Applications and Future Perspectives of CRISPR/Cas9 Gene Editing Technologies in CAR-T Cell Therapy

Menghan Liu *

Division of Biosciences, University College London, Gower Street, London, WC1E 6BT, United Kingdom

* Corresponding Author Email: Jacqueline.liu.24@ucl.ac.uk

Abstract. A recent major breakthrough in cancer immunotherapy is the Chimeric antigen receptor-T cell (CAR-T cell) therapy, which has shown significant clinical efficacy in haematological malignancy treatment. Nonetheless, its application in a more general way is limited by a number of challenges, such as T-cell exhaustion, off-target associated toxicities, and the difficulty of personalised manufacturing. Recently, new opportunities have come into solving these problems with the introduction of clustered regularly interspaced short tandem repeats (CRISPR)-Cas9 genome editing, which has made it possible to perform precise and combinatorial genetic editing in CAR-T cells. The important applications of CRISPR in CAR-T cell engineering, which include the disruption of inhibitory immune checkpoints to enhance antitumour activity, the generation of universal allogeneic CAR-T cells by deletion of T-cell receptor and human leukocyte antigen (HLA) genes, and the modulation of cytokine signalling pathways to reduce toxicity are discussed in this review. Moreover, novel approaches, including targeted CAR integration and multiplex gene editing, are discussed as having the potential to enhance the therapeutic efficacy and scalability. In spite of these improvements, there are issues of off-target effect, delivery efficacy, genomic instability, and unaddressed issues of long-term safety. CAR-T cell therapies are likely to be improved further in future through advancements in genome editing technology, delivery methods, and synthetic biology. In general, CRISPR/Cas9-based engineering is a promising way of developing the next generation of precision cancer immunotherapy.

Keywords: CRISPR/Cas9, CAR-T cell therapy, genome editing, cancer immunotherapy, immune checkpoint, PD-1, cytokine release syndrome, gene knockout.

1. Introduction

Over the last decades, the field of cancer immunotherapy has largely transformed the outlook of the field of oncology by interfering with the ability of the immune system to detect and destroy the malignant cells. A significant advancement in these methods has been the CAR-T cell therapy, which entails genetic modification of T cells derived out of the patient to express artificial receptors that specifically bind to tumour-related antigens. It has been demonstrated through clinical studies that CAR-T cell therapy can be used to produce significant efficacy in haematological malignancies, specifically in B-cell leukaemia and lymphoma, where lasting remission has been documented in patients who are otherwise limited in terms of treatment options [1, 2]. Although these achievements have been made, the wider use of CAR-T therapy is constrained by a number of obstacles that include treatment-related toxicities, T-cell exhaustion, antigen escape, and little success in solid tumours [3].

The conventional CAR-T cell engineering relies mostly on gene transfer by means of viral vectors, which allows the stable expression of CAR constructs but does not allow a good control of the genomic location of the integration sites. Then, heterogeneous expression of CAR can be achieved by randomly inserting transgenes, with no guarantee of cellular behaviour or possible safety hazards like insertional mutagenesis. Additionally, traditional methods have a low multiplexing genetic capability, which limits the capacity to target multiple biological barriers to immunological inhibition, cytokine dysregulation, and tumour microenvironment-mediated repression at once [4]. Such constraints have necessitated the pursuit of more specific and multifunctional genetic engineering approaches to maximise the functionality of CAR-T cells.

The novelty of gene-editing technologies like CRISPR/Cas systems that have arisen to address these issues. CRISPR is based upon a bacterial immune system, offering specific and efficient editing of particular loci of the genome using customized RNA-guided nucleases. CRISPR is more precise, scalable and operates quickly and cheaply compared to previous programmable platforms, e.g., ZFNs and TALENs [5, 6]. Notably, CAR-T cell therapy can be adapted to CRISPR-based strategies especially well, with T cells being edited in vitro, which enables strict quality control and safety evaluation before being reinfused into the patients [7].

