

# Using CRISPR/Cas9 Genome Editing to Improve the Efficacy of CAR-T Therapy in B-Cell Acute Lymphoblastic Leukemia

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B-cell Acute Lymphoblastic Leukemia (B-ALL) is a deadly cancer within the bone marrow. Despite modern cancer fighting approaches such as chemotherapy, radiation therapy, and stem cell transplants, this disease still has high recurrence rates and is unable to be cured in many patients. With the recent emergence of Chimeric Antigen Receptor T-cell therapy (CAR-T therapy), B-ALL can be treated using new and improved strategies. CAR-T therapy relies on engineered T-cells to target specific cancer cells that the body normally is unable to detect. In order to further improve CAR-T therapy and increase its effectiveness in fighting B-ALL, the breakthrough genome editing tool CRISPR/Cas9 has been used to genetically modify these cells. In this paper, we consider how CRISPR/Cas9 can be used to generate an off-the-shelf CAR-T resource, improve CAR-T cell persistence, and reduce CAR-T associated toxicities.

## Introduction

Around 6,000 cases of Acute Lymphoblastic Leukemia (ALL) are diagnosed annually in the United States, including 1,390 deaths every year resulting from this disease<sup>1</sup>. ALL accounts for 34% of all cancers in children, making it the most common childhood cancer<sup>2</sup>. B-Cell Acute Lymphoblastic Leukemia (B-ALL) is the most common subtype, causing 75% of ALL cases in adults. In the past few decades, treatment methods including remission-induction therapy, consolidation therapy, and allogeneic hematopoietic stem-cell transplantation (HSCT) have significantly increased the 5-year survival rate for patients from 10% to 65% in 2023<sup>3,4</sup>. Although complete or partial remission can be achieved using these methods, many patients experience relapse, refractory leukemia, or other unwanted side effects such as Graft versus Host Disease (GvHD) during stem cell transplants<sup>3</sup>. This has led researchers to search for novel methods to overcome these challenges. In recent years, a new suitor for cancer immunotherapy has emerged: Chimeric Antigen Receptor T-Cell therapy (CAR-T therapy). This approach uses patient-derived T-cells that are genetically engineered to target specific antigens found on cancerous cells, therefore killing them. CAR-T therapy is versatile because these cells can be designed to express a wide array of receptors, each of which can target distinct proteins on the surface of a cancer cell. CAR-T cells in patients with B-ALL are directed against CD19, a cancer antigen on most B-ALL cells<sup>5</sup>. CAR-T therapy has been FDA-approved to treat several hematological malignancies including lymphomas, some forms of leukemia, and multiple myeloma<sup>6</sup>. However, CAR-T therapy has its limitations such as its as-

sociated toxicities, the inconvenience of manufacturing autologous CAR-T cells, and CAR-T cell exhaustion<sup>7</sup>. With the help of genome editing tools such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), new and improved techniques have been developed to advance the engineering of CAR-T cells and overcome these restrictions. Initially discovered in bacteria as an adaptive immune system, CRISPR and its associated protein Cas9 have been harnessed over the years to be able to precisely cleave and edit DNA in humans. This review provides an overview of current applications for using the CRISPR/Cas9 system to improve the efficacy of CAR-T therapy, specifically in B-ALL.

## B-Cell Acute Lymphoblastic Leukemia

B-ALL is a progressive cancer in which lymphoid progenitor cells undergo mutations and excessive proliferation<sup>3</sup>. As a result, the bone marrow produces too many immature B-lymphocytes, leading to the inability to make enough healthy blood cells<sup>8</sup>. This can have severe effects such as an increased risk of developing infections, bleeding, joint pains, and clotting. In approximately 50% of childhood cases, B-ALL arises from leukemic cells with hyperdiploidy (more than 50 chromosomes per cell) and the fusion of the TEL gene on chromosome 12 with the AML1 gene on chromosome 21<sup>9</sup>. The AML1 transcription factor controls the expression of many genes essential for proper hematopoietic stem cell growth. The fusion of the TEL and AML1 genes inhibits AML1 function, causing abnormal hematopoietic stem cell differentiation and the promotion of B-ALL development<sup>9</sup>. Other less common genetic abnormalities that give rise to B-ALL in-

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clude hypodiploidy (fewer than 45 chromosomes per cell), fusion of the MLL and AF4 genes, and BCR and ABL genes<sup>9</sup>. Like AML1, the proteins encoded by these genes are crucial for gene expression and various signaling pathways in B-lymphocytes. Translocations of these chromosomes disrupt the normal function of these proteins, resulting in impaired B-cell growth, excessive proliferation, and thus B-ALL<sup>9</sup>.

The standard treatment for B-ALL is chemotherapy, consisting of three phases: remission induction, consolidation therapy, and long-term maintenance<sup>3</sup>. Chemotherapy drugs are infused into the patient to kill the fast-growing cancer cells. The initial remission induction therapy aims to destroy all the leukemia cells, but it often leaves behind some cells that need to be targeted through more intense approaches. In the consolidation phase, treatment is intensified to kill off any remaining leukemic cells. If the leukemia is still present after the first two phases, a combination of drugs may be added in maintenance therapy<sup>10</sup>. Unfortunately, chemotherapy is not completely effective, as 10-15% of children and adolescent patients will experience severe relapse and only 30-40% of adult patients will achieve long-term remission<sup>3</sup>.

