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Uses of CRISPR/Cas9 gene editing in chimeric antigen receptor T cell therapy to treat B-cell acute lymphoblastic leukemia

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Abstract
B-cell acute lymphoblastic leukemia (B-ALL) is a hematological malignancy that affects almost 500,000 people per year, approximately two-thirds of them being children and adolescents. While current treatments such as chemotherapy and hematopoietic stem cell transplantation have significantly reduced the death rate of leukemia, there remain many challenges to overcome. Scientists have discovered therapies that aim to overcome these challenges, including chimeric antigen receptor (CAR) T cell therapy. CAR-T cell therapy is a relatively new treatment that aims to increase survival in relapsed/refractory B-ALL. This review paper will discuss the mechanisms of chimeric antigen T cells and their current limitations. It will present the mechanistic details of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR associated (Cas)9 protein genome editing system, its DNA editing mechanism, and its use to modify human cells. These applications of CRISPR/Cas9 to CAR-T cell therapy to solve current limitations will be discussed. CRISPR can improve CAR-T cells by limiting T cell exhaustion, limiting antigen escape, and preventing graft-vs-host disease. This paper will also present data from clinical trials and discuss possible solutions in the future with respect to CAR-T cells' use in B-cell leukemia. CAR-T cell therapy is a developing field that is viewed by many as the next paradigm in B-ALL treatment, and its limitations can be overcome with the CRISPR/Cas9 gene editing complex. Using CRISPR to manufacture CAR-T cells has the potential to significantly increase survival rates of leukemia and other cancers.

Keywords
Immunotherapy, Leukemia, B cell lymphoblastic leukemia, Gene editing, Cancer, CAR-T cells, CRISPR/Cas9, T-cell exhaustion, Antigen escape, Graft versus host disease

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Introduction

The field of gene editing has expanded significantly due to the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR associated (Cas)9 protein. In the ten years since its discovery, researchers have started to focus on the potential applications of this efficient, versatile system. This review aims to cover mechanistic details of the CRISPR/Cas9 genome editing system and its use to improve human CAR-T cells, specifically exploring applications for B-cell acute lymphoblastic leukemia (B-ALL).

Leukemia

B-cell acute lymphoblastic leukemia is a cancer in children and adults where lymphoid cells in the bone marrow undergo mutations and rapid proliferation that cause disease (1). According to the American Cancer Society, the estimated number of new cases in the United States in 2021 was 5,690. Among all cases, approximately two thirds occur in children and young adults (2). Some factors that predict clinical outcome of treatment outcome include age, leukocyte count at time of diagnosis, genetic abnormalities in the leukemia cells (type of leukemia), and factors in the host such as responses to drugs like methotrexate (3).

The main treatment for ALL is chemotherapy, which can be used alone or with other targeted drug therapies. Chemotherapeutic drugs circulate through the bloodstream and kill cells that are in the process of replicating (4). The aim of this treatment in ALL is to destroy the leukemic cells. Cancer cells replicate faster than normal body cells, hence chemotherapy is more likely to kill them, but it also damages healthy tissues that are constantly growing such as hair and the lining of the digestive system. Remission is achieved when no cancer cells can be detected in the blood or bone marrow, and treatment needs to continue for several months after remission to prevent relapse. Another newer treatment is hematopoietic stem cell transplantation (HSCT), where a patient receives stem cells from the bone marrow of a healthy donor to replace the ones that have been destroyed by radiation or chemotherapy. However, HSCT does come with the risk of graft-versus-host disease (GVHD), a condition that occurs when donated cells see healthy tissues in the patient's body as foreign and attack them (5).

Treatments developed over the past 40 years have increased 5-year survival rates in children and adolescents to > 80%. However, approximately 15-20% of these patients experience relapse (6). The development of genetically modified T cells with chimeric receptors, to allow selective killing of cancer cells holds significant promise in treating relapsed patients.

