OXFORD JOURNAL
OF STUDENT
SCHOLARSHIP

Improving Cancer Immunotherapy with CRISPR/Cas9-Mediated Gene Knockout of A2AR, PD-1, and CTLA-4 in T Cells

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ABSTRACT

Cancer is the second leading cause of death in the United States overall and the leading cause among individuals younger than 85 years. T lymphocytes (T cells) are important for regulating and directing immune responses in the adaptive immune system to keep the body healthy and functional. However, T cells show limited efficacy when killing solid cancer cells. T cells become dysfunctional due to immunosuppression in the tumor microenvironment (TME), which results in reduced T cell persistence, reduced cytokine production, and reduced cytotoxicity. CRISPR/Cas9, a precise gene editing tool, may be used to knock out genes associated with immunosuppression and boost antitumor immunity in T cells. In this review, we examine single-gene knockouts of three major contributors to immunosuppression in T cells within the TME: *A2AR*, *PD-1*, and *CTLA-4*. To determine the effects of the immunosuppressive gene knockouts, we analyze studies that use *in vitro* experiments and *in vivo* xenografts of immunodeficient mice. Our review suggests that CRISPR-mediated gene knockout of *A2AR*, *PD-1*, and *CTLA-4* results in enhanced T cell proliferation, improved persistence, elevated cytotoxicity, and increased cytokine production, ultimately boosting T cell antitumor immunity and combating solid tumors. However, clinical trials are needed to demonstrate the long-term safety, immunity, and persistence of the recently proposed therapy.

INTRODUCTION

T lymphocytes (T cells) are major immune cells in the adaptive immune system responsible for regulating and directing immune responses to deter infiltrating cells, such as cancers and pathogens, that impede homeostasis in the body. T cells recognize antigens using T cell receptors (TCRs) and antigen presenting cells (APCs), cells that expose antigens to the immune system, activate naive T cells. The T cells regulate and direct an immune response to destroy cancerous or infected cells. The most common types of T cells are CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells. CD8+ T cells secrete cytotoxic molecules, such as granzymes and perforin, for cytotoxicity against the cancerous or infected cells, while CD4+ T cells help activate other immune cells, such as CD8+ and B cells, to produce a more efficient immune response. (Sun 2023).

Cancer is a disease in which cells proliferate uncontrollably and are unresponsive to important regulatory death and repair signals, such as cell cycle checkpoints and apoptosis (Huang 2021). Surgery, chemotherapy, and radiation therapy are current cancer treatments in use to inhibit tumor growth. However, chemotherapy has restricted efficacy and a high risk of toxicity (Gutteridge 1985), while radiation therapy has limited precision and is not accessible in some areas. Additionally, while surgery may remove most of the cancer tissues without affecting healthy cells, surgery cannot comprehensively remove all of the cancer due to the intricate structure of the human body (Zafar 2025). T cells detect and attack cancerous cells with cytotoxic abilities and antitumor immune responses, playing a pivotal role in antitumor immunity. Specifically, TCRs recognize and bind to antigens, and CD4+ T cells activate CD8+ T cells by producing cytokines. CD8+ T cells release cytotoxic molecules like granzymes to kill cells presenting the antigens. However, these T cells need to target recurrent antigens for prolonged durations to combat cancer. This eventually leads to T cell dysfunction, where T cells are overstimulated and lose proliferation, cytotoxicity, and cytokine production, inhibiting T cells' ability to detect and kill cancer cells. As highlighted in this study, the immunosuppressive tumor microenvironment (TME), caused by checkpoint receptors and suppressive molecules, is a key factor for T cell exhaustion and a reduced immune function.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) is a gene editing tool that can alter a region of DNA that may carry a specific disease-causing gene (Stadtmauer 2020). CRISPR/Cas9 is composed of the Cas9 protein and a guide RNA (gRNA). The gRNA directs the Cas9 protein to the targeted gene location, and the Cas9 protein cuts the DNA at specific regions. Because checkpoint receptors and suppressive molecules inhibit antitumor immunity in T cells, scientists can use CRISPR/Cas9 to delete the genes associated with the inhibitory responses in T cells. T cells are crucial for anti-tumor immunity, and CRISPR gene editing may improve immunotherapy by removing immunosuppressive genes (Stadtmauer 2020).

In this review, *in vitro* refers to studies conducted outside living organisms in controlled laboratory settings. *Ex vivo* refers to studies conducted on cells and tissues extracted from and observed outside the living organism. In contrast, *in vivo* refers to studies conducted and observed within living organisms.

In this review, we aim to examine CRISPR-mediated gene editing for improved immunotherapy in solid tumors. Our goal is to analyze the safety, efficacy, and improved antitumor immune responses associated with the deletion of three major immunosuppressive genes in T cells: *A2AR*, *PD-1*, and *CTLA-4*. While cancer mortality rates are declining, cancer remains a leading cause of death in the United States (Siegel 2025), demonstrating the importance for further improvements in cancer immunotherapy.