A more effective approach to fighting B-ALL is allogeneic hematopoietic stem cell transplantation. This method involves the transfer of healthy blood stem cells from a donor to the cancer patient. Before the patient can receive healthy stem cells, they must receive chemotherapy and radiotherapy to deplete the unhealthy blood cells and to keep the body from rejecting the foreign cells. Once this is completed, the healthy stem cells can be infused into the bone marrow where they can begin producing healthy blood cells<sup>11</sup>. The major risk with this approach is Graft versus Host Disease due to the allogeneic cells attacking the foreign tissues of the patient. GvHD is life-threatening and can cause global inflammation, shock, and organ damage<sup>11</sup>.

Although a variety of treatment methods have significantly improved survival rates for patients with B-ALL, they have been unable to permanently cure this disease, and there remains a large percentage of patients who never achieved complete remission. In the past decade, immunotherapy in the form of CAR-T cell therapy has shown promise to treat cancer like never before.

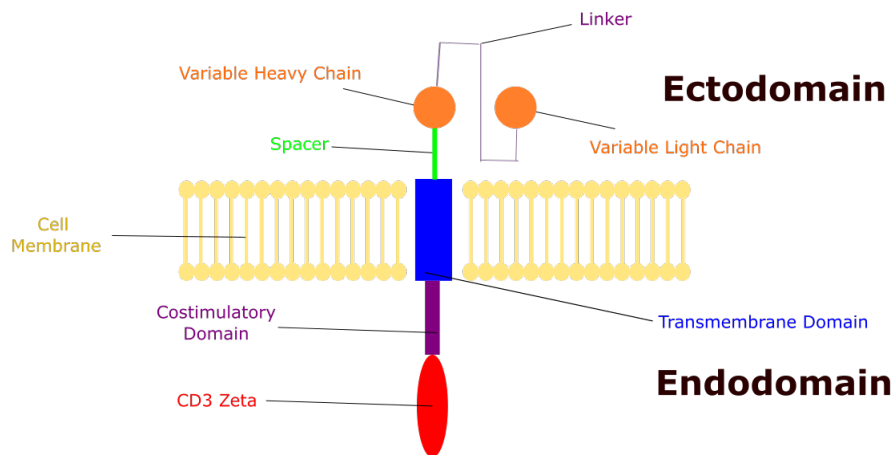
### CAR-T Therapy

Chimeric Antigen Receptor T-cells are genetically engineered T-lymphocytes with special receptors to target specific antigens on the surface of cancer cells. T-lymphocytes normally function by recognizing cells with abnormal proteins, which can include virus-infected cells and cancerous cells. Following recognition, they have the potential to kill these cells to stop the infection or proliferation of the cancer. However, cancer cells can evade T-lymphocytes through reduced im-

munogenicity, hence the need for a manufactured CAR on the T-cells to bind to cancer antigens. Following recognition of the cancer-specific antigen, the CAR-T cell is activated to kill the cancer cell without causing damage to healthy cells. In B-ALL, the most commonly manufactured CAR targets the antigen CD19 because of its expression on most malignant B-cells. The structure of a CAR protein is composed of three parts: an endodomain, a transmembrane domain, and an ectodomain (Figure 1). The ectodomain (extracellular) is the antigen-binding domain derived from a monoclonal antibody (a protein that binds to antigens). This domain is a single-chain variable fragment made up of variable heavy and light chains connected by a linker<sup>12</sup>. A spacer combines the ectodomain with the transmembrane domain. This domain spanning the cell membrane is used to fix the CAR to the cell. Finally, the intracellular signaling domain, or endodomain, is used to transduce a signal to the T-cell, triggering the release of cytokines to promote apoptosis (cellular death) in the cancer cell. CAR-T cells have gone through four generations based on varying intracellular domains. In the first generation, the endodomain consisted of the CD3 Zeta receptor alone. To further improve cytokine production and in vivo persistence of these CAR-T cells, costimulatory domains such as CD28 and 4-1BB were added in later generations<sup>13</sup>.

To manufacture these chimeric antigen cells, T-cells must be extracted from either the patient (autologous) or from a donor (allogeneic). In studies using CAR-T cells, lentiviral vectors have been the main source of adding a specific CAR gene into the extracted cells. The CAR-T cells are cultured in vitro after modification, screened for the presence of an effective CAR, then expanded to sufficient numbers to be infused back into the patient. This process can take weeks, and researchers are still looking for ways to make these manufacturing procedures more efficient.

Although CAR-T therapy has shown great promise in patients with cancer, a study has shown that the relapse rate of CD19-targeted CAR-T therapy is as high as 30%<sup>3</sup>. One of the ways that cancer can evade CAR-T therapy is through antigen escape. This occurs when leukemic cells display loss of the target antigen due to the downregulation of the gene encoding it. Cancerous cells without the targeted antigen are favored by natural selection against cells expressing high levels of the antigen, allowing them to pervade against the CAR-T cells. To overcome this challenge, CAR-T cells have been rolled out to target multiple antigens in case of downregulation, such as CD19/CD20 bivalent CAR-T cells. Another limitation of this therapy lies within the autologous CAR-T manufacturing process. When T-cells are extracted from the patient's own body, the remaining T-lymphocytes are poor in quality and quantity. This can lead to rapid disease progression in the patient, who has to wait an additional 3-4 weeks before receiving their engineered cells back<sup>7</sup>. To address these limitations with current



**Fig. 1** Structure of a Chimeric Antigen Receptor. A CAR is made of an ectodomain (top) containing the variable heavy and light chains (orange) from a monoclonal antibody, a small spacer (green) connecting to the transmembrane domain (blue), and an endodomain (bottom) that contains a costimulatory domain (purple) fused to a portion of CD3 Zeta (red).