CRISPR in combination with CAR-T cell editing has enabled the creation of new designer T cells that have improved therapeutic capabilities. They are the violation of inhibitory immune checkpoint genes to enhance T-cell persistence, precise insertion of T-cell CAR elements into particular genetic sites, and the control of cytokine signalling pathways to lessen treatment-linked toxicity. Moreover, CRISPR has made it possible to produce universal allogeneic CAR-T cells, which have the promise of producing therapies with greater accessibility and lower production costs [3]. In this review, the recent applications of CRISPR-based genome editing and new advances in the enhancement of CAR-T cell therapy are reviewed and analysed.

2. CRISPR-Cas9 Engineering Mechanism

The CRISPR-Cas system dates back to an immune defence system in bacteria and archaea to defend them against invading viruses. In the process of phage DNA invocation into the spacers, protospacer adjacent motifs (PAM) sequences are important to target DNA recognition [8]. The system may be divided into six major types. It is important to note that the *Streptococcus pyogenes* (SpCas9) is the origin of the Cas9 nuclease [9].

The CRISPR/Cas9 platform is fundamentally made up of two parts, the single-guide RNA (sgRNA) and the Cas9 endonuclease. CRISPR array transcription leads to the production of pre-crRNA which can be further processed to produce crRNA that can create a duplex with transactivating crRNA (tracrRNA). The tracrRNA:crRNA duplex is loaded into Cas9 to enable it to introduce double strand breaks (DSBs) within the target DNA. The Cas9 comprises two major lobes: the nuclease (NUC) lobe for cutting DNA and the recognition (REC) lobe for binding the gRNA and the targeted DNA. In the NUC lobe, the RuvC domain is split into three subdomains scattered along the protein but only has one active site that is responsible for cutting the non-target strand (NTS) and HNH domain cleaves the target strand (TS). Besides, the C-terminal domain within the REC lobe predominates PAM binding [10].

The sgRNA is an engineered fusion of tracrRNA and crRNA that contains a short nucleotide sequence that is complementary to the target DNA sequence. After it has been introduced into a cell, the sgRNA complexes with Cas9. This ribonucleoprotein (RNP) complex surveys genomic DNA of a PAM sequence on NTS, typically, 5'-NGG-3' on SpCas9 [10]. When bound to the proper site, Cas9 creates a DSB in the DNA which triggers the cellular repair processes. Non-homologous end joining (NHEJ) is a mutagenic process that often causes insertions or deletions (INDELs). The mechanism is typically employed to silence genes, including inhibitory immune checkpoint receptors in CAR-T cells. Conversely, homology-directed repair (HDR) involves the application of a homologous DNA template to permit accurate insertion or substitution of genes, which may be used to insert a CAR construct into a known locus. Moreover, in drafting the repair template, the two homology arms are to be acquired on both sides of the sequence of the donor and the sequence should be modified to disrupt sgRNA binding, and avert cleavage by Cas9 [11].

Furthermore, it has been shown that Cas9 has been applied in CRISPR-edited immune cells and CRISPR elements can be introduced into cells in different ways [12]. Past experiments have demonstrated that the lentiviral transduction can be successfully used to perform practical CRISPR/Cas-mediated gene editing [13]. Nevertheless, the risk of off-target mutation may increase with permanent integration of the Cas9 gene. Consequently, it is usually desired that Cas9 be transiently expressed. CRISPR components may be transfected either as DNA, RNA or RNP complex.

It does not need intracellular transcription or translation and therefore is immediately active even though RNPs need further preparation [14]. CRISPR components are most frequently delivered to cells through electroporation or lipofection to obtain cellular delivery. Electroporation is the process of using pulses to form temporary holes in the membrane that allows negatively charged RNA and Cas proteins to enter the cytoplasm. The technique is linked with fast and very effective genome editing as well as may be accompanied by significant cellular toxicity. Conversely, lipofection is based on the use of cationic lipid particles to deliver CRISPR components and enter cells by the process of endocytosis. Although lipofection is not as detrimental to cells as electroporation, it is less effective in transfection than electroporation [15]. The CRISPR delivery strategy, therefore, should be selected with due consideration to their limitations. Altogether, CRISPR as a protocol reuses genome editing, which is programmable in eukaryotes, to form the basis of its revolutionary application in current cancer immunotherapy.