CRISPR-mediated *A2AR* Knockout in T cells

CRISPR/Cas9 editing can be used to delete adenosine A2A receptors (*A2AR*) in Chimeric Antigen Receptor T (CAR-T) cells, allowing the CAR-T cells to overcome immunosuppression and combat solid tumors (Giuffrida 2021; Li 2020). CAR-T cells are T cells engineered with a CAR, a synthetic receptor

introduced to strengthen T cells' antitumor immune response. On the P4 CAR, a type of CAR, the single-chain variable fragment (scFv) recognizes antigens (such as those on cancer cells), while the intracellular signaling domains CD28, which produces costimulatory signals, and CD3ζ, which produces activation signals, activate the T cell to attack the recognized targets (Li 2020). Currently, CAR-T cells are effective in treating hematological malignancies, which are cancers beginning in the bone marrow, blood, or lymphatic system. However, CAR-T cells have limited efficacy against solid tumors (Leone 2018; Beavis 2012; Giuffrida 2021; Li 2020). Adenosine, a prominent factor for immunosuppression in the TME, is a product of adenosine triphosphate (ATP) that undergoes hydrolysis through CD39 and CD73 (Li 2020; Antonioli 2013). Extracellular ATP promotes inflammation in the TME, but when ATP undergoes hydrolysis, the adenosine byproduct causes immunosuppression, suppressing immune cells such as CAR-T cells (Allard 2017). Of the four adenosine receptors, *A2AR* is chiefly responsible for inhibiting solid tumor immunity (Li 2020).

By measuring cell persistence and cytokine levels, Lauren Giuffrida and collaborators determined a way to efficiently delete *A2AR* with the use of CRISPR/Cas9 in murine and human CAR-T cells (Giuffrida 2021). It was previously known that adenosine is one key metabolite responsible for immunosuppression in the TME, and *A2AR* is the main receptor influencing the immunosuppressive behavior. However, Giuffrida did not know whether targeting *A2AR* would work efficiently on patients with solid tumors, whether the effects of *A2AR* knockout T cells were dose-dependent, or how to target *A2AR* for deletion with minimal side effects. Based on previous research, the research group hypothesized that deleting *A2AR* using CRISPR/Cas9 would enhance CAR-T cell anti-tumor immunity to treat solid tumors. The study group conducted *in vitro* and *in vivo* experiments to compare the original CAR-T cells with both short hairpin RNA- (shRNA) and CRISPR-mediated *A2AR* knockouts in CAR-T cells. Only a complete knockout of *A2AR* significantly enhanced CAR-T cell function as determined by measuring cytokine production, cytotoxicity, and persistence of CAR-T cells with different *A2AR* reduction rates. When the team compared shRNA and CRISPR-mediated *A2AR* knockouts *in vivo* in mice, both resulted in increased IFN-γ and TNF cytokine production due to upregulated levels of cytokine producers IRF4 and Granzyme B. After analyzing the mice's spleen nine days after shRNA-edited CAR-T cell infusion, the research team found a significantly lower number of shRNA-edited CAR-T cells compared to the initial amount induced; only the CRISPR-mediated knockout maintained CAR-T cell persistence as measured by flow cytometry analysis. The research team then used CRISPR editing to target *A2AR* for deletion in immunocompetent mice, with murine and human CAR-T cells. Both human and murine CRISPR-edited CAR-T cells retained persistence and showed improved immunity, specifically an increase in cytokine levels compared to cytokine levels before the deletion. This suggests CRISPR/Cas9 is a more persistent tool than shRNA to delete *A2AR* in CAR-T cells and to enhance CAR-T cells' anti-tumor immune response while maintaining persistence (Giuffrida 2021).

By measuring killing capabilities, cytokine levels, and cell exhaustion rates *in vitro* and then measuring efficacy *in vivo*, Na Li and collaborators demonstrated that CRISPR/Cas9-mediated *A2AR* deletion in CAR-T cells greatly enhances anti-tumor immunity of the CAR-T cells (Li 2020). The research team hypothesized that adenosine is a major molecule responsible for immunosuppression in CAR-T cells, and

deleting *A2AR* using CRISPR/Cas9 could reduce the immunosuppressive effects of adenosine signaling, enhancing CAR-T cell efficacy. The research team compared the CRISPR-edited CAR-T cells with the original CAR-T cells *in vitro*. Of the four adenosine receptors, *A2AR* and A2BR were primarily expressed in the CAR-T cells. To verify *A2AR* was the main receptor allowing adenosine-mediated immunosuppression, the scientists conducted CRISPR-mediated *A2AR* knockout and A2BR knockout in CAR–T cells. In the absence of 2 chloroadenosine (CADO), a molecule used to mimic adenosine, both CAR-T cell types had similar cytotoxicity. However, in the presence of CADO, the cytotoxicity of original CAR-T cells and A2BR targeted CAR-T cells was significantly lowered: only the CAR-T cells with *A2AR* knockout had higher cytotoxicity. Before the central study, the researchers conducted an experiment through in vitro testing suggesting that A2AR plays a significant role in adenosine-mediated suppression. Additionally, *in vivo* testing using two models- a CDX model, in which human lung cancer is implanted in immunodeficient mice, and a PDX model, in which patients' tumor tissue is implanted into immunodeficient mice- compared CRISPR-edited CAR-T cells with original CAR-T cells. In both models, the CRISPR-edited CAR-T cells could more successfully kill tumor cells as measured by the size of the tumor: in the PDX model with CRISPR-edited CAR-T cells, the tumor shrank to less than 30% of the tumor's original volume, while the tumor reduction rate in the CDX model was mentioned but not specified. The CRISPR-edited CAR-T cells in the PDX model had a higher CD8 to CD4 ratio than the original CAR-T cells, which may indicate that the PDX and CDX models had different side factors contributing to the results. However, CRISPR-edited CAR-T cells in both models had unchanged CD3+ persistence as determined by comparing the quantity of CRISPR-edited and original CAR-T cells. Altogether, CRISPR-mediated *A2AR* deletion in CAR-T cells has the potential to overcome immunosuppression and enhance CAR-T cell antitumor immunity (Li 2020).