CAR-T cell approaches, researchers have begun applying the breakthrough gene editing system CRISPR/Cas9 to directly improve the efficacy of CAR-T therapy.

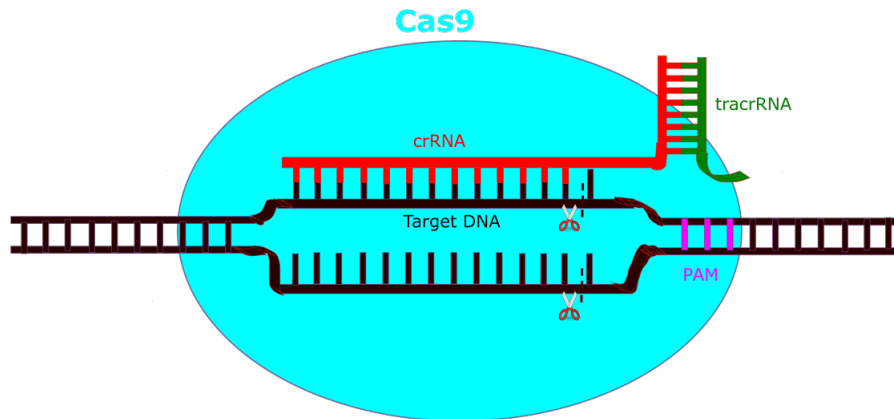
## CRISPR Gene Editing

In the late 1980s a young scientist, Francisco Mojica, began to examine the DNA of an archaeal microbe with extreme salt tolerance, *Haloferax mediterranei*<sup>14</sup>. In the first DNA fragment he examined, he noticed multiple copies of a short palindromic sequence, separated by unique spacers. These sequences of DNA would later be called clustered regularly interspaced palindromic repeats (CRISPR). Interestingly enough, different spacers within the fragment of DNA were complementary to the DNA of different viruses the microbe was resistant to. Mojica realized that the sequence of the CRISPR DNA played a role in the bacterial immune system of *Haloferax mediterranei*<sup>14</sup>.

By the mid-2000s, scientists had found that CRISPR systems were present in many bacteria, and they understood the role of CRISPR in the adaptive immune system of bacteria. The defense system relies on particular proteins to cleave a fragment of DNA from an invading bacteriophage, called a protospacer, and insert this genetic material into the spacer regions of the bacteria's CRISPR locus. Hence, the spacers are fragments of foreign virus DNA, with palindromic repeats separating them. When a similar bacteriophage returns to inject its viral DNA, the bacteria transcribes the protospacer DNA into precursor CRISPR RNA (pre-crRNA) molecules, which get processed into CRISPR RNA (crRNA)<sup>15</sup>. The crRNA binds to the nuclease CRISPR-associated protein 9

(Cas9), and this complex scans the invading bacteriophage DNA for a complementary target sequence to the crRNA strand. However, this target recognition system alone is insufficient to produce DNA breaks. The protospacer adjacent motif (PAM) is a short DNA sequence that must be present on the target DNA next to the site of crRNA complementary binding. For Cas9 to cleave DNA, a PAM sequence of NGG (N being any nucleotide, G being Guanine) must be adjacent downstream of the target DNA<sup>15</sup>. If the crRNA binds to a complementary sequence and the PAM is present, Cas9 introduces a double-stranded break (DSB) in the viral DNA, destroying the virus genome and ending the infection before it can take place. In nature, there are three major types of CRISPR/Cas9 systems<sup>16</sup>. The type I and type III systems use multiple Cas proteins to process the pre-crRNAs into mature crRNAs, which can then bind to a multi-protein Cas complex capable of introducing a DSB in DNA<sup>15</sup>. On the other hand, type II systems use a trans-activating crRNA (tracrRNA) to process the pre-crRNAs. The tracrRNA binds to the pre-crRNA and acts as a binding scaffold, triggering the maturation of pre-crRNA to crRNA<sup>15</sup>. The tracrRNA-crRNA complex then binds to Cas9, ready to produce DSBs in DNA (Figure 2). The mechanism in type II systems has proven to be much more convenient for genome editing because of its ability to create DSBs with Cas9 alone, instead of a multi-protein Cas complex.

In 2012, scientists Jennifer Doudna and Emmanuelle Charpentier explored the possibility of producing cuts within a specific gene not found in bacteriophages using the CRISPR components<sup>15</sup>. Instead of designing a tracrRNA-crRNA complex to guide the Cas9, they engineered a chimeric RNA that fused the ends of the separate molecules into a single-stranded structure that could direct Cas9 to a complementary sequence in a



**Fig. 2** Schematic of a Type II CRISPR System. A crRNA (red) strand bound to the tracrRNA (green) pairs with the complementary target DNA (black) upstream of the PAM (pink) site, allowing the Cas9 protein (blue) to produce a DSB at the cleavage site.

target gene. In Figure 2, separate crRNA and tracrRNA's are shown; the novel approach combines the two free ends of the RNAs to make a chimeric RNA. The researchers programmed five different chimeric RNAs to target different regions of the gene encoding the green fluorescent protein (GFP). In each of the five cases, Cas9 produced a DSB at the target site next to a PAM<sup>15</sup>. This breakthrough was a clear indication that an RNA-programmed Cas9 could target and possibly edit any gene in living organisms.