CAR-T cells

T cells are leukocytes that coordinate adaptive immunity, including responses to tumors, pathogens, and allergens (7). T cells express receptors that can recognize antigens on the surface of virus infected and cancer cells and induce cell death in cells that express non-self or abnormal antigens. They can also maintain immunological memory. Both patients' (autologous) and healthy donors' (allogeneic) T cells can be engineered to recognize and kill cancer cells by adding chimeric antigen receptors (CARs). CAR-T cells are genetically modified T cells with receptors that allow the leukocytes to attach to cells expressing surface antigens common in cancer cells (1). In the case

of B- ALL, leukemic B cells express more CD19 receptors than non-cancer cells, so most studies using CAR-T cells for ALL target the CD19 receptor. The CAR-T cell can be engineered to recognize CD19 and target these cells for destruction. To treat hematological malignancies effectively, a CAR-T cell must be able to perform a long-lasting, durable clinical response.

A schematic of a CAR is shown in Figure 1. As the name suggests, a CAR is a chimera, or combination, of multiple proteins known to be involved in T cell recognition and activation. CARs are composed of an ectodomain that consists of a single chain variable fragment and a spacer, a transmembrane domain, and an endodomain made of signaling molecules (Figure 1). The variable fragment is made from parts of a monoclonal antibody that recognizes the antigen on the cancer cell's surface, while the spacer lends flexibility. Longer spacers provide extra flexibility, but CARs with shorter hinges can bind to membrane-distal epitopes more effectively. The signaling molecules in the endodomain induce T cell activation and lead to a more robust immune response. These molecules include CD3 zeta and, in later generations, a costimulatory domain such as CD28 or 4-1BB.

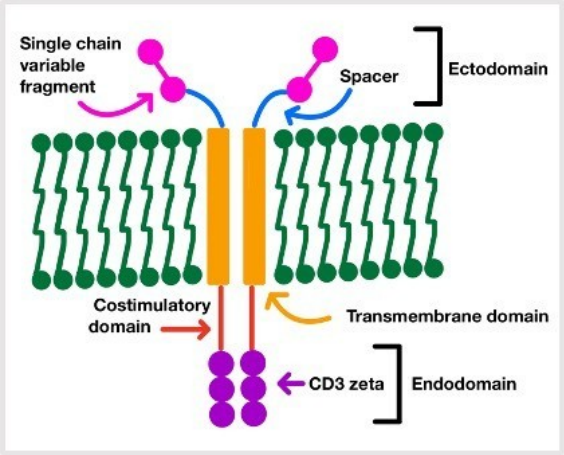

Figure 1: Structure of a Chimeric Antigen Receptor
A CAR is composed of an ectodomain (top) made up of a single chain variable fragment (pink) and a spacer (blue), a transmembrane domain (orange), and an endodomain (bottom) made up of a costimulatory domain (red) and the signaling molecule CD3 zeta (purple).

CARs are manufactured with a target antigen in mind. The ideal target antigen is expressed homogeneously in tumor cells but not on normal tissues, so as to prevent toxicity. In the case of ALL, the antigen CD19 was chosen as an acceptable target due to its high expression on most leukemic cells, while at the same time lacking expression on hematopoietic stem cells (1). CD19 is, however, expressed on many non-cancerous B cells, putting patients at a high risk of B cell aplasia. To minimize this risk, researchers are working on CAR-T cells that only activate in the presence of two or more antigens, that deactivate in the presence of a specific antigen, or that activate in the presence of either targeted antigen (8).

Although CAR-T cell therapy has shown promise in patients with relapsed/refractory B-ALL, limitations have gradually emerged,

as patients are at a high risk of early relapse after achieving complete remission (9, 10). A major mechanism of CD19 relapse is antigen loss, which can occur *via* antigen escape or lineage switch. (11). Antigen escape occurs when B-ALL cells downregulate CD19 expression to avoid detection by CAR-T cells, and a lineage switch is the phenomenon when a patient relapses with a related but phenotypically different disease, such as B-ALL relapsing into acute myeloid leukemia (AML), which commonly expresses the surface markers CD13 and CD33, rather than CD19 (12).