Both studies share the same hypothesis: CRISPR-mediated *A2AR* deletion can improve immune functions in CAR-T cells. Giuffrida's study measured the dose-dependence of *A2AR* deletion, but did not confirm adenosine as the main cause of immunosuppression, with *A2AR* as the primary receptor responsible for these effects. However, in Li's study, the first two experiments aimed to confirm adenosine as a key cause of immunosuppression, with A2AR as the primary receptor involved, through *in vitro* comparisons measured by cytokine production levels and cell destruction rates. Furthermore, both research groups used different A2AR agonists to mimic the adenosine molecule: Giuffrida's study used NECA, while Li's study used CADO and CGS. A study conducted by B Johansson demonstrated that NECA more closely mimics adenosine (Johansson 1993). However, the difference in *A2AR* agonists does not have a significant effect on the study. Both CADO and NECA consistently upregulated antitumor immunity, demonstrating the effectiveness of adenosine suppression. In essence, Li et al. and Giuffrida both demonstrate that CRISPR-mediated *A2AR* deletion in CAR-T cells can improve anti-tumor immunity. While the preclinical studies demonstrated the feasibility and enhanced antitumor efficacy of CRISPR-mediated *A2AR* deletion, further studies are necessary to demonstrate the therapy's clinical safety and effectiveness (Giuffrida 2021; Li 2020).

CRISPR-mediated *PD-1* Knockout in T cells

PD-1 is a receptor found on the surface of immune cells that regulates and suppresses immunity by binding to the PD-L1 and PD-L2 ligands. Because *PD-1/PD-L1* immune checkpoint prevents overactive immune responses by sending signals that reduce immunity and prevent attacks on healthy cells in the body, *PD-1* deficiency could potentially lead to autoimmune diseases (Trautmann 2014). Numerous studies have suggested that *PD-1* deficient human TCR transgenic cells and CAR-T cells enhanced cytotoxicity in mice (Stadtmauer 2020), and anti-*PD-1* antibodies, such as pembrolizumab, have become common checkpoint inhibitors for *PD-1* knockout in cancer patients, especially non-small-cell lung cancer (NSCLC) patients (Lu 2020). Anti-*PD-1* antibodies block *PD-1* from binding to PD-L1, preventing suppression of T cells to strengthen immunity. However, these antibodies block the *PD-1* protein in all cells in the immune system, not just in T cells. Systematic *PD-1* knockout could upregulate T cell persistence uncontrollably, causing side effects and autoimmunity, because the *PD-1* gene typically prevents the immune system from attacking healthy, essential tissue (Su 2016). While scientists have conducted PD-1 blockade using antibodies and are aware of the potential benefits and side effects of the therapy, the safety, feasibility, and improved T cell efficacy of CRISPR-mediated PD-1 knockout in T cells still need to be demonstrated.

You Lu and colleagues induced CRISPR through electroporation to target *PD-1* in patients' T cells and evaluate the safety and feasibility for improving T cell anti-tumor immunity (Lu 2020). Moreover, earlier studies by Mary Keir et al. and Holbrooke Kohrt et al. had shown drugs like pembrolizumab have become a common anti-*PD-1* checkpoint inhibitor for NSCLC (Keir 2008; Kohrt 2005; Marabelle 2025). You Lu hypothesized that CRISPR-Cas9-mediated *ex vivo* disruption of the *PD-1* gene in T cells could be a safe and feasible treatment for patients with NSCLC. The researchers conducted a single-dose phase 1 clinical trial on twelve NSCLC patients who had already undergone multiple unspecified prior treatments that had failed. The CRISPR components, the gRNA and Cas9 protein, were induced into patients *ex vivo* through plasmid-based electroporation. The gRNAs, sgRNA1 and sgRNA2, targeted exon 2 of the *PD-1* gene. As determined by T7E1 cleavage assay, around 20% of T cells were successfully edited for *PD-1* targeting. Additionally, *PD-1* expression was decreased by 46.3% in T cells as determined by flow cytometric analysis. A significant increase of IFN-γ expression, a key cytokine that activates the immune response, was also found in CD8+ killer T cells. This showed that the edited T cells had enhanced immune responses, providing supportive findings for the study. To track the eighteen possible off-target mutation sites, the scientists first conducted next generation sequencing (NGS) with cSMART, then conducted whole genome sequencing (WGS). NGS detected that the off-target mutations mostly occurred in intergenic regions and introns (non-coding regions), which demonstrated low risk of harmful effects. Furthermore, the off-target mutations were mostly deletions or insertions; only a few were frameshift mutations, which can be more harmful. Most of the frameshift mutations were detected in on-target regions. Taken together, the detected mutations, whether on-target or off-target, were of low risk. Additionally, WGS detected eighty-four minor mutations; the researchers stated that the mutations were probably not caused by CRISPR-editing and were likely caused by factors unrelated to the study. Lu's team also tracked the patients' side effects. Of the twelve patients, eleven had mild side effects, and one patient had no side effects; the time period for the side effects ranged from fourteen weeks to a year depending on the patient. To measure T cell persistence, the researchers used NGS and observed *PD-1*

levels in the patients' blood samples after infusion. After introducing the edited T cells into the patients, the researchers also discovered additional T cell clones, which were not present before the infusion, by observing the blood samples; TCR diversity enhances immune responses. No major side effects were detected in the patients, but the T cell therapy did not shrink the tumor; the edited T cells only helped stop tumor growth for some time before the edited T cells died out. Because the T cells helped stop tumor growth, Lu's study is a successful start to further enhance T cell anti-tumor immunity. The researchers concluded that future studies should increase T cell input and extend the follow-up period to better assess long-term efficacy (Lu 2020).