3. Applications of CRISPR in CAR-T Cell Therapy

CAR-T cell therapy is a significant breakthrough in adoptive cellular immunotherapy, which entails genetic engineering of T lymphocytes to express synthetic receptors that enable the CAR-T cell to recognise tumour-associated antigens (TAA) without relying on major histocompatibility complex (MHC) presentation, thereby circumventing some of the immune evasion strategies that tumours use (Figure 1). CAR-T cell therapy has been mostly conducted through ex vivo engineering approach. The T cells of the peripheral blood of the patient are first obtained by leukapheresis. Such cells are genetically engineered to produce a CAR either with viral vectors or genome-editing technologies. After genetic modification, the engineered T cells are produced in large quantities and reintroduced into the patient where they recognise tumour antigens and trigger specific cytotoxic reactions. Having been reinfused, CAR-T cells attach to TAA of cancerous cells via their extracellular receptor domains. This engagement triggers signalling pathways within the cell that facilitates the proliferation of T cells, cytokine release and tumour cell destruction.

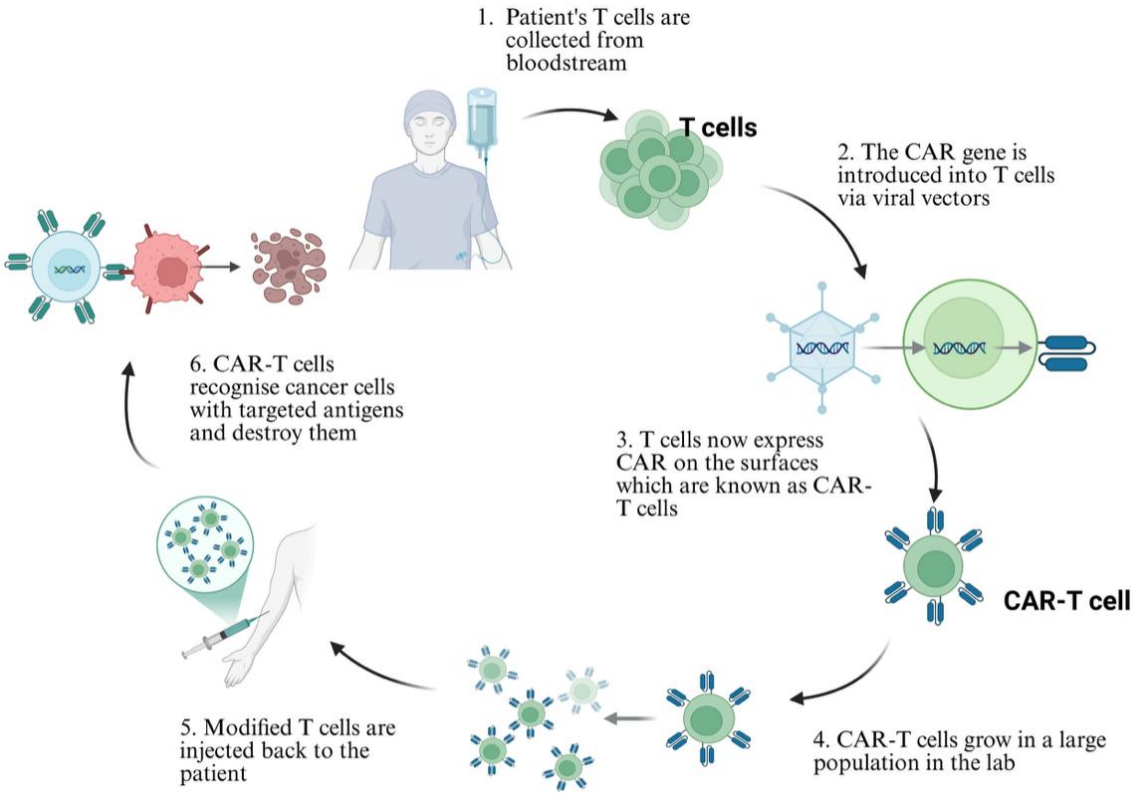

Figure 1. Schematic overview of the ex vivo CAR-T cell therapy process, including T-cell isolation, genetic modification, expansion, and reinfusion into patients (Original).