In addition, T cell exhaustion plays an important role in CAR-T cell therapeutic failure (13). It is widely believed that limited CAR T cell expansion and persistence in the hostile tumor microenvironment represents an additional barrier to effective CAR T cell responses and durable clinical remission (14). Inhibitory receptors such as PD-1, CTLA-4, and LAG-3 exhibit immunosuppressive signaling that play significant roles in T cell exhaustion. Exhausted T cells lose their effector functions, decreasing their efficacy in a therapeutic context (15).

Another important limitation of CAR-T cells is that autologous T cells are the most widely used in clinical trials, but they are cumbersome, time-consuming, and expensive to manufacture, possibly leading to a variable outcome depending on factors in the patient such as quantity and quality of harvested cells (15, 16). Using allogeneic T cells has the potential to overcome these limitations. However, the endogenous T cell receptor (TCR) on allogeneic T cells could recognize antigens on the recipient cells that, in turn, may cause graft-versus-host disease (GVHD).

Gene editing techniques such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and, most recently, clustered regularly interspaced short palindromic repeats (CRISPR) have the potential to attenuate the above mentioned limitations of CAR-T cell therapy. Gene editing can improve CAR-T cell characteristics by increasing their specificity and effectiveness, and reducing the costs and risks associated with the treatment.

CRISPR

Designer nucleases such as CRISPR/Cas9 are efficient genome-editing tools that hold great promise for experimental biology and therapies (17). The recognition of the first short palindromic repeats is credited to molecular biologist Francisco Mojica, who discovered them in the microbe *Haloferax mediterranei* (18). By the mid 2000s, scientists had discovered that CRISPR was an evolved adaptive immune system that uses a system called Cas to make RNA-guided double-stranded breaks (DSBs) in DNA. In 2020, the Nobel Prize in Chemistry was awarded to Jennifer Doudna and Emmanuelle Charpentier for their development of gene editing using CRISPR, highlighting its importance in biology and medicine.

CRISPR and its associated Cas proteins originate from bacteria and archaea, which have evolved these systems to protect themselves from invading pathogens. Bacteria and archaea integrate short bits of foreign DNA sequence from pathogens into their CRISPR array. These pieces of DNA are called protospacers, which are then transcribed into CRISPR RNA (crRNA) molecules that

can recognize complementary protospacer sequences in an invading virus or phage.

There are three types of CRISPR/Cas systems: Type I, Type II, and Type III. Cas proteins of Type I and Type III assemble into multi-protein complexes capable of recognizing and cleaving nucleic acids. Type II systems, however, use trans-activating crRNAs (tracrRNAs) that are complementary to the repeat sequences in crRNA and use the Cas9 endonuclease to trigger processing by RNase III. Most researchers focus on Cas9 because it is known to be the sole protein responsible for silencing foreign DNA. (19). Mature crRNA is paired to tracrRNA, which guides Cas9 to induce DSBs in the targeted DNA, as outlined in Figure 2. Site-specific cleavage occurs at locations determined by both base-pairing complementarity between the crRNA and the target protospacer DNA and a protospacer adjacent motif (PAM) juxtaposed to the complementary region in the target DNA. At a cleavage site, the Cas9 HNH nuclease domain cleaves the complementary strand and the RuvC domain cleaves the noncomplementary strand. In plasmid DNA, cleavage usually produces blunt ends approximately three base pairs upstream of the PAM (Figure 2).

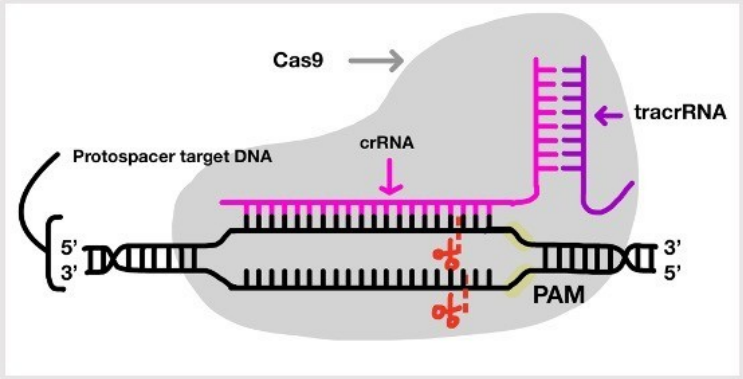

Figure 2: Association of CRISPR RNA, Cas9, and Template DNA to form the CRISPR/Cas9 complex. Schematic representation of crRNA (pink), tracrRNA (purple), and protospacer target DNA (black). Recognition of the PAM (yellow highlight) is driven by complementary base pairing and Cas9 (gray) cuts 3 bp upstream of the PAM.