Shu Su and the research team demonstrate that CRISPR-Cas9–mediated knockout of *PD-1* in human primary T cells using a non-viral electroporation method enhances their antitumor function and offers a clinically feasible strategy for adoptive cell therapy (Su 2016). CRISPR editing has been proven to efficiently target genes in mice, rats, and even monkeys based on previous studies (Li 2013; Shen 2013; Ma 2014; Niu 2014), but because there isn't sufficient evidence for CRISPR editing's efficacy on humans, the direct clinical use of CRISPR/Cas9 may be risky due to unknown factors, such as long-term safety and the immune response. Su and colleagues have hypothesized that *PD-1* knockout in T cells with CRISPR/Cas9 editing by plasmid-based electroporation for *in vitro* insertion is feasible and efficient, enhancing T cell anti-tumor immunity. Su and colleagues designed gRNAs to target exon 2 and cut the human *PD-1* gene. Using flow cytometry and microscopy to detect GFP expression, an indicator of stable Cas9 expression produced by plasmids, the research team determined the most persistent electroporation method. Throughout the study, the researchers used T cells from healthy donors as well as late-stage cancer patients. Using fluorescence microscopes, the researchers also checked GFP expression in T cells from healthy donors and patients. By comparing the two types of donors, the researchers evaluated that the patients' T cell efficiency was less than the healthy T cells as determined by cytokine levels and cytotoxicity. Furthermore, the researchers conducted a PCR and T7EN1 assay to confirm that the *PD-1* deletions were successful at the target sites seven days after induction. By tracking and comparing the control and edited T cells, Su and the team found that initial *PD-1* expression in both T cell types was similarly low. However, twenty-one days after being cocultured with IL-2 T cell antagonist, the edited T cells' expression only grew from 2.44% to 4.48%; the control T cells' expression of *PD-1* grew slightly more, from 3.4% to 17.5%. Additionally, the team noticed that CRISPR-mediated *PD-1* knockout in T cells had no effect on T cell population growth or T cell clones. The researchers further tested specific CD3+ T cell activation markers and found that the therapy did not alter the T cells' stability or memory. Using ELispot assay, the researchers also detected an enhanced cytokine IFN-γ production in the edited T cells of both healthy donors and cancer patients when cocultured with LMP2a tumor antigen. To further prove the therapy's success, the scientists measured the edited T cells' cytotoxicity. The edited T cells were cocultured with PD-L1-expressed M14 melanoma tumor cells, and cytotoxicity was measured with CFSE/PI cytotoxicity assay. T cell cytotoxicity and cytokine production with *PD-1* knockout were dose-dependent. In general, the CRISPR-mediated *PD-1* knockout in T cells provided more cytotoxicity even when exposed to PD-L1, more cytokine production, and better immunity than control T cells. The edited T cells showed no alterations in general T cell functions or growth. Because healthy T cells showed

slightly better results, patient T cells may show slightly lower efficacy of the therapy. The researchers concluded that *PD-1* knockout in T cells improves anti-tumor immunity (Su 2016).

By observing cocultures with NY-ESO-1 tumor cells *in vivo* and *ex vivo* on three cancer patients, the study found that CRISPR/Cas9 multiplex editing to delete *PD-1* receptors can improve T cell anti-tumor immunity, enhance persistence, and reduce T cell exhaustion (Stadtmauer 2020). One major concern is that edited TCRs could cause mispairing with the wild-type TCRs, potentially leading to harmful effects, such as autoimmunity, rather than beneficial ones. Another challenge is that editing T cells may increase T cell exhaustion rates, leading to lowered efficacy. Numerous studies have proven that *PD-1* deficient human TCRs and CAR-T cells enhanced cytotoxicity in mice, but it is still unclear if the same results can be applied on humans. In this study, Edward Stadtmauer et al. aimed to conduct a Phase 1 first-in-human clinical trial to show that multiplex CRISPR-editing for *PD-1* deletion can safely improve T cell function in patients with NY-ESO-1 tumor antigens. T cells were engineered to become NYCE T cells (NY-ESO-1 transduced CRISPR 3X-edited cells), in which CRISPR targeted the TRAC, TRBC, and PDCD1 genes. TCR-engineered T cells, instead of CAR-T cells, were used because CAR-T cells caused more side effects, likely due to factors unrelated to gene-editing; the researchers wanted to observe side effects specific to CRISPR editing. When the NYCE T cells and the wild-type T cells were cocultured with HLA-A2+ tumor cells engineered to express NY-ESO-1, the NYCE T cells showed greater cytotoxicity and removed expression of the normal TCRs. The NYCE T cells were then injected into three cancer patients for *in vivo* evaluation. Using iGUIDE, a tool that finds Cas9 cuts in DNA, the research team evaluated on-target and off-target effects. The on-target cuts were successfully made where the researchers intended. Moreover, the off-target cuts were minor and of low risk. Using the TALENs method, the researchers also found that around 4% of T cells had translocations. With prior knowledge that even healthy individuals carry translocations, showing no problems for months, the researchers considered the translocations to be nothing major. To measure T cell persistence, the scientists expanded edited T cells in blood samples of all three patients three to nine months after infusion and cocultured the T cells with NY-ESO-1 tumor cells *in vitro* in a dish. All three patients showed tumor killing capabilities, which showed that the edited T cells remain active for months in patients. This is a good sign that the T cell therapy works. Because patient UPN39 showed the most T cell persistence and cytotoxicity, Stadtmauer and the team tracked the patient's T cells at three stages: before infusion, ten days after infusion, and four months after infusion of the edited T cells. The number of edited T cells only dropped slightly, which showed that the NYCE (edited) T cells are persistent and can survive. Furthermore, the edited T cells started to look like central memory T cells i*n vivo* after four months. This finding is consistent with previous studies without gene editing by Theodore Nowicki et al.: the previous finding had observed the same effects, but the T cells in this study were found to be longer lasting and stronger, with reduced cell exhaustion. Additionally, *PD-1* deletion in mice posed risks for genotoxicity, T-cell related cancer, and scientists also predicted autoimmune problems. Luckily, there were no signs of autoimmune problems or genotoxicity in any of the three patients. One of the three patients died due to aggressive myeloma spread, not from major side effects of the therapy. Overall, these findings conclude that gene editing helps with T cell persistence and strength. It also proves that multiplex CRISPR-editing of genes is a safe and doable method. Earlier concerns about rejection of the edited T cells are cleared, but