Chimeric antigen receptors have a modular structure that consists of functional domains. An antigen-binding domain, typically a single-chain variable fragment (scFv) of an antibody, is found in the extracellular region, and binds a specific antigen on tumour cells. The linking of this region to the cell membrane is made by the form of a hinge and transmembrane domain that gives it structural flexibility and stability. The intracellular region of the CAR contains the CD3ζ signalling domain, which is a product of the T cell receptor complex, and leads to cytotoxic reactions on antigen recognition. Moreover, the co-stimulatory domains, including CD 28 and OX40 are included to increase the growth and survival of T cells. These domains in combination define the functional characteristics of the CAR-T cells [16].

The CAR-T cell technology has gone through various generations depending on the number of co-stimulatory domains that the cell has. The initial generation CARs only had the CD3ζ signalling domain and had low clinical activity because of a lack of T cell activation. Second-generation CARs had one co-stimulatory domain and CD3ζ, which significantly increased antitumor activity. Most of the currently approved CAR-T therapies are based on these constructs. Third generation CARs have two co-stimulatory domains which reinforces intracellular signalling and stability. Most recently T cells Redirected to Universal Cytokine Killing (TRUCKs) have been engineered to release immune-modulating cytokines on activation by antigen recognition. Fifth-generation CARs combine gene-editing mechanisms to control endogenous signalling pathways [3]. Although the initial versions of CAR-T therapies were remarkably effective, they have multiple shortcomings, such as T-cell exhaustion and insufficient persistence. Therefore, the creation of CRISPR/Cas9 system has dramatically widened the opportunities of CAR-T cell therapy because of the possibility to make strict genetic adjustments.

3.1. Enhancing CAR-T Cell Antitumor Activity

CAR-T cell therapy has shown remarkable clinical success in cancer treatment. However, T-cell exhaustion, a dysfunctional state that arises during persistent antigen stimulation in tumours or chronic infections, often influences the effectiveness of the therapy. Tumour cells frequently exploit immune checkpoint pathways to suppress T cell function and evade immune surveillance. Receptors such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), and lymphocyte activation gene 3 (LAG-3) transmit inhibitory signals that reduce T cell proliferation and cytokine production and contribute to T-cell exhaustion. Although checkpoint inhibitors have improved outcomes in cancer immunotherapy, systemic antibody blockade may cause toxicity and lacks the potential to fully restore CAR-T function. Targeted deletion of those genes encoding inhibitory receptors can boost the antitumor activity.

A phase I trial have demonstrated that immune checkpoint inhibition of mesothelin-targeted CAR-T cells, specifically PD-1 blockade, significantly enhances modified-T cell activities against tumour cells and suppresses tumour progression, leading to higher median OS in patients of malignant pleural mesothelioma [17]. Given that immune checkpoint inhibitors can extend and promote CAR-T cell activities, researchers intended to validate whether depletion of immune checkpoint genes can enhance CAR-T cell functions. In a pioneering preclinical study, the CRISPR/Cas9-mediated disruption of PDCD locus has resulted in an improved long-term persistence and functionality in CAR-T cells. Employing a Cas9 RNP approach, the primary murine T cells isolated from OT-I transgenic mice were edited ex vivo by nucleofection with Cas9 RNPs and CAR constructs were delivered by retroviral transduction. This approach inserted the DSBs in the PDCD1 gene that is repaired with the help of NHEJ leading to gene inactivation. As a result, the edited CAR-T cells lost the expression of PD-1 proteins and were adoptively transferred on a model of sustained antigen stimulation. As Dötsch et al. were able to show, CD19 targeting PD-1 KO CAR-T cells remained over a year in vivo and maintained antigen-specific function with no signs of malignant transformation or immune homeostasis [18].