Although the tracrRNA:crRNA mechanism works efficiently in nature, the possibility of a single RNA-guided Cas9 is appealing due to its streamlined use for programmed DNA cleavage and genome editing. Jinek et al, engineered five different chimeric guide RNAs to target a portion of the gene encoding green-fluorescent protein (GFP) and tested their efficacy against a plasmid carrying the GFP coding sequence in vitro (19). A chimeric guide RNA combines tracrRNA and crRNA to create a synthetic single-guide RNA that can direct the Cas9 protein to the PAM. (20). In every case, Cas9 programmed with chimeric RNAs was able to cleave the plasmid at the correct target site, indicating that any known DNA sequence with a PAM could be targeted using Cas9, in principle. Cong et al, were the first to codon-optimize Cas9 and fuse it to a nuclear localization signal to transfect different combinations of CRISPR/Cas9 components into the nucleus of mammalian cells (21). They found that DSBs occurred with three required components: Cas9, tracrRNA, and crRNA, defining an efficient three-component system for genome modification in mammalian cells.

Their other main finding was that chimeric gRNA was able to lead to both gene knockout and gene insertion. Since these early experiments, CRISPR/Cas9 has been widely used in molecular biology and medicine to edit cell DNA in a variety of contexts, including in CAR-T cells.

CRISPR/Cas9 is a system that can make specific, accurate breaks in DNA, but precise genome-editing also relies on the repair of nuclease-induced DSBs by either homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) (17). Both are cellular processes that occur naturally in response to DNA damage, so the primary challenge is to induce a DSB into the desired location and then let the cell take over. HDR is a precise repair mechanism that uses homologous donor DNA to repair DNA damage, whereas NHEJ is an error-prone mechanism in which broken ends of DNA are joined together, often resulting in a heterogeneous pool of insertions and deletions. Therefore, for producing targeted gene knock-in or other specific mutations, DSBs should preferably be repaired by the HDR pathway (22). In contrast, NHEJ can be induced to cause various insertion or deletion mutations (indels) that lead to gene knockout. DSBs caused by Cas9 can repair using either the NHEJ or the HDR pathway, but they are more commonly repaired by NHEJ (22). Therefore, it requires specific engineering in order to favor the HDR pathway.

A method of promoting HDR over NHEJ is by timing the delivery of the CRISPR/Cas9 system to the S and G2 phases of the cell cycle, as these stages are typically when HDR occurs (22). Lin et. al. used timed delivery of Cas9 complexes in human fibroblasts and embryonic stem cells and found that rates of HDR increased from undetectable to 33% efficiency (23). These results also presented with significantly higher rates of HDR than those using TALENs. However, further increase of efficiency beyond 33% requires manipulation of the proteins involved in both pathways.

A more common method of promoting HDR is to inhibit important enzymes of the NHEJ pathway such as DNA ligase IV (22). Maruyama et. al. investigated the effects of Scr7, an inhibitor of ligase IV, on the efficiency of HDR, and found that Scr7 increased the efficiency of HDR-mediated genome editing in every gene examined (24). Inclusion of Scr7 also improved insertion efficiency of DNA fragments, which is important because donor DNA insertion is a necessary step of HDR. Additionally, Chu et. al. found that expressing the ligase IV inhibitor proteins E1B55K and E4 or f6 improved HDR efficiency up to eightfold and significantly decreased NHEJ activity in human and mouse cell lines (25).

In addition to using inhibitory molecules, HDR can also be promoted with small interfering RNA (siRNA) to inhibit expression of the Ku protein (22). Li et. al. used siRNA to suppress Ku70 and Ku80 in pig fetal fibroblasts and found that downregulation of Ku70 and Ku80 resulted in the promotion of multiple HDR pathways, implying that Ku70 or Ku80 is an essential protein in the NHEJ pathway, therefore its inhibition can suppress the NHEJ pathway and promote HDR (26). An advantage of siRNA is that it results in the temporary downregulation of proteins, and once the desired effect has occurred, normal protein function is restored. This can be

considered an advantage because complete knockout or mutation of the Ku proteins is often associated with an increased risk of cancer.