further studies are needed with more patients, longer tracking durations, and healthier participants to evaluate the safety of the therapy. Overall, the finding deemed the hypothesis as correct and the study as successful (Stadtmauer 2020).

While all three studies aimed to enhance antitumor immunity by using CRISPR/Cas9 to knock out *PD-1* in T cells, each study used a different method and targeted a different type of cancer. Lu's study targeted NSCLC through a single *PD-1* knockout in patient T cells. Stadtmauer's study sought to treat cancers that expressed NY-ESO-1 by conducting a triple gene knockout (*PD-1*, *TRAC*, and *TRBC*) on TCRs. Su's study targeted chronic myeloid leukemia (CML), blood cancer, by conducting a *PD-1* knockout of cytotoxic T cells (CD8+) before coculturing the T cells with K562 leukemia cells, cells which model but are not actually CML patient cells. While Stadtmauer and Lu edited T cells *in vitro* before infusing the T cells back into patients and observing effects of the therapy *in vivo*, Su's study edited and observed T cells only *in vitro*. Unlike the other two studies, which focused on solid tumors, Su's study tested cytotoxicity against leukemia cells *in vitro* only. All three studies delivered CRISPR/Cas9 into T cells using plasmid-based electroporation, which effectively introduced the gene edits. While Su's study focuses on editing healthy CD8+ T cells and Stadtmauer's study focuses on editing cancer patient T cells, Lu's study edits both T cells from healthy donors and patients, to directly compare results from both types of T cells. All three studies showed progress in T cell antitumor immunity, but in different ways and amounts. Su's *in vitro* study showed an increase in IFN-γ production and increased cytotoxicity when co-cultured with K562 leukemia cells. Su's study focused more on whether *PD-1* deletion is possible and increases T cell efficacy, rather than observing the therapy's safety. Lu's *in vivo* study observed both feasibility and safety of the T cell therapy. Lu's study showed that CRISPR-mediated PD–1 knockout produces minimal off-target mutations and side effects. Lu's study was the first *in vivo* trial for using CRISPR to target *PD-1*: in the study, the T cells helped inhibit tumor growth, but did not shrink the patients' tumor. As Lu's study was conducted on last-stage patients, the researchers stated that more *in vivo* trials with more edited T cells on healthier patients are needed to confirm the positive outcomes of this therapy. Stadtmauer's study was a proof-of-concept study, and the first *in vivo* multiplex-CRISPR-editing study. Stadtmauer's study suggested *PD-1* knockout with the use of multiplex CRISPR editing was safe, with minimal side effects and off-target mutations only found on moderately expressed or non-coding regions of the DNA. Stadtmauer's study also suggested that the therapy increased T cell persistence and was feasible. However, Stadtmauer's study alone is not a safe way to confirm the viability and positive results of the hypothesis, as Stadtmauer conducted experiments on only three patients. Overall, all three studies taken together suggested that *PD-1* deletion enhances antitumor immunity. CRISPR-mediated *PD-1* knockout in T cells could potentially be used clinically for patients with solid tumors.

CRISPR-mediated *CTLA-4* Knockout in T cells

The immune system is balanced with inhibitory and stimulatory signals. Increased costimulatory signaling of the immune system can lead to autoimmunity, while excessive inhibition, most often caused by inhibitory signals such as *CTLA-4* and *PD-1*, suppresses antitumor immunity (Agarwal 2023). Furthermore, a naive T cell is activated through two distinct costimulatory signals, one of which occurs

when the CD28 receptor, a stimulatory receptor, binds to the B7 protein on an antigen-presenting cell (APC). However, the *CTLA-4* receptor, an inhibitory receptor with a structure similar to CD28, acts as a competitive inhibitor by binding to the B7 protein with a stronger tendency than CD28, preventing CD28-mediated costimulation. CTLA-4 upregulates coinhibitory signaling, which reduces proliferation, lowers IL-2 production, and increases T cell apoptosis, ultimately causing immunosuppression in the TME. Antibodies, such as the anti-*PD-1* pembrolizumab, which blocks *PD-1*, and anti-*CTLA-4* ipilimumab, which blocks *CTLA-4*, are frequently used to block the inhibitory receptors clinically in patients' T cells. However, Agarwal suggested CRISPR/Cas9-mediated knockout of the immune checkpoints is more durable as a permanent gene knockout because it provides long-term persistence and improved immunity without repeated redosing (Agarwal 2023).

