CRISPR-edited CAR-T cells: Using CRISPR-Cas9 to Improve CAR-T Therapy

Tatchapon Ajavavarakula *

School of Life Sciences, Tsinghua University, Beijing, China

* Corresponding Author Email: liaomq20@mails.tsinghua.edu.cn

Abstract. One of the cornerstones of cancer immunotherapy, chimeric antigen receptor T cell (CAR-T) immunotherapy is a treatment comprising of T cells transfected with artificial receptors that target a specific tumor antigen, potentiating tumor destruction. Despite the effectiveness of this technique in treating hematopoietic malignancies, efficacy against other cancers leaves much to be desired. CAR-T therapy's anti-tumor effectiveness, safety, and accessibility are hampered by issues such T cell exhaustion, toxicity, and ineffective production techniques. With the advent of CRISPR-Cas9 technology, allowing ease of genome editing, it is now possible to address these challenges. By introducing a double-strand break at a particular genomic location, this gene editing technology can be utilized to target inhibitors of T lymphocyte function, directed to specific loci to produce a less toxic product, and engineer allogeneic CAR-T cells. However, CRISPR-Cas9 confers its own limitations, including off-target editing. This review introduces the applications of CRISPR technology to CAR-T therapy and evaluates how the technology can optimize the effectiveness, safety, and product availability of this cancer immunotherapy. This paper also addresses some of the potential drawbacks of CRISPR-edited CAR-T cells.

Keywords: CAR-T Therapy, CRISPR-Cas9, T Cell Exhaustion, Toxicity, Allogenic CAR-T Cells.

1. Introduction

Cancer accounts for 1 in every 6 deaths worldwide, second only to cardiovascular disease. In 2020, estimated cancer deaths amount to 9.9 million globally. In 2040, the number is expected to reach 16.2 million [1]. By applying effective preventive, early detection, and treatment measures, a significant number of lives could be spared each year. With the exception of non-melanoma skin cancer, 42 percent of new cancer cases in the US can be prevented, including the 18 percent of cancer cases brought on by obesity and 19 percent of cancer cases brought on by smoking [2]. Better cancer screening can also increase early detection and treatment. Efficacious and safe treatment options are also key to increasing survival. There are many treatments for cancer, one which has much potential is CAR T therapy (Figure 1).

CAR-T immunotherapy, a prevalent activated cell therapy, is immunotherapy where T cells are modified with CARs to target cancer cells more effectively. CARs consist of three domains: ectodomain, endodomain, and transmembrane domain. On recognition of an antigen, a signal is transduced from the ectodomain to the endodomain, triggering downstream signaling pathways that activate T cells to affect its function and kill tumor cells. On paper, this therapy seems to be capable of effectively and specifically killing cancer cells, however, there are many adverse effects. Much effort has been put to increase its safety and efficacy. Currently, the therapy is at its fifth generation of development, wherein it contains a STAT3-binding domain and a truncated peptide from IL-2Rβ in addition to TCR and co-stimulatory domains. Besides the activation signal from TCR (signal 1) and costimulatory domain (signal 2), the third structure is capable of cytokine signaling (signal 3) through the JAK-STAT pathway. All 3 signals combine to proficiently promote T cell activation. Despite advances over the years, there are still limitations of CAR-T therapy that need to be overcome, limitations such as exhaustion, toxicity, and bottlenecks in manufacturing. Fortunately, with the increasing ease of gene editing, made possible by the CRISPR technology, scientists are now better equipped to deal with challenges present in CAR-T therapy.

CRISPR-Cas systems are composed of gRNA and Cas protein that, together, form a molecular scissor that can perform a double-stranded break in a sequence of DNA of interest. With the advent of this technology, there has been a massive increase in implementing genetic modifications as a method to counteract a variety of genetic disorders and malignancies. This technology can be used to correct known mutations of a genetic disorder. Experimental models can be induced using CRISPR technology to study disease development. Combinatorial genetic screens can also be conducted to study high-order gene interactions. In order to increase T lymphocyte persistence and effector function, lessen toxicity, and create allogenic CAR-T cells that are commercially available for purchase, CRISPR-Cas9 applications in T cell-based immunotherapies are currently being investigated. (Figure 1). This paper reviews these applications, including optimization of CAR-T therapy through enhancing function, mitigating toxicity, and producing allogeneic CAR-T cells. This is then followed by discussing the limitations in its present application and the potential solution.