After a CRISPR edit has been made, it is necessary to physically sort pools of edited and unedited cells to ensure that treatment is highly effective. A method of sorting cells is flow cytometry, which sorts cells based on scattered light signals from lasers that are pass through a single-cell stream (27). For example, Osborn et. al. used this method to collect cells with knockout of the endogenous TCR (16). Antibodies that can recognize the TCR on the cell surface are labeled with chemical fluorophores, and then added to the pool for the purpose of staining cells with a TCR. When CRISPR knocks out the TRAC gene in cells, researchers can incubate cells with an antibody that recognizes the TCR, pass the pool of cells through the flow cytometer, and sort for cells that do not have detectable levels of TCR. This is a way to confirm that the pool of cells consists solely of edited cells.

CRISPR Applications to CAR-T cells

CRISPR is an efficient, versatile gene-editing tool that can potentially solve many of the problems plaguing medicine. As discussed above, CAR-T cells are a prospective solution to the problem of refractory/relapsed ALL, but they also have many limitations. Table 1 presents a general overview of how CRISPR can be used to eliminate these limitations and make CAR-T cells a more efficient and effective treatment.

Table 1 shows a brief overview of the limitations of CAR-T cells (left) and possible corresponding solutions with CRISPR (right), described in more detail in the section below.

Table 1. The advantages of CRISPR with CAR-T cells at a glance

T-cell exhaustion	Knock out inhibitory signaling molecules to decrease the number of pathways to exhaustion
Antigen escape	Add multiple CARs to the same T cell to target more than one antigen on cancer cells so cancer cells cannot downregulate a sole antigen and escape
Risk of GVHD and HLA rejection with allogeneic transplants	Knock out the endogenous TCR on allogeneic CAR-T cells

As mentioned above, T cell exhaustion is a major barrier in CAR-T cell efficacy (13). The generation of T cells that are resistant to inhibitory pathways is expected to improve CAR-T cell efficacy, and CRISPR/Cas9 has been used to knock out inhibitory signaling molecules such as PD-1, CTLA-4, and LAG-3. Co-inhibitory receptors maintain immune tolerance in a normal context, but when cancer cells exhibit PD-L1 on their surfaces, the tumors can shut down the immune response and proliferate further (28). Multiple studies have shown that CRISPR/Cas9 PD-$1^{k/o}$ CAR-T cells have better antitumor effects against PD-$L1^{+}$ tumor models compared to unmodified CAR-T cells in vitro (29-32). In addition, Tang

et. al. found that the presence of an endogenous TGF-ß receptor accelerated the exhaustion of CAR T cells by upregulating PD-1, but knocking out TGF-ß receptor II (TGFBR2) with CRISPR completely abrogated this negative effect and significantly improved CAR-T cell efficacy (33). Checkpoint inhibitory drugs such as Keytruda®, Opdivo®, or Yervoy® are an alternative approach to block checkpoint inhibitors rather than using CRISPR. For example, in one clinical trial, 33% of patients exhibited clinical benefit after PD-1 blockade with Keytruda®, and in another, an objective response was reported in 87% of patients after treatment with Opdivo® (34, 35).

Another major barrier in CAR-T cell efficacy, related to T-cell exhaustion, is stress-induced senescence. Senescent T-cells are usually characterized by a loss of costimulatory molecules such as CD27 and CD28, and another important marker is telomere shortening (36, 37). Akin to methods described above with cellular exhaustion, reversing premature senescence could be a pathway to increasing the efficacy of CAR-T cells. However, studies have shown that, as senescence mechanisms are related to DNA damage, reversing senescent cells by selective blockade can contribute to the development of uncontrolled proliferation, leading to an out-of-control immune response (38- 40). There is currently no literature studying the effects of CRISPR-Cas9 on CAR-T cells in reversing premature senescence, therefore, this area needs to be studied further.