In addition to PD-1, other inhibitory receptors are being targeted with CRISPR technology in order to increase the efficacy of CAR-T. CTLA-4 deletion has been observed to augment the proliferation

of CAR-T cells and enhance antitumor responses in preclinical models of leukaemia and myeloma. The CTLA-4-deficient CAR-T cells in in vitro models of CD19-targeted CAR-T dysfunction showed higher proliferation, cytotoxic activity, and cytokine production than wild-type cells. Higher expression of surface CAR levels was also found in these edited cells in repeated antigen stimulation, avoiding the progressive downregulation of CAR levels observed in T-cell exhaustion. Mechanistic studies have shown that deletion of CTLA-4 augmented the CT28 co-stimulatory signalling because CTLA-4 competitively interacts with the B7 ligands CD80 and CD86 than CD28. CRISPR-mediated CTLA-4 KO CAR-T cells in xenograft mouse models of B-cell acute lymphoblastic leukaemia decreased tumour burden and improved survival by restoring the CD28 co-stimulation [19]. Likewise, LAG-3 KO CAR-T cells demonstrated strong anti-tumour response on the basis of antigen specificity in cells and in the xenograft murine model as opposed to normal CART19 cells [20].

Along with membrane-bound inhibitory receptors, intracellular immune checkpoints have also been discovered as a potential target of CRISPR-mediated editing. Protein tyrosine phosphatase non-receptor type 1 (PTPN1) is one of such molecules, and it prevents the activation of T-cells by suppressing the signalling of STAT5. The deletion of PTPN1 induced by CRISPR/Cas9 has been shown to increase the activation of STAT5 to stimulate T-cell proliferation and acquisition of effector functions [21]. These results indicate the possibilities that lay in combining extracellular and intracellular regulatory pathways to optimize CAR-T cell activity.

3.2. Generation of Allogeneic CAR-T Cells

The autologous CAR-T cell production still is a labour-intensive, expensive, and time-consuming procedure, restricting its availability to patients with fast-progressing disease. The CRISPR-based genome editing has made possible the production of allogeneic CAR-T cells as “off-the-shelf” products by removing determinants of alloreactivity [22]. CRISPR is an important gene essential in facilitating allogeneic CAR-T cell production. Graft-versus-host disease (GVHD) is one of the major challenges facing donor-derived cell therapy and the result of the donor T cells perceiving the host tissues as foreign. On the other hand, the infused T cells can be quickly destroyed by the host immune cells when they identify the HLA molecules of the donor on the engineered T-cells. To avoid this complication, the CRISPR is applied to knock out genes that encode the endogenous T cell receptor (TCR), therefore, eliminating alloreactivity. Moreover, HLA expression genes can be engineered to become less recognizable by the immune cells of the recipient and the chance of immunological rejection decreases [22].

A common method is disruption of the T-cell receptor alpha constant (TRAC) gene which removes the expression of the endogenous TCR complex. TRAC knockout using CRISPR/Cas9 inhibits the recognition of host antigens by donor T cells and, thus, this inhibition has been shown to greatly decrease the risk of GVHD. Simultaneously, the removal of the 2-microglobulin (B2M) may be carried out to remove surface expression of major histocompatibility complex class I (MHC-I) molecules, and, thus, reduce recognition by host cytotoxic T lymphocytes [12]. The protocol involved lentiviral delivery of CAR19 and electroporation of TRAC/B2M-targeted sgRNA using Cas9 mRNA. Multiplex CRISPR editing methods have produced CAR-T cells, which have lower alloreactivity but retain strong antitumor capability in preclinical models. In another method, the CAR transgene is inserted into the TRAC locus by the HDR with the help of recombinant adeno-associated virus (AAV) [23]. The TRAC KO CAR-T cells result in the more physiologically regulated expression of CAR and enhanced T-cell functions. In a like manner, a study employing PD-1/TRAC KO engineered T cells presented specific cancer-antigen constructs including NY-ESO and LAGE-1 to permit specific tumour recognition [4, 24]. The results from this trial displayed 9-months persistence in engineered T cells without any observed toxicities to CRISPR modifications. However, off-target effects appeared in CRISPR-edited CAR-T cells. Furthermore, CRISPR-mediated modification can be combined with the expression of HLA-E to reduce natural killer (NK) cells–mediated clearance of HLA-deficient CAR-T cells [25]. Moreover, induced pluripotent stem cells (iPSCs) have been investigated as a renewable source for generating allogenic TCR KO CAR-T cells. In an early phase