Long Shi and colleagues measured cytotoxicity, cytokine production, and apoptosis levels *in vitro*, and measured survival and tumor control *in vivo* in mice to discover the feasibility of CRISPR-mediated *CTLA-4* knockout (Long 2017). Antibodies, such as ipilimumab, targeting *CTLA-4* on animals enhanced the cytotoxicity of T cells in previous studies by Jonathan Espenschied, Sara M Mangsbo, and Y F Yang (Espenschied 2003; Mangsbo 2010; Yang 1997). It is known that *CTLA-4* is a key immune inhibitor, but whether gene editing to delete *CTLA-4* in T cells can enhance antitumor immunity is still uncertain. Shi and the team wanted to determine if using CRISPR/Cas9 to knock out *CTLA-4* in T cells improved the T cells' antitumor immunity, persistence, and proliferation, and whether it was attainable using in vitro assays and in vivo xenografts. The researchers derived PBMCs, a mix of immune cells including T cells, from healthy donors' blood samples. Shi and the team used Ficoll-Paque density gradient centrifugation and conducted RPMI culturing to separate PBMCs and mature the cells into immune cells. By adding cytokines and proteins, adherent cells were matured into dendritic cells (DCs), while the suspension cells were matured into CIKs (Cytokine-induced killer cells), immune cells with increased cytokine production and cytotoxicity that have traits of both T cells and natural killer cells. The CIKs and DCs were then cocultured to produce antigen specific CTLs, targeting HCT116 cancer cells. Plasmid-based lentiviruses were then used to infuse CRISPR components into CTLs, some containing non-targeting sgRNA and others containing sgRNAs targeting *CTLA-4* for comparison. The *CTLA-4* knockout CTLs also had more cytotoxicity as determined by WST-1 assay. As determined by flow cytometry, the HCT116 cancer cells cocultured with the edited CTLs showed more apoptosis than the HCT116 cancer cells cocultured with the control CTLs. Because cytokine production is directly linked to enhanced T cell immunity, the scientists conducted an ELISA (enzyme-linked immunosorbent assay) to measure TNF-α and IFN-γ cytokine levels. The *CTLA-4* deficient CTLs produced more TNF-α and IFN-γ than the control CTLs did. Shi and the research team also used SCID (Severe Combined Immunodeficiency) mice to test survival and tumor growth *in vivo* by comparing the control CTLs and edited CTLs. While using immunodeficient mice may prevent attacks against human cells foreign to the immune system of the mice, results may not fully be applicable to humans because of the complexity of the human immune system and the dependence of T cells on other immune cells for full function. HCT116 cancer cells were injected subcutaneously to form tumors, and CTLs were then injected intravenously to measure the CTLs' antitumor immunity. The researchers found that all mice infused with control CTLs died by day 62, while only 10% of the mice infused with edited CTLs died by that time; the remaining 90% of mice infused

with *CTLA-4* knockout CTLs survived past that time period. All things considered, CRISPR-mediated *CTLA-4* knockout in T cells appeared to enhance antitumor immunity preclinically, and Shi's study suggested the potential feasibility of the therapy. Further clinical trials are needed to assess the therapy's clinical safety (Long 2017).

Sangya Agarwal et al. conducted a CRISPR-mediated *CTLA-4* knockout in CD19-directed CAR-T cells (CART19) in CLL patients (Agarwal 2023). The researchers evaluated the feasibility and efficacy enhancement of the therapy in vitro under chronic antigen exposure (CAE) and *in vivo* using mouse xenografts. CART19 cells are used to treat B cell cancer, but the treatment has reduced efficacy in some patients, especially CLL patients. The reduced efficacy of the therapy is likely due to T cell exhaustion, apoptosis, and increased expression of checkpoint receptors, which ultimately lead to CAR-T cell dysfunction. In this study, Agarwal and the team first used CRISPR/Cas9 to compare single and dual *PD-1* and *CTLA-4* knockouts to enhance the already existing CART19 treatment by deleting checkpoint receptor genes to improve efficacy and persistence. The study particularly conducted *PD-1* and *CTLA-4* deletions because they are major checkpoint receptors that suppress T cell antitumor immunity. Of all the gene-edited CAR-T cells, only the *CTLA-4* knockout CART19 cells demonstrated enhanced antitumor immunity based on *in vitro* stress-test experiments and *in vivo* testing in NSG mice. The researchers hypothesized that CRISPR-mediated *CTLA-4* knockout in CART19 cells can improve the efficacy of T cells derived from CLL patients. To observe if *CTLA-4* knockout CART19 cells had improved T cell fitness, CLL patients' dysfunctional T cells were edited *in vitro*: some became wild-type CART19 cells, while others became *CTLA-4* knockout CART19. Compared to the control CART19 cells, the *CTLA-4* knockout CART19 cells had improved persistence, proliferation, and cytotoxicity as measured by *in vitro* stress test cocultures and *in vivo* xenograft models. As demonstrated by *in vitro* stimulation assays, the improvements were more drastic in non-respondent T cells than partial and complete respondent T cells, because the partial and complete respondent T cells already exhibited better baseline cytokine production, cytotoxicity, and proliferation. The *CTLA-4* knockout CART19 cells also had greater IFN-γ cytokine production than the control CART19 cells. Other cytokines that enhance immunity, such as IFN-α and IL-2, were not reported to increase in the study, which may suggest not all key cytokines associated with immunity were raised. CAR expression is the presence of the CAR on the T cell that helps recognize and attack tumor cells. Because CAR expression is a key factor for improved T cell cytotoxicity, survival and increased cytotoxicity, improved persistence of the CAR expression is associated with improved therapeutic outcomes. Maintained CAR expression indicates that T cell activation is persistent and functional, while the loss of CAR expression results in T cell dysfunction. The scientists used flow cytometry during the CAE assay and found that only *CTLA-4* knockout CART19 maintained CAR expression, unlike CART19 with *PD-1* knockout, dual knockout, and control CART19 cells. To confirm the *in vitro* findings, the researchers conducted *in vivo* experiments on mouse xenografts. The study used NSG, immunodeficient, mice and injected acute lymphoblastic leukemia (ALL) cancer cells. The mice were then treated with different CAR-T groups: *CTLA-4* knockout, *PD-1* knockout, dual knockout, and control CAR-T cells. Consistent with *in vitro* studies, only the *CTLA-4* deficient CAR-T cells had improved tumor control, T cell growth, and longer survival. When comparing wild-type and knockout CART19 cells from CLL patients in the mice, the non-respondent edited T cells had improved activity,