Another major barrier in the efficacy of CAR-T cells is antigen downregulation and escape. A method of overcoming antigen loss after CAR-T cell therapy is to target more than one antigen on cancer cells (11). In the case of B-ALL, this could mean targeting CD22, as CD22 CAR-T cells have demonstrated significant clinical efficacy after CD19 loss (41, 42). Fry et. al. established the clinical possibilities of a CD22 CAR in B-ALL, as potency against B-ALL was comparable to that of CD19 CAR (43). Hu et. al. were able to mitigate CD19 antigen escape by developing a universal CD19/CD22 dual-targeting CAR-T cell with its TRAC gene knocked out by CRISPR/Cas9 (44). Qin et. al. demonstrated the comparable efficacy of CD19/CD22 CAR-T cells in eliminating ALL cells that lacked one or the other receptor and provided a pathway for the construction of multiple antigen- targeting cells (45) (28). However, to reduce the risk of the antigen downregulating both antigens at the same time, a tumor biopsy should be screened for the presence of CD19/CD22 to ascertain if dual-targeting CAR-T cells would be effective. This procedure can help decide which type of CAR-T cell to manufacture, so as to avoid selective pressure on the tumor cells to enable escape.

To combat the labor-intensive and expensive process of modifying autologous T cells, researchers are looking for ways to implement allogeneic CAR-T cells without causing GVHD or HLA-mediated rejection. Since the endogenous T cell receptor in the allogeneic cells is what causes these conditions, the CRISPR Cas9 system can potentially be used to silence the endogenous T cell receptor. Osborn et. al. knocked out the TCRα chain (TRAC) in the endogenous TCR with ZFNs, TALENs, meganucleases (MNs), and CRISPR to compare the efficacy of each method (16). The CRISPR/Cas9 system showed the highest levels of TRAC disruption and did not appear to cause off-target edits. They also

demonstrated that CRISPR edited primary T cells possessed a significantly greater viability than TALEN edited cells, and they were successful in obtaining adequate numbers of cells such that the dose was within the range necessary for T cell therapy. In addition, Eyquem et. al. used CRISPR to insert a CD19-specific CAR into the TRAC locus (46). They compared their gene-edited cells to conventional retroviral encoded CARs and found that the targeting of CARs to a TCR locus minimized the risks of oncogenic transformation and TCR-induced autoimmunity, yielded constant CAR expression as opposed to variegation, and provided a more potent T cell by delaying T cell exhaustion. Using these strategies, Ren et. al. accomplished highly efficient multiplex genome editing of CAR-T cells using CRISPR (47) Allogeneic CAR-T cells were created by knocking out endogenous TCR and HLA class I on the allogeneic cells, demonstrating the possibility of a simple and cost- effective scale-up method for clinical application. Another option for using CAR cells without causing GVHD is by transplanting natural killer (NK) cells, as NK cells lack a TCR (48). Liu et. al. transplanted allogeneic NK cells without HLA matching and found that a majority of patients experienced complete remission (49). Allogeneic cells are the future of CAR-T cell therapy and there are multiple ways to use CRISPR to solve challenges in manufacturing allogeneic cells.

Safety concerns

CRISPR/Cas9 generally disrupts its intended target sites reliably, but an important caveat to consider is to what extent these nucleases induce off-target cleavage events, especially in therapeutic applications (47). Compared to ZFNs and TALENs, CRISPR theoretically has a lower specificity because it is a monomer, and thus binds to a shorter target sequence (50). Several studies have examined the off-target effects of CRISPR, with one showing that it can induce off- target mutations at sites that differ by up to five nucleotides, implying that there may exist thousands of potential off-target cleavage sites in the human genome (51).

Before using cells in patients, it is necessary that every cell is correctly edited and there are no harmful off-target mutations. One widely used assay for this determination is the T7 endonuclease 1 mismatch detection assay (52). However, this method often incorrectly reports sgRNA activity. Three other commonly used methods are Tracking of Indels by Decomposition (TIDE), next-generation sequencing (NGS) and Indel Detection by Amplicon Analysis (IDAA). Sentmanat et. al. found that NGS, TIDE, and IDAA predict similar editing efficiencies, but TIDE and IDAA can report false alleles in edited cells (52). Targeted NGS seems to be the most effective pool for screening edited cells because it provides accurate estimates.