Following a cut by Cas9, there are two pathways by which the DNA can be repaired in human cells: non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the NHEJ pathway, the cell attempts to join the broken ends of the DNA back together. However, this process is error-prone, often resulting in insertions and deletions of random DNA (indels)<sup>17</sup>. These mutations can disrupt normal gene function and lead to the gene no longer being expressed (gene knockout). In the HDR pathway, the cell uses a donor DNA template to fill the gap in the damaged DNA. By designing the donor DNA template to contain novel genes or a corrected gene sequence, HDR can be utilized to add specific genetic information or correct pre-existing mutations. Thus, CRISPR/Cas9 may be used to knockout genes, add in new ones, or correct underlying mutations.

There are a few methods to introduce the CRISPR components into cells. In studies involving CRISPR in CAR-T cells, the engineered chimeric RNA and Cas9 (extracted and purified from a bacterial source) are mixed beforehand into ribonucleoproteins (RNPs). These RNPs are advantageous over viral transductions because they are transient and because of reduced off-target effects<sup>18</sup>. RNPs can then be transferred into cells by electroporation or lipofection. In electroporation, an electrical current is sent through the cells, introducing pores in

the plasma membranes for the RNPs to enter through. Lipofection uses lipid vesicles embedded with RNPs that enter the cell by endocytosis.

Before the discovery of CRISPR/Cas9 gene editing, the main gene-editing tools were zinc-finger nucleases (ZFNs) and transcription activator-like-effector nucleases (TALENs). ZFNs and TALENs relied on chimeric proteins to recognize DNA target sequences and produce DSBs<sup>7</sup>. Although they became more precise over time, the protein-based DNA recognition mechanisms were inefficient, meaning that they introduced many off-target effects, and the manufacturing process was time-consuming. In contrast, CRISPR is much cheaper, easier to manufacture, and more precise. By engineering a chimeric RNA and attaching it to Cas9, the complementary nucleotide matching system between RNA and DNA in CRISPR gene editing is much less error-prone and more efficient.

## CRISPR Applications to CAR-T Therapy

Because CRISPR gene editing is so versatile and CAR-T cell generation involves the genetic engineering of T-cells, there are many applications for utilizing CRISPR to improve CAR-T therapy. CRISPR can be used to generate off-the-shelf CAR-T cells, improve CAR-T cell persistence in vivo, and reduce CAR-T associated toxicities.

### Off-The-Shelf CAR-T Cells

Current CAR-T autologous approaches have many limitations. These disadvantages include the extensive time it takes to manufacture, high cost, and rapid disease progression in the patient due to poor T-lymphocyte quality<sup>7</sup>. A proposed solu-

**Table 1** Summary of ongoing clinical trials using CRISPR to improve CAR-T cells.\*

Clinical Trial Number	Disease	CAR Target	CRISPR Target	Start Date	Estimated Completion Date
NCT04557436	B-ALL	CD19	TRAC, CD52	August 12, 2020	June 30, 2022
NCT03166878	B-Cell Leukemia	CD19	TRAC, $\beta$ 2M	June, 2017	May, 2022
NCT03545815	Mesothelin solid tumors	Mesothelin	TRAC, PD-1	March 19, 2018	December 30, 2020
NCT04035434	B-ALL	CD19	TRAC, $\beta$ 2M	July 22, 2019	August, 2026
NCT03398967	B-Cell Leukemia	CD19, CD20, CD22	TCR	January 2, 2018	May 20, 2022
NCT04037566	B-ALL	CD19	HPK1	August, 2019	August, 2024
NCT04244656	Multiple Myeloma	BCMA	TRAC, $\beta$ 2M	January 22, 2020	January, 2027
NCT04637763	B-NHL	CD19	TRAC, PD-1	May 26, 2021	September, 2025

tion is the development of a universal allogeneic CAR-T cell. This would provide an off-the-shelf resource where modified T cells from one individual could be given to any other individual at therapeutic doses without side effects. However, there are two major obstacles to this approach. The first issue is the risk of GvHD due to the recognition of foreign antigens by the endogenous T cell receptor (TCR) on the allogeneic CAR-T cell<sup>19</sup>. The endogenous TCR on the donor cells might recognize the patient's cells as foreign, resulting in this unwanted side effect. Another challenge with this option is the possible rejection of the allogeneic CAR-T cells by the patient's immune system. This happens due to host recognition of non-self human leukocyte antigen (HLA) molecules expressed on the donor cells. Any remaining functional T-cells from the patient may recognize and target these foreign cells for destruction, preventing them from eliminating the cancer. In the following sections, we will consider current approaches to overcome these problems using CRISPR editing of CAR-T cells.