but the complete respondent T cells showed no difference in mice survival and tumor clearance, which is again consistent with the *in vitro* experiments conducted earlier in the study. In the *in vitro* dual gene knockout that was conducted as a baseline experiment, *PD-1* deletion negated the increased proliferation, enhanced CAR expression, improved memory phenotype, and better antitumor efficacy caused by *CTLA-4* knockout. Agarwal et al. suggested CRISPR-mediated *CTLA-4* knockout is feasible and enhances antitumor immune functions both *in vitro* and *in vivo* in mice. Further clinical trials are also needed to confirm the safety of the therapy in patients (Agarwal 2023).

Studies by Long Shi et al. and Sangya Agarwal et al. both aimed to demonstrate the feasibility and improved efficacy of CRISPR-mediated *CTLA-4* knockout in T cells, but used distinct methods. Shi used normal CTLs from healthy donors to conduct CRISPR-mediated *CTLA-4* deletion, and Agarwal first modified CLL patient T cells into CART19 cells *in vitro* to conduct CRISPR-mediated *CTLA-4* deletion. Because CART19 cells are already FDA-approved and clinically in use to treat cancers, Agarwal's study provides stronger therapeutic relevance for positive outcomes in future cancer treatments with *CTLA-4* knockout T cells. Agarwal conducted the gene editing directly on CART19 treatment cells of CLL patients *in vitro*, which models clinical conditions needed to confirm the therapy's safety and feasibility. However, the use of healthy donor T cells in Shi's study does not fully reflect therapeutic use for cell dysfunction in cancer patients. Nevertheless, both studies demonstrated that CRISPR-mediated *CTLA-4* deletion leads to enhanced *in vitro* cytokine production, cytotoxicity, T cell persistence, and proliferation in addition to *in vivo* tumor shrinkage in mice. Taken together, the results of both studies suggest the improved antitumor immune functions of the therapy in preclinical models. Neither study conducted essential clinical experiments to confirm safety of CRISPR-mediated *CTLA-4* knockout in T cells (Shi 2017;Agarwal 2023). Moreover, clinical studies in cancer patients are necessary to confirm the safety and enhanced efficacy of the therapy.

Scientists may be hesitant to further investigate gene editing due to ethical concerns. Particularly, clinical applications may cause off-target mutations, which can lead to harmful consequences after long durations. These side effects not only affect the individuals that are given the therapy, but also future generations if germline genes are affected. All individuals need to be aware of potential long-term consequences of gene editing before the technology is therapeutically introduced. Furthermore, if gene editing becomes a popular tool for immunotherapy, individuals may start to misuse it for aspects beyond treatments and cures. CRISPR-mediated gene editing may be a promising cancer immunotherapy if used carefully with limitations.

CRISPR-Mediated Gene Knockout Summary

Study	(Guiffrida 2021)	(Li 2020)	(Lu 2020)	(Su 2016)	(Stadtmauer 2020)	(Shi 2017)	(Agarwal 2023)
Gene Target	*A2AR*	*A2AR*	*PD-1*	*PD-1*	*PD-1*	*CTLA-4*	*CTLA-4*
Research Type	*In vitro + ex*	*in vitro + in*	Phase 1	*in vitro*	Phase 1	*in vitro*	*in vitro + in*

	vivo preclinical trial	*vivo* preclinical trial	clinical trial	preclinical trial	clinical trial	preclinical trial	*vivo* preclinical trial
T Cell Type	CAR-T from murine and patient T cells	CAR-T from patient T cells	T cells (CD4, CD8+) Healthy donor + patient T cells	T cells (CD4, CD8+) Patient T cells	T cells (CD4, CD8+) Patient T cells	T cells (CD8+) Healthy donor T cells	CAR-T-19 from patient T cells
Cancer Type	E0771-Her2 murine breast cancer cells and unspecified human xenograft model	CRL-5826 human pancreatic cancer human xenograft model	NSCLC lung cancer patients	NALM6 leukemia cancer human xenograft model	refractory hematologic cancer patients	A375 melanoma cancer cells cocultured	ALL and CLL leukemia cancer human xenograft model
Safety & Off-target Evaluation	Assayed predicted off-target sites only	Assayed predicted off-target sites only	Assayed predicted off-target sites only	WGS, NGS comprehen-si ve off-target evaluation	Assayed predicted off-target sites only, clinical tracking	Assayed predicted off-target sites only	Assayed predicted off-target sites only
T Cell Improvements (Results)	Proliferation, cytokine production, cytotoxicity	Proliferation, cytokine production, cytotoxicity, tumor size	Proliferation, cytokine production, cytotoxicity, persistence	Proliferation, cytokine production, cytotoxicity, persistence	Proliferation, cytotoxicity, persistence, tumor shrinkage	Proliferation, cytokine production, cytotoxicity	Proliferation, cytotoxicity

DISCUSSION

In this review, we analyzed how CRISPR/Cas9 gene editing can improve T cell immunotherapy for enhanced antitumor immune responses. We inspected three major immunosuppressive gene knockouts: *A2AR*, *PD-1*, and *CTLA-4*. CRISPR-mediated knockout of the three immunosuppressive genes resulted in increased persistence, proliferation, cytokine production, and cytotoxicity of the edited T cells, offering possible positive outcomes for future treatments.