of a clinical trial, iPSC-derived CAR-T cells exhibit extensive proliferative capacity and can be precisely modified using CRISPR technology to knock in CAR19 constructs in the TRAC locus via AAV and produce TCR KO CART19 cells [26].

Overall, as ongoing clinical trials continue to evaluate the safety and efficacy of these engineered cells, CRISPR-based donor-derived CAR-T therapies hold significant promise for overcoming the manufacturing and accessibility limitations associated with current autologous CAR-T treatments despite the fact that the therapy still require verification from more clinical experiments to assess the persistence and real-life effectiveness.

3.3. Modulation of Cytokine Signalling and Toxicity

While cytokines play a central role in CAR-T cell activation and antitumour responses, excessive cytokine release can lead to severe toxicities, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). The high concentrations of pro-inflammatory cytokines like interleukin (IL)-6, interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are the major causes of these adverse events. It has been found that tocilizumab, which is an IL-6 receptor block, enhanced patient outcomes in cases of CART-mediated CRS, however they do not completely eliminate toxicity and can impair therapeutic effect [27]. Thus, CRISPR/Cas9 system provides a direct method of cytokine signalling pathways genetic regulation. GM-CSF gene is a central mediator that has been implicated both in CRS and neurotoxicity, which has been overwhelmed to significantly inhibit the production of inflammatory cytokines without affecting the proliferation and cytotoxicity of CAR-T cells. Ablation of GM-CSF gene in CART19 cells mediated by CRISPR leads to a significant reduction in GM-CSF secretion, but does not affect T-cell persistence. The xenograft model has shown that GM-CSF–deficient CAR-T cells did not interfere with the generation of other major cytokines that mediate normal CAR-T cell activities, which increase the overall survival relative to wildtype CART19 [28].

In addition, CRISPR technology has been used to overcome on-target off-tumour toxicity where CAR-T cells identify antigens expressed on both cancerous and normal tissues. One such example is the targeting of CD33 in acute myeloid leukaemia (AML) in which the expression of CD33 on normal hematopoietic stem cells (HSCs) causes dose-limiting toxicity. To deal with this CRISPR/Cas9 has been employed to silence CD33 in HSCs, enabling selective elimination of malignant cells without impairing normal haematopoiesis. These findings have demonstrated that the HSC progeny that is depleted of CD33 is resistant to CART33 as compared to the HSC control [29]. Fratricide is another issue of this therapy which is observed in T-cell malignancies where the CAR-T cells attack themselves because of the expression of shared antigens. An example is that CAR-T cells targeting CD7 may be eliminated by them, which restricts their proliferation and performance. The Ablation of CD7 in CAR-T cells induced by CRISPR, avoiding fratricide, allows strong expansion and long-term antitumor responses. On the same note, multiplex editing approaches of TRAC, and CD7 have been applied to generate fratricide-resistant universal CAR-T cells with improved therapeutic potential [30].

Overall, the results indicate that CRISPR/Cas9-based gene editing is an effective tool to tune cytokine signalling and reduce the toxicities of CAR-T cell therapy. CRISPR will be used to create safer and more efficient CAR-T products by selectively disrupting pro-inflammatory cytokines, removing shared antigen expression, and enhancing target specificity. With the further development of these approaches, combining the toxicity modulation with other genetic improvement and anti-tumour therapies will be key to the optimization of CAR-T therapy in a variety of malignancies.