**0.01 CRISPR To Knockout Endogenous TCR and HLA** CRISPR/Cas9 has been used to improve off-the-shelf CAR-T cells by disrupting the endogenous TCR and HLA on the donor cells. Without these proteins, the CAR-T cells have a much lower risk of rejection and unwanted attacks on the patient's immune system. In one study, a CAR targeting CD19 was inserted into allogeneic T-cells through a lentivirus<sup>20</sup>. The CAR-T cells were then electroporated to introduce RNPs targeting the gene encoding  $\beta$ 2-microglobulin ( $\beta$ 2M).  $\beta$ 2M is an essential protein required for the expression of HLA. Once the RNPs were introduced, a knockout of the  $\beta$ 2M gene was generated after a CRISPR/Cas9 mediated cut at the gene locus and subsequent NHEJ repair to introduce mutations. By disrupting the HLA, the investigators hoped for reduced rejection of the CAR-T cells. After a successful knockout of  $\beta$ 2M, the CAR-T cells were electroporated again with RNPs targeting the T-cell receptor alpha chain (TRAC) gene. TRAC is a subunit of the TCR. These RNPs generated a knockout of the TRAC gene through a similar process as

the  $\beta$ 2M knockout. By disrupting the TCR, the investigators hoped for the elimination of GvHD. Following successful generation of the double-knockout CAR-T cells, they were evaluated for function in vivo and in vitro. The results from different tumor models indicated that the double-knockout CAR-T cells maintained regular antitumor function with reduced alloreactivity and no evidence of GvHD<sup>20</sup>.

One safety concern with the disruption of  $\beta$ 2M is the risk of natural killer (NK) cell activation against these cells. NK cells are lymphocytes regulated by interaction with HLA proteins<sup>21</sup>. When HLA expression is disrupted in CAR-T cells, a mismatch may occur with an NK cell, which is then activated to kill it. One solution that has shown promise to prevent this is forced expression of the HLA-E subunit by the addition of its gene<sup>22</sup>. This can be achieved using the HDR pathway with the HLA-E gene serving as the donor template.

There have been several clinical trials with allogeneic CRISPR-edited CAR-T cells lacking endogenous HLA and/or TCR (Table 1). In one study, patients with refractory multiple myeloma and sarcoma were treated with TCR knockout T-cells targeting cancer antigens NY-ESO-1 and LAGE-1. No toxicities related to the CRISPR modifications were observed, and the T-cells persisted for up to nine months<sup>23</sup>. One year after the trial began, all three patients, who failed to respond to previous treatments, had progressed, and two of them were able to survive. Additionally, one patient had a substantial reduction in the size of their tumor.

**0.02 CAR Transgene Integration at Gene Knockout Loci** Another approach using CRISPR to generate off-the-shelf CAR-T cells involves the HDR pathway to introduce the CAR transgene<sup>24</sup>. Instead of adding the CAR and RNPs separately, this technique inserts the RNPs into T-cells along with the CAR gene. The CAR gene contains regions on either side that are complementary to the TRAC locus, allowing it to serve as a template for homologous recombination. The RNPs target a specific location within the TRAC locus, creating a DSB, which is then repaired with HDR through the integration of the CAR template DNA. There are multiple benefits

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of this strategy. First, the CAR is placed under the control of the endogenous TRAC promoter, meaning that its expression will be closer to physiological levels as compared to the random genomic insertion achieved by a lentivirus. Second, the insertion of the CAR at the TRAC locus will disrupt the endogenous TRAC gene, meaning that the only TCR produced in the cell will be the CAR. This eliminates the possibility of the endogenous TCR recognizing the patient tissues as being foreign and initiating a GvHD response<sup>24</sup>.

Using CRISPR methods to disrupt the TCR and HLA genes, researchers can generate CAR-T cells that lack these proteins. This means that these cells will have a lower risk of GvHD and rejection by the patient. These CRISPR/Cas9 edits could be used in the generation of CAR-T cells that could be infused into any patient with B-ALL, representing a major improvement over current approaches that require the use of patient-derived CAR-T cells. Although this technology is not fully developed yet, results suggest that the future of off-the-shelf CAR-T cells is closer to becoming a reality, which would have major implications for the treatment of B-ALL.

### Improving CAR-T Cell In Vivo Persistence

There are numerous factors that limit CAR-T expansion and persistence in vivo. One of these is CAR-T cell exhaustion, a condition in which the engineered cells lose the ability to kill the cancer cells. CAR-T cell exhaustion is often caused by inhibitory checkpoint receptors and death receptors<sup>25</sup>. Currently, there are several strategies for implementing CRISPR to overcome CAR-T therapeutic resistance and increase in vivo antitumor function.

