocyte activation gene 3 (LAG3) and T-cell immunoglobulin and mucin domain-3 (TIM3) are typical markers of T-cell exhaustion along with PD1 and CTL4, characterized by a significant decrease of TNF-α and IFN-γ [48]. LAG3 is a high-affinity ligand for major histocompatibility class II (MHC II) molecules. Its synergy with PD1 is crucial for preventing autoimmunity [49], and it is known to enhance the immunosuppressive function of regulatory T cells (Treg cells) [50]. It was previously reported that the dual antibody-induced blockade of LAG3 and PD1 reverses T-cell exhaustion and enhances therapeutic activity in preclinical models of cancers and chronic infections in a synergistic manner [51–57]. Zhang et al. managed to knockout LAG3 in CAR-T cells by CRISPR/Cas9 system, although no improvement in the resistance to T-cell exhaustion was shown. Apart from technical considerations, it was hypothesized that a single knockout of LAG3 may not be sufficient, while a double knockout with PD1 should be studied [58]. Similarly, it was proved several years before that the simultaneous antibody-induced blockage of PD1 and TIM3 was more effective than targeting each immune checkpoint alone in order to restore IFN-γ production and stop tumour growth [59]. TIM3 is expressed in a wide range of T-cell types [60], being essential to explore this knockout in CAR-T cells.

Another resourceful approach to evade immunosuppressive environment is based on the knockout of transcription factors associated with T-cell exhaustion. Chen et al. showed that T-cell depletion promoted by PD1 and TIM3 exhibits similar expression profiles which are related to the secondary activation of nuclear receptor transcription factors from nuclear receptor 4A (NR4A) family [61]. Next, they studied a triple knockout of NR4A1, NR4A2 and NR4A3 in CAR-T cells and evaluate functionality and viability. The results showed prolonged survival and enhanced antitumor activity. Furthermore, the accessible chromatin regions were enriched for binding motifs for transcription factors such as NF-κB and AP-1, which are essential for immune activation and function [61].

Importantly, immunosuppressive soluble proteins are produced by the tumour microenvironment. Stromal healthy cells that support the expansion of the tumour can release different factors to the surroundings of the malignancy such as transforming growth factor β (TGFβ), leading to the differentiation of effector T cells into Treg cells [62]. TGFβ seems to be the key immunosuppressive cytokine in solid tumours as it has been shown to dramatically suppress T-cell proliferation, inhibit the maturation of T helper cells and reduce their effector functions by inhibiting TCR signalling [63–65]. To prevent the systemic administration of TGFβ antagonists and side effects, Vong et al. tried two different approaches to specifically disrupt TGFβ signalling pathway in B-cell maturation antigen (BCMA)-specific CAR-T cells: the first one involved the CRISPR knockout of TGFβR2, whereas the second was based on overexpressing a dominant negative TGFβR2. Both strategies led to insensitivity to the inhibitory effects of TGFβ *in vitro* and prevented the apparition of TGFβ-induced gene expression profile *in vivo* [65]. TGFβ-induced inhibition can be encountered by targeting diacylglycerol kinase (DGK) too, which is specifically upregulated in xenograft mouse models and tumour-infiltrating anergic T cells from patients with renal cancer [66–68]. This enzyme catalyzes the conversion of diacylglycerol (DAG) into phosphatidic acid (PA). Since DAG interacts with important effectors from TCR signalling pathway such as Ras-activating protein, its conversion to PA results in downregulation of extracellular signal-related kinases 1/2 (ERK1/2) among other effector molecules. By disrupting *DGK* gene with CRISPR/Cas9 technology, not only the enhancement of CAR-T cell proliferation and functionality was achieved by upregulating ERK pathway but also the resistance against TGFβ-induced inhibition and other immunosuppressive factors [69].

CRISPR/Cas9 system as gene-editing tool for CAR-T cell improvement

CRISPR/Cas9 system has emerged in the last few years as one of the best options for gene editing. Discovered in bacteria as a defence mechanism against viruses, the system is composed by an endonuclease (Cas9) and a single-guide RNA (sgRNA) that redirects the endonuclease to a certain locus in the genome by base complementarity. Next, Cas9 cuts the target, and the host cell has to repair the DNA breakage. If a donor template with homologous arms is available, homology-directed repair (HDR) can occur, resulting in precise editing of the genome. On the contrary, non-homologous end joining (NHEJ) is more likely to solve the break, which usually implies insertion or deletion of nucleotides (indels) that may disrupt the reading frame of the gene. The result is a very powerful editing system with several positive assets, including simplicity, high efficiency and wide applicability [70]. Potential applications are gene editing for gene therapy and the development of animal models for research and drug screening [71].