4. Limitations of CRISPR-Engineered CAR-T Cells

Even though there is a transformative potential of CRISPR/Cas9-mediated genome editing in improving CAR-T cell therapy, a number of limitations are critical and need to be overcome before this technology can be used in large-scale clinical practices. These hurdles cut across the areas of genomic specificity, delivery efficacy, immunogenicity, and long-term safety. One of the most important issues with the CRISPR/Cas9 technology is the presence of off-target effects, when a cleavage of the DNA can occur at the loci with partial sequence homology to the target site. These off-target mutations can either disrupt tumour suppressor genes or activate oncogenic pathways. The architecture of sgRNA is also crucial as it may cause INDEL formation at a particular site to produce unwanted gene mutation. In spite of the fact that the sgRNA design has been improved through sequence truncation, nucleotide modulation and optimization of GC content to enhance specificity and minimize mutations, it is still difficult to entirely prevent off-target activity. It is also indicated that the addition of guanine nucleotides can enhance most of the promoter transcriptions to a large degree. Besides, it has been reported that overexpression of Cas9 nuclease leads to overcleavage, which highlights the need to rigorously monitor the conditions of editing [3]. The other critical constraint is the effectiveness and accuracy of genome editing and it is largely affected by the choice of Cas9 variants. Editing efficiency variability may cause heterogeneous CAR-T cell products, which may influence the therapeutic consistency and efficacy. Alternative nucleases like Cas12a (Cpf1) that require a single Polymerase III promoter and engineered Cas9 variants are more specific and smaller, although their clinical application remains being actively pursued [31].

Introduction of CRISPR/Cas9 into target T cells is another step of bottlenecking. Existing methods are viral vectors, electroporation of RNP complexes, lipid nanoparticles, and extracellular vesicles. Viral delivery systems are also effective but pose a problem of the issue of insertional mutagenesis, long-term expression of Cas9 and immunogenicity. Electroporation is a non-viral technique that can be used to minimize integration risks, but is commonly linked with cellular toxicity and diminished viability. New delivery systems, such as lipid-based nanoparticles, exosome-mediated, and others, are designed to enhance safety and targeting precision but need to be optimized further [3]. CRISPR/Cas9 components have the potential to be immunogenic, which is also a potential barrier. It has been reported in humans that pre-existing immunity to bacterial-derived Cas9 proteins, e.g. Streptococcus pyogenes or Staphylococcus aureus can cause immune-mediated clearance of edited cells or unwanted inflammatory responses. To overcome these risks, epi-tope masking, orthologous Cas proteins, or transient expression systems are under investigation [31].

Moreover, CRISPR-induced DSBs may trigger DNA damage response pathways, such as p53 signalling, which may cause apoptosis or p53-deficient cells to be selected. This also portends the risk of clonal proliferation of genetically mutated cells having oncogenic potential. Moreover, when the DSBs are improperly repaired using NHEJ, the rearrangements of the chromosomes may occur, such as chromosomal translocations, which increase the likelihood of genomic instability further [31].

Lastly, gene editing in CAR-T cells has not been fully comprehended in terms of long-term functional outcomes. As an example, immune checkpoint disruption could potentially contribute to immune homeostasis inefficiency, which could result in overactivation, exhaustion, or autoimmunity. The trade-off between increased efficacy and long-lasting T-cell fitness must be weighed as pointed out in the recent studies.