In addition, more sensitive deep sequencing assays are needed to identify low frequency off-target mutations. Accurately predicting off-target cleavage sites is a challenge because most algorithms are unable to cover all potential off-target sites (53). One such method is termed genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-Seq). In this method, DSBs are tagged in the genome, amplified, and then deep sequenced. Another method, called *in situ* breaks labelling, enrichments on streptavidin, and next generation sequencing (BLESS), labels *in situ* breaks and uses deep sequencing to identify off target sites.

Multiple studies have demonstrated a low incidence of off-target mutagenesis using CRISPR/Cas9. Ren et. al. reported rare off-target mutagenesis when targeting TRAC and TRBC with Cas9 (11). Wang et. al. and Li et. al. both demonstrated no significant off-target effects using CRISPR/Cas9 to knock out CCR5 (54) (26). However, while off-target effects are not common, they can occur in higher amounts due to prolonged Cas9 expression. To mitigate this effect, a variety of newly discovered protein inhibitors such as AcrIIA4; encoded by *Listeria monocytogenes* prophages; can block Cas9 from cleaving DNA (55). Timed addition of these inhibitors limits the amount of time that Cas9 is active, thus decreasing the probability of off-target gene editing.

Another method to reduce the risk of off-target edits is by choosing a delivery mechanism of Cas9 such that the protein is expressed within a carefully defined window. There are three formats of delivering the CRISPR/Cas9 complex into a cell (56). Using the DNA format, DNA enters the nucleus, is transcribed, and is translated into protein, and the Cas9 protein returns to the nucleus to do editing. This method has a relatively long half-life, with the Cas9 peaking around 24-48 hours and sometimes taking weeks before expression returns to normal levels. This length of time comes with the risk of random integration of the Cas9 plasmid into the genome, which would allow for permanent expression of the protein. The mRNA format is directly translated into a protein, and since this is more efficient, Cas9 expression peaks around 5-7 hours. Finally, the ribonucleoprotein format is where a pre-assembled complex made up of purified Cas9 protein and gRNA is transfected. This method is the fastest, most efficient, and therefore has the lowest chance of off-target effects (57, 58).

A third potential method to reduce off-target effects is to simply use a truncated guide RNA. Fu et. al. reports that target regions that are less than 20 nucleotides in length can reduce the risk of off-target effects by as much as 5000 times without sacrificing on-target efficiency (59). This may be explained by the idea that in normal gRNAs, there is excess RNA/DNA binding energy, hence reducing this binding energy may poise the CRISPR/DNA complex to be more sensitive to mismatches (53) (60).

Other than off-target effects, another potential safety concern is that in the drive to upregulate T effector cells, there may be a loss of T regulatory cell function, causing an increase in the propensity for autoimmune diseases, cytokine release syndrome (CRS), and tissue damage (61-63). To combat these diseases, it is necessary to find predictive markers that inform of the risk of an unwanted immunological response (64, 65). For example, a biomarker that is considered to be a predictor of severe CRS after CD19 CAR-T cell therapy is high monocyte chemoattractant protein 1 (66). Recognizing these signals can influence decision-making on whether to continue infusing cells, pre- emptively treat a side-effect, or end treatment immediately.

In addition, as genetic edits are inherently heritable to T-cell progeny, the long-term effects of CRISPR-edited CAR- T cells must be considered. CAR-T cell persistence is a major focus of many researchers, and many strategies for improving CAR-T cell treatment involve improving T-cell persistence (67). Studies of CCR5 k/o CD4+ T-cells showed

healthy engraftment and gave rise to more CCR5 k/o T-cells (68-70). Other studies demonstrate the capability for CAR-T cells to proliferate after gene knockout (71-73). Therefore, some sort of CRISPR/Cas9 off switch needs to be discussed. It is possible to engineer suicide genes into CAR-T cells that allow them to be killed when their target is in remission, potentially preventing any negative long-term effects (74-76). Another new technology is CRISPRoff and CRISPRon, which can silence or activate most genes while leaving the genes unchanged, in contrast to CRISPR/Cas9, which physically cuts the DNA (77).