CRISPR/Cas9 technology has been tested in many different types of cells from diverse organisms. As a matter of fact, it has already been used in the development and improvement of CAR-T cells (Figure 1) as well as other genome editing tools [28, 32, 44, 70, 72, 73]. Besides, CRISPR/Cas9 modification is present in clinical trials for CAR-T cell therapy improvement (Table 2). Before the development of CRISPR/Cas9 technology, other genome-editing mechanisms like zinc finger nucleases (ZFNs) or TALENs were implemented. However, CRISPR/Cas9 has surpassed all of them in terms of affordability and feasibility [74, 75]. In addition, it enables the multiplex gene editing, a very interesting feature that allows the generation of universal CAR-T cells by long-term repression of endogenous TCR, HLA class I molecules and inhibitory checkpoints including CTLA4 and PD1 [71, 74–76]. Furthermore, it is a solid alternative to the classical lentiviral insertion of the CAR since it avoids random integration in the genome and uncontrolled expression of the construct [77].

Despite its virtues, CRISPR/Cas9 system is not infallible and has its own limitations. Off-target effects are known to occur

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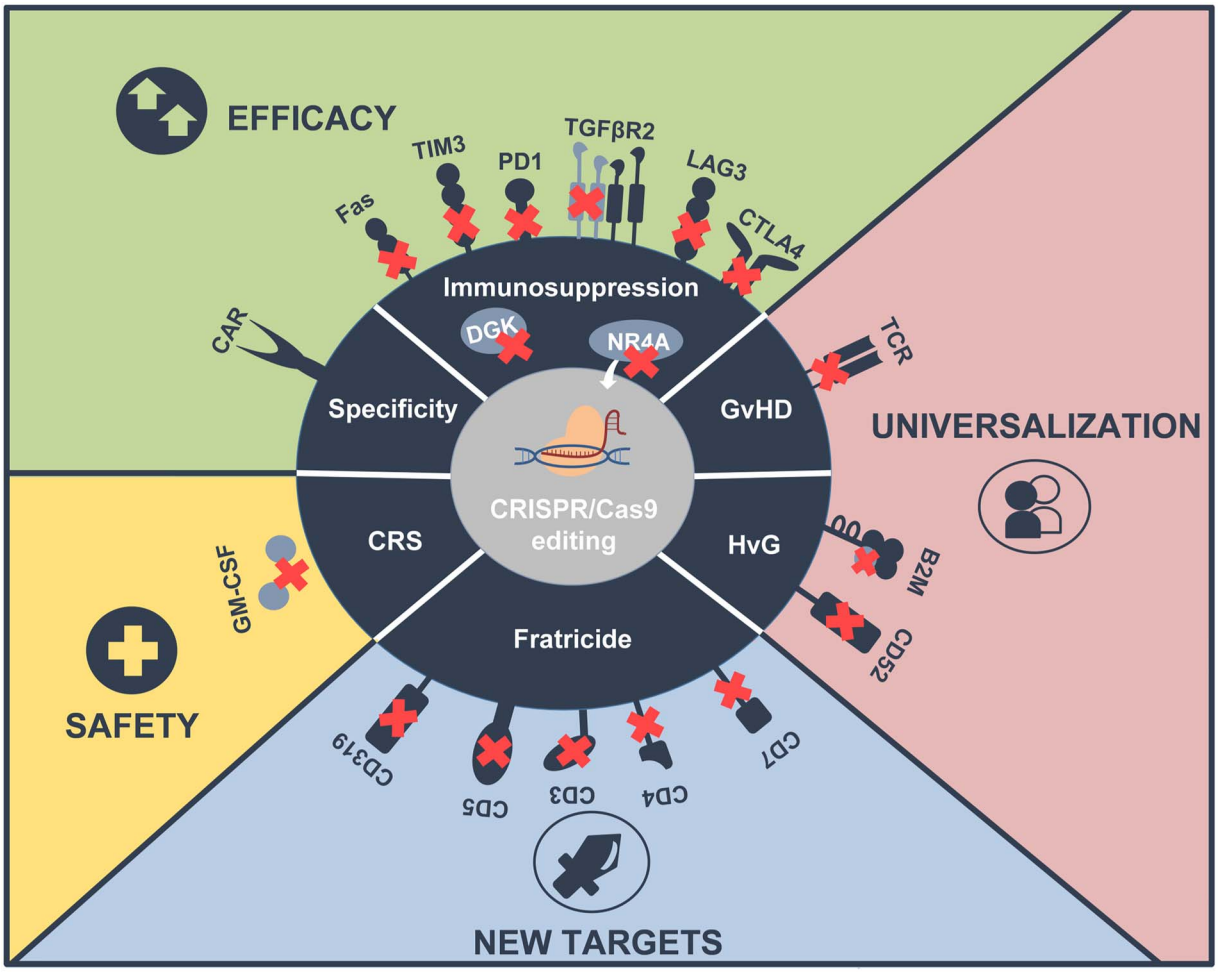