5. Future Perspectives

Further advances in CRISPR-based CAR-T cell therapy will be based on ongoing developments in genome editing methods, delivery models, and cell engineering models. The development of next-generation editing tools, such as high-fidelity Cas9 versions, base editors, and prime editing systems, which promise the potential of introducing specific genetic mutations without causing any double-strand DNA breaks, thus reducing the risk of chromosomal rearrangements and p53-mediated toxicity, is likely to enhance precision and reduce off-target activity and DNA damage. Simultaneously,

attention to the delivery methods optimisation is one of the most important issues. Although the viral vectors are effective in gene transfer, there are risks linked with using the vectors, which include immunogenicity and insertional mutagenesis. Consequently, non-viral methods, such as electroporation of RNP complexes, lipid nanoparticles, and extracellular vesicles, provide the potential alternatives that can make the procedures much safer and allow more controlled editing. Another important direction is the production of universal allogeneic CAR-T cell products. The advantage of CAR-T cells is that donor-related or off-shelf cells can be used to circumvent the logistical and economic challenges of autologous manufacturing. The endogenous TCR and HLA disruptions by the CRISPR, along with other cell sources like iPSCs, offers a possible basis of scalable cost-effective therapies. Nevertheless, issues of immune compatibility, in vivo persistence and functional durability have yet to be overcome before it can be widely used clinically.

Notably, the long-term effects of the disruption of checkpoint genes are not fully comprehended. As an example, despite the potential beneficial effects of PD-1 or CTLA-4 knockout in increasing short-term cytotoxicity, sustained activation can increase T-cell exhaustion or memory formation, which can potentially limit the long-term effect of therapy. Hence, subtler approaches, including reversible gene modulation, might be necessary instead of irreversible gene alteration. New approaches that combine CRISPR technology with synthetic biology and precision medicine will probably further increase the therapeutic space. The selective response of T cells to complex tumour antigen patterns can be facilitated by programmable and logic-gated CAR constructs, which can enhance specificity and minimise off-tumour toxicity. Moreover, the use of multiplex editing strategies to stimulate cytokine signalling pathways, metabolic regulators, and exhaustion-related genes can also be used to further improve T-cell fitness in adverse tumour microenvironments [31]. A combination therapy is also a promising direction. CRISPR-based CAR-T cells, when used together with immune checkpoint inhibitors, cytokine regulation approaches, or targeted therapy, can enhance clinical responses in a wider spectrum of malignancies. These methods can be especially relevant in overcoming resistance in hard tumours.

6. Conclusion

To conclude, the development of CRISPR/Cas9 genome editing and CAR-T cell therapy has brought a tremendous improvement to the cancer immunotherapy arena through the flexibility and accuracy of genetic engineering. This has been further enhanced by the introduction of CRISPR/Cas9 system which has allowed more precise and multiplex genetic modifications that overcome major limitations of traditional CAR-T strategies. According to this review, CRISPR-based engineering allows interfering with inhibitory immune checkpoints, producing off-the-shelf CAR-T cells derived from donors, CAR-integration specificity, and cytokine signalling regulation to alleviate treatment-related toxicity. Collectively, these breakthroughs highlight the importance of genome editing in the creation of new CAR-T therapies with enhanced functionality and production. In spite of these developments, there are still various obstacles that restrict the wider clinical usage. The most significant obstacles, especially in solid tumours, include insufficient CAR-T cell persistence, antigen escape and the suppressive tumour microenvironment. Even though CRISPR-mediated multiplex editing provides a way of addressing these challenges by improving T-cell fitness and resistance to inhibitory signalling, these methods add another layer of complexity and may be associated with safety issues. More specifically, widespread genome editing can raise the risk of unintended genomic editing and dys-regulated immune responses, and the challenges of production and the long-term effects of widespread genetic modification remain poorly characterized, particularly the issue of T-cell exhaustion and persistence.

The long-term safety of CRISPR-engineered CAR-T cells is important to ensure. The off-target editing risks, chromosomal rearrangement, and triggering of DNA damage response pathways require stringent genomic evaluation and standardised procedures. Complete monitoring plans will be needed to assist clinical translation. In conclusion, the CRISPR/Cas9 system is transforming the future of

CAR-T cell therapy as it allows more and more specific and flexible cellular engineering. The ongoing development of editing technologies, delivery methods, and integrative therapy approaches will continue to increase the clinical utility of CAR-T cells and develop the next generation of cancer immunotherapy.

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