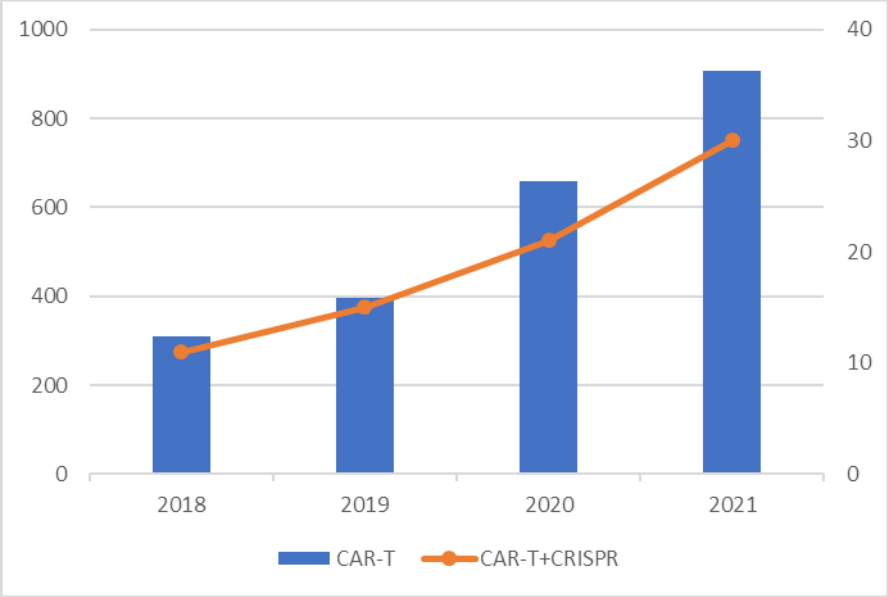


Figure 1. Number of research papers on CAR-T therapy (blue) and CAR-T+CRISPR (orange) from 2018 to 2021. The amount of CAR-T+ CRISPR research papers accounts for a significant portion of total articles about CAR-T. The general trend has seen an increasing ratio over the last few years. It is shown that there is increasing interest in applying the CRISPR technology in this therapy.

2. Enhancing CAR-T cell effector function and persistence

An extensive issue in CAR-T therapy, the state of exhaustion devastates the effectiveness of CAR-T cells. It is believed to be an intrinsic negative feedback mechanism of T cells that results from tonic CAR signaling. Epigenetic remodeling, as shown by Weber et al., allows exhausted CAR-T cells to regain activity when tonic CAR signaling is temporarily inhibited. This is accomplished by either inhibiting CAR signaling kinases or forcing suppression of CAR. The phenotypic and epigenomic changes in T cell fatigue can be prevented or reversed by inducing rest before or after they occur. Because transient CAR-T cell rest reinvigorates T cell functionality, engineering modulation of targets, either genetic or epigenetic, that promote exhaustion by employing CRISPR-Cas9 has the ability to amplify T lymphocyte persistence and effector function.

Studies have demonstrated that genetic ablation of T cell persistence and effector function suppressors such as CTLA4, LAG-3, and PD1 using CRISPR-Cas9 strengthens T cell effectiveness [3]. This presents the first tractable point of intervention. More recently, it was found that exhaustion is epigenetically controlled and exhibits a unique chromatin profile. A time course study of alterations of the epigenome revealed that changes in chromatin profile precede the manifestation of exhaustion phenotypes. These changes include alteration of chromatin accessibility at sequences upstream to

genes such as PDCD1, CTLA-4, and HAVCR2, which are exhaustion-marked genes. In an in vitro model, deletion of PDCD1 exhaustion-specific enhancer reduces PD-1 expression. Modification of exhaustion-specific enhancers, but not the gene itself, leaves the gene open to expression under the control of other enhancers in non-exhausted states. This suggests an alternative method of enhancer editing using CRISPR-Cas9 as a potential path to preventing or reversing T cell exhaustion to improving T cell effector function [4]. However, more studies into the potential effects of these deletions are required.