Figure 2. CRISPR/Cas9 targets for improving CAR-T cell features. The integration of CAR can be performed by CRISPR/Cas9-induced insertion. On the contrary, the knockout of most of the targets is performed to assess greater efficacy and effectiveness in the treatment by evading the reception of immunosuppressive signals, mostly by blocking immune checkpoints and their signalling pathways. The universalization of CAR therapy is ensured by knocking out receptors such as TRAC and B2M, preventing GvHD and HvG effects. Other receptors are specifically disrupted for the sake of the personalized therapy in order to avoid self-destruction (fratricide) and guarantee the compatibility of the antitumoral treatment, allowing broader options of CARs and new targets. Safety concerns are usually solved by systemic administration of blocking antibodies, although the expression of soluble factors such as GM-CSF may be disrupted, so the immunological response is ameliorated, and iatrogenic effects such as CRS are prevented. Abbreviations: B2M, β-2-microglobulin; CAR, chimeric antigen receptor; Cas9, CRISPR-associated protein 9; CD, cluster of differentiation; CRISPR, clustered regularly interspaced short palindromic repeats; CRS, cytokine release syndrome; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DGK diacylglycerol kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GvHD, graft-versus-host disease; HvG, host-versus-graft effect; LAG3, lymphocyte activation gene 3; NR4A, nuclear receptor 4A; PD1, programmed cell death protein 1; TGFβ, transforming growth factor β; TGFβR2, TGFβ receptor 2; TIM-3, mucin domain-3; TCR, T- cell receptor.

and can affect the fitness of the cell, but several strategies involving a precise sgRNA design, truncated sgRNA and chimeric DNA-RNA-based sgRNA and using different Cas9 variants can prevent it [71, 76, 78–81]. Next, the frequency of HDR is very low, so both gene correction and gene addition are considered as a rare event. However, it is possible to promote them by HDR enhancers and NHEJ inhibitors [76, 82–84]. In conclusion, these limitations can be overcome to generate suitable CAR-T cells (Table 3). The delivery of CRISPR/Cas9 into the T cell can be done following different approaches: plasmid DNA, messenger RNA or complexed in ribonucleoproteins (RNP). The efficiency of gene editing is very variable independently from the cell type or donor, but it seems that both mRNA and RNP delivery are suitable for achieving high indel rates [76]. This would allow a higher accuracy and proper editing of CAR-T cells.

Although traditional CAR-T cell generation is based on viral transduction of CARs, the possible side effects of using integrative viruses might lead to severe complications [85]. Due to this reason, targeted transgene integration into the *TRAC* gene by CRISPR/Cas9 system is the preferred option for several research groups, achieving high integration rates through the delivery of the repair template by AAV6 transduction or long ssDNA electroporation [77, 86]. Another type of delivery is by means of integrase-defective lentiviruses (IDLV), due to their large genome carry capacity, low pathogenicity and capability of transducing both, dividing and non-dividing cells [87, 88]. However, no preclinical trials have been performed up to now.

Conclusions

CAR-T cell-based antitumor immunotherapies enable innovative and powerful treatments for cancer patients. Despite the high expectations, improvements are required to maximize their efficacy and reduce potential risks. As outlined, many advances

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can be achieved by editing the T-cell genome: universalization, immunosuppressive resistance, wider possibilities for CAR choice and safer therapies are the key players in CAR-T cells' future. To pursue these goals, CRISPR/Cas9-based gene-editing technologies are available to target several genes of interest simultaneously with great efficiency (Figure 2). The simplicity and reduced cost that present this methodology have raised CRISPR/Cas9 among the rest of the gene-editing tools which are currently available. Furthermore, the standardization and automatization of the process can be performed by cutting-edge technologies such as CliniMACS Prodigy®. This biomedical platform offers good manufacturing practice production of CAR-T cells for clinical application in a closed, controlled system [89–91] and is compatible with CRISPR/Cas9 transfection protocols. We conclude that the balanced synergy of CRISPR/Cas9 and CAR-T cell therapies might bring hope and cure for many cancer patients in the near future.

Key Points

- CAR-T cell therapies have become one of the most efficacious and innovative anticancer treatments.
- Additional genetic modifications are needed to overcome the current limitations of CAR-T cell therapy beyond B-lineage malignancies.
- CRISPR/Cas9 technology is postulated as one of the major allies in the development of universal, more effective and safer CAR-T cell therapies by improving T-cell features.

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