In B-ALL, one of the most important immune escape mechanisms is the overexpression of the immune checkpoint receptors Programmed cell death protein 1 (PD-1) and Programmed Cell Death Ligand 1 (PD-L1)<sup>26</sup>. PD-1 is a protein on the surface of all T-cells, and PD-L1 is the binding protein expressed on most B-ALL leukemic cells. The binding of PD-1 and PD-L1 inhibits T-cells from killing the cancer cells by reducing T-cell activity and proliferation. In a healthy person, PD-1 and PD-L1 play important roles in regulating the body's immune system by preventing T-cell targeting of B-lymphocytes. B-ALL cells overexpress PD-L1 to exploit the PD-1/PD-L1 pathway and promote cancer immune escape<sup>26</sup>. To effectively increase antitumor function in CAR-T cells, many studies have incorporated CRISPR to knockout PD-1. One study utilized RNPs targeting the PD-1 gene in anti-CD19 CAR-T cells via electroporation<sup>27</sup>. A DSB at the gene was generated, followed by NHEJ, mutations, and subsequent PD-1 knockout. These PD-1 knockout CAR-T cells were no longer impaired by the high levels of PD-L1 on the B-ALL cells because they no longer expressed the binding PD-1. Therefore, the CAR-T cells could proceed with the recognition and killing

of the cancerous cells as if the PD-L1 was absent. The results from xenograft tumor models indicated that these cells had enhanced antitumor efficacy in vivo and in vitro. Another study used CRISPR/Cas9 to knock out multiple genes encoding TRAC,  $\beta$ 2M, and PD-1 to improve antitumor efficacy using mouse models<sup>28</sup>. These triple-knockout CAR-T cells had approximately 50% higher lytic activity than normal CAR-T cells.

Other T-cell inhibitory receptors such as CTLA-4 and LAG-3 play important roles in CAR-T cell exhaustion. When CTLA-4 binds to the protein B7, T-cell persistence and function decrease. Similarly, LAG-3 can bind to several proteins on cancer cells to inhibit T-cell activation. Using CRISPR to disrupt the expression of these inhibitory receptors on CAR-T cells is still being tested in clinical trials but has shown promise to significantly improve antitumor function.

In addition to inhibitory receptors, CAR-T cells possess death receptors. The most well-known death receptor in CAR-T cells is Fas, which can activate the apoptosis pathway in T-cells by binding to its ligand FasL, a protein expressed on cancer cells. A study using CRISPR to generate Fas knockout CAR-T cells against leukemia found prolonged survival, apoptotic resistance, and enhanced CAR-T function<sup>29</sup>.

Altogether, CRISPR/Cas9 can be utilized to target multiple genes within CAR-T cells encoding proteins that limit their effectiveness. By removing inhibitory and death receptors, harmful pathways can be disrupted, allowing CAR-T cells to persist longer and overcome exhaustion. These approaches have great promise in improving the effectiveness of CAR-T therapy in future treatments of B-ALL.

### Reducing CAR-T Associated Toxicities

One of the biggest problems with CAR-T therapy is its associated toxicities, some of which can be life-threatening. The most notable of these include cytokine release syndrome (CRS), neurotoxicity, and off-target toxicity<sup>7</sup>. These toxicities can lead to organ damage, fever, fatigue, and severe inflammation. Although the release of cytokines is crucial for CAR-T antitumor function, an excess of specific cytokines can lead to CRS and neurotoxicity. These cytokines include interleukin-2 (IL-2), IL-6, IL-8, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF)<sup>30</sup>. The disruption of genes encoding these cytokines using CRISPR has shown potential to overcome CAR-T related toxicities.

GM-CSF is a cytokine associated with the development of CRS and neurotoxicity in anti-CD19 CAR-T therapy. To effectively treat B-ALL and prevent toxicities, one study experimented with GM-CSF knockout CAR-T cells<sup>31</sup>. RNPs targeting the GM-CSF gene were introduced into anti-CD19 CAR-T cells, allowing a DSB and subsequent NHEJ repair leading to mutations and inactivation of the gene. After the generation of

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these cells, they were tested for function with xenograft models. These cells had enhanced antitumor function *in vivo*, increased survival compared to regular anti-CD19 CAR-T cells, and significant prevention of CRS and neurotoxicity<sup>31</sup>. Additionally, the GM-CSF knockout did not impair the release of any other essential cytokines. These results suggest that CRISPR/Cas9 targeting of harmful cytokines may be a viable strategy to combat the risk of CRS and neurotoxicity.

Off-target toxicity occurs because of the presence of the CAR-T cell targeting antigen on normal cells. CD19 is mostly present on leukemic cells in B-ALL, yet it is present on some healthy B-lymphocytes. As a result, the anti-CD19 CAR-T cells may kill CD-19 expressing B-lymphocytes in what is known as B-cell aplasia<sup>3</sup>. To mitigate this issue, researchers have explored using CRISPR to insert safety switches, or suicide genes, into the CAR gene. Activation of safety switches can initiate apoptosis in CAR-T cells in case of potential off-target toxicities<sup>32</sup>. Thus, the CAR-T cells can terminate themselves before killing healthy B-lymphocytes. One safety switch in particular, inducible caspase-9 (iC9), has demonstrated its ability to eliminate anti-CD19 CAR-T cells in various tumor models<sup>33</sup>. One patient with refractory B-ALL was relieved of neurotoxicity and off-target toxicities after the addition of the iC9 safety switch, which subsequently reduced the amount of CAR-T cells in the body by 90% within 24 hours<sup>34</sup>.

Treatment with CAR-T therapy is associated with many deadly toxicities, but new methods harnessing gene editing to reduce the side effects have emerged. CRISPR/Cas9 can be used to target harmful cytokines and implement safety switches. In the future, CAR-T cells with these edits may have increased effectiveness against B-ALL by minimizing the risk for related toxicities in human patients.