Exhausted T cells also exhibit increased access to genes of the inhibitory transcription factors AP-1 family proteins, which have been demonstrated to potentiate exhaustion. Previous studies indicated that c-Jun, a classical AP-1 factor, is highly expressed in CAR-T cells, making these cells more exhaustion-resistant. CRISPR-Cas9 can be utilized to introduce a transgene containing an element that up-regulates the expression of c-Jun. Preliminary data suggested that CRISPR-Cas9 disruption of inhibitory AP-1 factors can also promote T cell effector function [5]. Alternatively, catalytically inactive Cas9 (dCas9) attached to chemical epigenetic modifiers (CEMs) can be utilized to stimulate or inhibit certain genes to increase CAR T cell persistence. CEMs are designed to modulate gene expression by recruiting in endogenous chromatin remodeling machinery [6].

3. Cytokine modulation to reduce toxicity and potentiate CAR-T cell function

Cytokines are an integral part of immune responses. It also has a huge influence on CAR-T cells. An elevated level of certain cytokines may lead to toxicities, while subpar concentration hinders function. Therefore, using CRISPR/Cas9-based technology knock-ins or knock-outs to modulate cytokines presents another obvious point of intervention.

There are several unique toxicities that accompany CAR-T therapy. A major and very lethal toxicity is cytokine release syndrome (CRS), which is defined as overexpression and secretion of immune-modulatory molecules including GM-CSF, IL-1, and IL-6. The first of which is a prime contributor to cytokine storm, while the subsequent storm of cytokines released from bystander immunocytes is reinforced by IL-1 and IL-6 (the first storm of cytokines being released from CAR-T cells) [7]. Mutation of GM-CSF gene and insertion of anti-IL-6 scFv and IL-1RA in CAR-T cells has shown reduced toxicity. More specifically, knock-out of GM-CSF mitigates CRS caused by CAR-T cell GM-CSF cytokine secretion, while knock-in of anti-IL6 scFv and IL1RA blocks IL-6 and IL-1 produced by bystander immune cells [7]. Additionally, specific cytokines can be placed under the control of the promoter of choice to control cytokine expression, using CRISPR-Cas9 to insert a cytokine-encoding sequence at a specified genomic location. In a study, the IL-13 gene sequence was replaced by the IL-15 gene, thus placing the control of the cytokine IL-15 secretion under the endogenous IL-13 regulatory gene sequence, thereby achieving activation-dependent regulation of IL-15 secretion [8]. Aside from reduced toxicity, GM-CSF-negative CAR-T cells retain their functionality as well as show potent anti-cancer activity [9].

4. Engineering off-the-shelf allogenic CAR-T cells

Autologous CAR-T therapies, since their invention, remains one of the most important advancement in our fight against cancer. It is highly effective against hematological malignancies such as B cell malignancies. However, bottlenecks in manufacturing autologous CAR-T cells present a weak point that hampers treatment efficacy. Current approaches to manufacturing autologous CAR-T cells are associated with high costs, lengthy production time, and dependence on patient fitness. Off-the-self CAR-T cells produced using T lymphocytes from healthy donors offer a compelling approach to overcome these barriers. Large-scale manufacturing can potentially reduce production costs while having a readily available product “off-the-shelf” forgoes delay for production, which may prove valuable for individuals suffering from highly proliferative illnesses. Moreover, using

donated cells from healthy individuals dispels concerns regarding the quality and fitness of patient T cells, which are affected by previous treatments.