Across the different studies focused on *A2AR*, *PD-1*, and *CTLA-4*, some edited natural T cells, while others used engineered CAR-T cells. Moreover, the cancer cells that were used to test the hypotheses of each study also varied from melanoma and leukemia cancer cells, to solid tumors like NSCLC. While all of the analyzed studies used patient-derived T cells, whether naturally or after implanting a CAR, the studies varied between *in vitro* experiments, *in vivo* xenografts, and *in vivo* clinically, on patients or healthy donors. The diversity of T cell and cancer cell types suggests the potential therapy has been examined in multiple biological contexts, though clinical studies are needed to determine the long-term safety, persistence, and consistent results of CRISPR-mediated immunosuppressive gene knockout. Some studies showed greater positive differences in the edited T cells than other studies when compared to control T cells. The differences in the studies' outcomes may be linked to uncontrolled variables or the differences in the T cell and cancer cell types used in each study. Future studies should look into these variations that may affect the results of CRISPR-mediated immunosuppressive gene knockout.

Because CRISPR-mediated knockout of *A2AR*, *PD-1*, and *CTLA-4* in T cells were all supported by *in vitro* and *in vivo* experiments and showed overall improvement in antitumor immunity- namely, T cell proliferation, cytotoxicity, tumor apoptosis, and increased cytokine production- CRISPR/Cas9 may be used as a future treatment for solid cancer patients. Though the studies analyzed in this review have many strengths that show positive outcomes for CRISPR-mediated gene editing, the studies also have some limitations. The studies mentioned the concerns for off-target mutations and potential side effects like autoimmunity. The concerns for off target mutations were comprehensively evaluated in *PD-1* knockout T cells using WGS and NGS(Su 2016), but were only assayed for predictable off-target sites in *A2AR* and *CTLA-4* single gene knockout T cells(Li 2020 ;Shi 2023; Agarwal 2023). Though all of the studies showed that the off-target sites were only located on non-coding DNA regions, *A2AR* knockout and *CTLA-4* knockout need to be clinically and comprehensively tested for off-target mutations. Additionally, it is imperative that future studies focused on the safety of this therapy are conducted, tracking the patients and testing the off-target mutations for longer durations to fully demonstrate that the therapy does not cause major harmful side effects. Moreover, though the three studies by Lu, Su, and Stadtmauer demonstrated enhanced antitumor immunity after *PD-1* deletion , namely increased T cell proliferation, cytotoxicity, persistence, and cytokine production, Sangya Agarwal did not observe the same positive results when comparing *PD-1* knockout with *CTLA-4* knockout (Lu 2020; Su 2016; Stadtmauer 2020). Though no negative effects were seen, the *PD-1* knockout T cells did not result in significant improvement when compared to the control T cells. One possible explanation may be that Lu, Su, and Stadtmauer used natural T cells, but Agarwal used CART19 cells, which possibly do not rely on *PD-1* deletion for immunity. Another reason may be that Agarwal and the team used NALM6 cancer cells, which already lack *PDL1/2* expression, to test the edited CAR-T cells, but more research is needed to understand the inconsistent results of different checkpoint blockades in T cells (Agarwal 2023).

As *A2AR* and *CTLA-4* knockouts have not yet been tested clinically, it is crucial that further studies demonstrate the therapeutic safety, feasibility, and efficacy, namely tumor shrinkage and T cell proliferation, cytotoxicity, and cytokine production, as demonstrated by the preclinical trials (Li 2020). Clinical trials are needed to evaluate the long-term persistence and exhaustion rates of *PD-1* deleted T

cells in different kinds of T cell targeting different cancer types, as Agarwal's study contradicted studies by the other three research groups (Lu 2020; Agarwal 2023). Furthermore, researchers are evaluating the feasibility, safety, and enhanced efficacy of multiplex immunosuppressive gene knockouts, such as *PD-1* and *CTLA-4* dual knockout (Stadtmauer 2020; Shi 2017). These studies should include evaluation of off-target mutations, persistence, proliferation, cytokine production, and tumor shrinkage in the TME. While the therapy shows great potential for future treatments, further evaluation of safety and efficacy is necessary with an upscale of participants to determine if other factors, such as age, sex, area of residence or immunity levels (patients vs. healthy donors), contribute to the enhanced responses. Upscaling participants for future studies is crucial, as only three patients were used in Stadmauer's clinical trial. Moreover, future studies should evaluate the use of CRISPR/Cas9 for immunotherapy on diseases beyond cancer, such as strokes and heart diseases. Most importantly, future studies should track donors for extended durations to observe the long-term effects of CRISPR-mediated immunosuppressive gene knockouts to synthesize a stronger method to mitigate T cell exhaustion (Su 2016), as T cell exhaustion was a consistent issue.

Scientists may be hesitant to further investigate gene editing due to ethical concerns. Particularly, clinical applications may cause off-target mutations, which can lead to harmful consequences after long durations. These side effects not only affect the individuals that are given the therapy, but also future generations if germline genes are affected. All individuals need to be aware of potential long-term consequences of gene editing before the technology is therapeutically introduced. Furthermore, if gene editing becomes a popular tool for immunotherapy, individuals may start to misuse it for aspects beyond treatments and cures. CRISPR-mediated gene editing may be a promising cancer immunotherapy if used carefully with limitations.

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