Applications in Clinical Trials

The first human trial of CRISPR modified CAR-T cells infused modified CAR-T cells into patients with refractory cancer to test the safety of genome engineering for anticancer purposes (78). Autologous CAR-T cells were infused into patients after CRISPR-Cas9 modification of the TRAC, TRBC, and PDCD1 loci. The researchers found that editing efficiency was consistent, rare off-target effects were observed, and the T cells in all 3 patients persisted at stable levels for 9 months. These results demonstrate that genome engineering is feasible and safe in human patients using CRISPR-Cas9.

Currently, while no clinical trials have been completed involving both CRISPR and B-ALL, there are a variety of current clinical trials applying CRISPR-edited CAR-T cells to B-cell acute lymphoblastic leukemia (79, 80). For example, a trial being performed at the University College in London is using CRISPR to edit the TRAC locus in anti-CD19 CAR-T cells manufactured from healthy adult donors (79). The study's aim is to induce remission in children with relapsed/refractory B-ALL before HSCT. In another ongoing trial, CD19-specific CAR-T cells were edited with CRISPR to decrease expression of HPK1, an immune inhibitor (81). Thus far, 30 days after infusion, there was a 72.7% complete remission rate, which is higher than that of standard CAR-T cell therapies. Although CRISPR editing is a recent development in clinical trials with CAR-T cells, it is reasonable to suggest that some new treatment methods will have increased success.

Discussion

There are currently no completed clinical trials in this field because research of CRISPR-edited CAR-T cells is still relatively new. The clinical trials in progress utilize CRISPR for the purpose of making off-the-shelf CAR-Ts. Based on the information presented in this paper, some predictions can be made for promising directions of this treatment in the future. CRISPR is used in cells to knock out endogenous TCRs and inhibitory signaling molecules to block T cell exhaustion and HLA-mediated immunity or GVHD. This allows for the production of a large number of widely applicable CAR-T cells that can be infused into any patient to fight off relapsed B-ALL. The most exciting possibility is to manufacture CAR-T cells that combine all the possible improvements mentioned in this paper such as dual-antigen recognition of CD19 and CD22 and using CRISPR to inhibit PD-1 and the TRAC gene.

However, the side effects of CRISPR editing must be considered as well. Off-target cleavage events could cause severe adverse reactions in a patient. Three possible solutions to these off-target effects are, to use a timed

addition of an inhibitor, using a ribonucleoprotein format to deliver the Cas9, or truncating the guide RNA. Other safety concerns to consider are loss of T regulatory cell function and long-term effects of CRISPR/Cas9 editing on T-cell progeny. These concerns need to be studied further in clinical trials.

Currently, approximately 20% of patients with ALL relapse and do poorly after treatment. In 2022, it is estimated that 6,660 people will be diagnosed with ALL, with approximately 6 out of every 10 of those people being children (82). Developing effective CAR-T cell treatments for relapsed/refractory and early-stage leukemia can benefit the majority of leukemia patients, as well as patients with other types of cancer. The results of CAR-T cell immunotherapy edited with CRISPR have been very encouraging in breast cancer therapy and have demonstrated safety and evidence for cytotoxic potency in malignant brain tumors like glioblastomas (83, 84).

Conclusion

CAR-T cell therapy is a developing cancer therapy with engineered chimeric T cell receptors that is viewed by many as the next stepping stone in B-ALL treatment. The current limitations to this therapy are antigen escape, lack of T cell persistence, and the burden of creating autologous cells. The use of the CRISPR/Cas9 gene editing complex can significantly attenuate these limitations. The next step in CRISPR/CAR-T cell therapy is to apply it successfully in clinical trials and develop a universal treatment that can be approved by the appropriate regulatory agencies such as the Food and Drug Administration. CRISPR/Cas9 gene editing may make it possible in the future to administer allogeneic CAR-T cells with CARs capable of recognizing and destroying any subtype of cancer.

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