In spite of these advantages, allogenic CAR-T cells confer impediments that interfere with the safety and effectiveness of the treatment. One such obstruction is graft-versus-host disease (GvHD), wherein endogenous TCRs on donor cells recognize HLA molecules on the extracellular membrane of the recipient's cell. This non-self HLA recognition elicits an immune reaction against the patient. To circumvent GvHD, CRISPR/Cas9 can be directed towards CAR-T cells to abrogate the presence of endogenous TCRs on the surface. CAR-transgene incorporation at the T cell receptor α constant locus was also extensively investigated [10]. Putting the gene under the control of the endogenous TCR promoter affords the ability to uniformly express CAR on the membrane, while simultaneously destroying endogenous TCRs, eliminating the risk of GvHD.

Conversely, host rejection of donor-derived T cells is another obstruction conferred by allogenic CAR-T, wherein host immune cells, on the identification of foreign HLA molecules on allogenic CAR-T cells, elicit an immune response. Similar to TCRs, genetic abrogation of both classes of MHC molecules of allogenic CAR-T cells can prevent host rejection [11].

5. Limitations of CRISPR-edited CAR-T cells and potential solutions

5.1. Off-target effects

Implementation of the CRISPR-Cas9 technology in T cell-based immunotherapies presents a serious obstacle to off-target editing. This is a major problem for they may contribute to mutations such as tumor suppressor gene inactivation, proto-oncogenes activation, or chromosome translocations. Additionally, the efficiency of gene editing is also under question. Since the CRISPR-Cas9 induces a double-strand break, this activates the p53 DNA repair pathway, resulting in inefficient editing. In contrast, cells with dysfunctional p53 pathways exhibit efficient gene editing, however, these cells are more prone to becoming malignant. Selection for these cells poses potential safety issues. Therefore, careful screening and identification measures for these phenomena are required, especially in clinical applications. Multiple methods, which include GUIDE-seq, SITE-seq, and CHANGE-seq, have been created for analyzing next-generation sequencing data and the off-target impacts of mutations brought on by CRISPR [12].

5.2. Immunogenicity

In the human body, pre-existing antibodies against some variants of Cas proteins have recently been found (SaCas9 and SpCas9). Therefore, the immunogenicity of the CRISPR-Cas system is another factor that needs to be considered when applying this technology to cancer immunotherapies. To combat against this pre-existing immunity, methods such as epitope masking can be implemented, wherein Cas9 immunogenic peptides are removed to prevent stimulation of the immune system [13].

5.3. Delivery

Safety and efficacy issues may also arise from CRISPR-Cas delivery methods. Viral or non-viral methods are the two broad kinds of delivery strategies. The most often used vectors for viral methods come from lentiviruses, adeno-associated viruses, or adenoviruses (ADVs, AAVs). ADVs as a vector has concerned regarding immunity in humans, whereas lentiviral vectors carry oncogenic potential. This could compromise the safety and effectiveness, wherefore there is increasing interest in non-viral delivery strategies [10].

6. Conclusion

CAR-T therapy represents a significant advance in cancer treatment. Similarly, CRISPR-mediated gene editing revolutionizes how scientists view and interact with biology. Naturally, a confluence of these two behemoths could potentially advance the field of cancer treatment and provide better cancer

healthcare. The utilization of the gene editing tool for CAR-T therapy could boost its anti-tumor potency while minimizing its toxicity. CRISPR-Cas9 can be used to target the enhancers of up-regulators, or the up-regulator itself, of T cell exhaustion. In the same sense, it can be employed to stimulate the expression of activators of T lymphocyte effector function as well. To mitigate toxicitthe y, regulation of cytokines through CRISPR-Cas9 can abate cytokine release syndromes and potentiate its cellular function. Furthermore, the development of allogenic CAR-T cells is also aided by this gene editing technology. Through combating GvHD as well as allorejection induced by allogenic CAR-T cells, CRISPR-Cas9 technology brings us closer to producing a commercially and readily available CAR-T cell product, making the treatment promptly available when needed. Despite the vast potential of this application, the CRISPR technology does come with certain baggage. Challenges such as off-target effects, immunogenicity, and delivery may present certain hold-backs in the application. Regardless, many are optimistic that the development of novel CAR-T therapies involving CRISPR-based technology will ultimately improve efficacy, safety, and availability.

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