## Discussion

Over the past few years, the groundbreaking genome editing system CRISPR/Cas9 has been applied to the innovative CAR-T cell therapy to treat B-ALL. Before immunotherapy, B-ALL treatment relied on chemotherapy or allogeneic stem cell transplantation, both methods that weren't completely effective. CAR-T therapy offers an approach driven by engineered T-cells that can target specific antigens on cancer cells and initiate cell death. The addition of a CAR to a T-cell leads to a novel way to fight cancer, but these cells have limitations. These include the inconvenient manufacturing process for autologous CAR-T cells, CAR-T cell exhaustion, and the deadly toxicities that are associated with this therapy. CRISPR/Cas9 can be used to fix each of these issues by generating an off-the-shelf CAR-T resource, disrupting inhibitory receptors to increase CAR-T survival, and removing harmful cytokines to minimize the risk of life-threatening toxicities. These solu-

tions will have great implications for future treatment of B-ALL with CRISPR-edited CAR-T cells.

As efficient as CRISPR/Cas9 has proven to be in the genome editing of CAR-T cells, there are still several limitations that need to be addressed to increase the safety and efficiency of the editing process. One of the main concerns is possible chromosome translocations due to the DSBs. If there are too many DSBs in the DNA at one time, there can often be translocations of DNA from different chromosomes, which can be very deadly to the CAR-T cells. As mentioned previously, chromosomal translocations in B-lymphocytes are the primary driver of B-ALL. Likewise, translocations in CAR-T cells may lead to excessive proliferation and the development of a new type of leukemia. One possible fix to this issue is sequentially adding the RNPs instead of simultaneously if multiple edits need to be made. This can reduce the risk of translocations arising because the CAR-T cell can focus on repairing one DSB at a time.

Another drawback to CRISPR-edited CAR-T cells is the risk of off-target cuts in the DNA by Cas9. Usually, Cas9 is extracted and purified from bacteria before it is ready to use in human cells. However, some variants of Cas9 aren't as specific in cleaving DNA as others, sometimes resulting in off-target effects. Instead, molecular evolution of Cas9 should be considered, in which small changes are made in the Cas9 structure, and then these different variants are tested for efficiency<sup>35</sup>. Only then should the most specific variant of Cas9 be selected for CAR-T cell therapy to ensure proper editing. Additionally, the use of nickases instead of the nuclease Cas9 has become a possibility. Unlike nucleases which produce DSBs, nickases produce a single-stranded break. Scientists could use two Cas9 nickases and design each to cut opposite strands at the same location. The end result is a DSB (repaired by NHEJ/HDR) after two single-stranded nicks in the DNA. One benefit of this approach is the odds of both nickases randomly cutting at some other part in the genome is much lower than one off-target cut by a nuclease<sup>36</sup>. Another benefit is that if there is an off-target nick, the DNA can be repaired without introducing mutations because only the DNA backbone needs repair.

In the future, CAR-T cells with CRISPR/Cas9 edits will be used to treat B-ALL. The most promising edits include the gene knockouts of TRAC, HLA, PD-1, and GM-CSF. These edits would lead to CAR-T cells that have a lower risk of GvHD, rejection, resistance, and developing toxicities. Many ongoing clinical trials are working out the best ways to combine these edits and effectively scale up CAR-T cell production. Finally, improvements to the CRISPR system itself can be made to make the entire editing process safer and more specific. CRISPR-edited CAR-T cells will be an essential tool in the treatment of B-ALL, and as they continue to improve, we may get closer and closer to a cure for this devastating disease.

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## References

- 1 Key Statistics for Acute Lymphocytic Leukemia (ALL), <https://www.cancer.org/cancer/acute-lymphocytic-leukemia/about/key-statistics.html>.
- 2 *Top 5 Pediatric Cancers: The Warning Signs*, Roswell Park Comprehensive Cancer Center.
- 3 M. Sheykhasan, H. Manoochehri and P. Dama, *Cancer Gene Ther*, **29**, 1080–1096.
- 4 <https://www.cancerresearchuk.org/about-cancer/acute-lymphoblastic-leukaemia-all/survival>, Survival.
- 5 U. Greenbaum, K. Mahadeo, P. Kebriaci, E. Shpall and N. Saini, *Front. Oncol*, **10**, year.
- 6 CAR T Cell Therapy — Penn Medicine. Penn Medicine - Abramson Cancer Center, <https://www.pennmedicine.org/cancer/navigating-cancer-care/treatment-types/immunotherapy/what-is-car-t-therapy>.
- 7 C. Manriquez-Roman, E. Siegler and S. Kenderian, *BioDrugs Clin. Immunother. Biopharm. Gene Ther*, **35**, 113–124.
- 8 *B Cell Acute Lymphoblastic Leukemia — OSUCCC — James. The James - OSUCCC*, <https://cancer.osu.edu/for-patients-and-caregivers/learn-about-cancers-and-treatments/cancers-conditions-and-treatment/cancer-types/leukemia/b-cell-acute-lymphoblastic-leukemia>.
- 9 C.-H. Pui and W. Evans, *Engl. J. Med*, **354**, 166–178.
- 10 *Treatment of Children with Acute Lymphocytic Leukemia (ALL)*, <https://www.cancer.org/cancer/leukemia-in-children/treating/children-with-all.html>.
- 11 *Stem Cell Treatment — Allogeneic Stem Cell Transplant — LLS*, <https://www.lls.org/treatment/types-treatment/stem-cell-transplantation/allogeneic-stem-cell-transplantation>.
- 12 R. Sterner and R. Sterner, *Blood Cancer J*, **11**, 1–11.
- 13 M. Honikel and S. Olejniczak, *Biomolecules*, **12**, 1303.
- 14 E. Lander, *Cell*, **164**, 18–28.
- 15 M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. Doudna and E. Charpentier, *Science*, **337**, 816–821.
- 16 K. Makarova, E. Koonin and C. CRISPR-Cas Systems, *Biol. Clifton NJ*, **1311**, 47–75.
- 17 Y. Miyaoka, J. Berman, S. Cooper, S. Mayerl, A. Chan, B. Zhang, G. Karlin-Neumann, B. Q. o. H. Conklin, N. E. Locus, Nuclease and C. T. Genome-Editing, *Rep*, **6**, 23549.
- 18 S. Zhang, J. Shen, D. Li and Y. Cheng, *Theranostics*, **11**, 614–648.
- 19 Immuno-Oncology, *CRISPR*, <https://crisprtx.com/programs/immuno-oncology>.
- 20 J. Ren, X. Liu, C. Fang, S. Jiang, C. June and Y. Zhao, *Clin. Cancer Res*, **23**, 2255–2266.
- 21 B. Duygu, T. Olieslagers, M. Groeneweg, C. Voorter and L. Wieten, *Front. Immunol*, **12**, 680480.
- 22 G. Gornalusse, R. Hirata, S. Funk, L. Riolobos, V. Lopes, G. Manske, D. Prunkard, A. Colunga, L.-A. Hanafi, D. Clegg, C. Turtle and D. Russell, *Nat. Biotechnol*, **35**, 765–772.
- 23 E. Stadtmayer, J. Fraietta, M. Davis, A. Cohen, K. Weber, E. Lancaster, P. Mangan, I. Kulikovskaya, M. Gupta, F. Chen, L. Tian, V. Gonzalez, J. Xu, I. Jung, J. Melenhorst, G. Plesa, J. Shea, T. Matlawski, A. Cervini, A. Gaymon, S. Desjardins, A. Lamontagne, J. Salas-Mckee, A. Fesnak, D. Siegel, B. Levine, J. Jadowsky, R. Young, A. Chew, W.-T. Hwang, E. Hexner, B. Carreno, C. Nobles, F. Bushman, K. Parker, Y. Qi, A. Satpathy, H. Chang, Y. Zhao, S. Lacey and C. June, *Science*, **367**, eaba7365, year.
- 24 J. Eyquem, J. Mansilla-Soto, T. Giavridis, S. Stegen, M. Hamieh, K. Cunanan, A. Odak, M. Gönen and M. Sadelain, *Nature*, **543**, 113–117.
- 25 A. Dimitri, F. Herbst and J. Fraietta, *Mol. Cancer*, **21**, 78, year.
- 26 T. Köhnke, C. Krupka, J. Tischer, T. Knösel and M. Subklewe, *J. Hematol. Oncol*, **8**, 111.
- 27 L. Rupp, K. Schumann, K. Roybal, R. Gate, C. Ye, W. Lim and A. Marson, *Sci. Rep*, **7**, 737.
- 28 X. Liu, Y. Zhang, C. Cheng, A. Cheng, X. Zhang, N. Li, C. Xia, X. Wei, X. Liu and H. Wang, *Cell Res*, **27**, 154–157.
- 29 J. Ren, X. Zhang, X. Liu, C. Fang, S. Jiang, C. June and Y. Zhao, *Oncotarget*, **8**, 17002–17011.
- 30 H. Murthy, M. Iqbal, J. Chavez and M. Kharfan-Dabaja, *ImmunoTargets Ther*, **8**, 43–52.
- 31 R. Sterner, R. Sakemura, M. Cox, N. Yang, R. Khadka, C. Forsman, M. Hansen, F. Jin, K. Ayasoufi, M. Hefazi, K. Schick, D. Walters, O. Ahmed, D. Chappell, T. Sahnoud, C. Durrant, W. Nevala, M. Patnaik, L. Pease, K. Hedin, N. Kay, A. Johnson and S. Kenderian, *Blood*, **133**, 697–709.
- 32 C. Li, H. Mei and Y. Hu, *Brief. Funct. Genomics*, **19**, 175–182.
- 33 I. Diaconu, B. Ballard, M. Zhang, Y. Chen, J. West, G. Dotti and B. Savoldo, *Mol. Ther*, **25**, 580–592.
- 34 M. Guercio, S. Manni, I. Boffa, S. Caruso, S. Cecca, M. Sinibaldi, Z. Abbaszadeh, A. Camera, R. Ciccone, V. Polito, F. Ferrandino, S. Reddel, M. Catanoso, E. Boccheri, F. Del Bufalo, M. Algeri, B. Angelis, C. Quintarelli and F. Locatelli, *Front. Immunol*, **12**, 755639, year.
- 35 J. Lee, E. Jeong, J. Lee, M. Jung, E. Shin, Y.-H. Kim, K. Lee, I. Jung, D. Kim, S. Kim and J.-S. Kim, *Nat. Commun*, **9**, 3048.
- 36 R. Gopalappa, B. Suresh, S. Ramakrishna and H. Kim, *Nucleic Acids Res*, **46**, e71, year.