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Enhancing the effectiveness of Chimeric Antigen Receptor (CAR) T cells against tumors through CRISPR/Cas9-mediated PD-1 disruption

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Abstract---Immunotherapies involving chimeric antigen receptor (CAR) T cells and checkpoint inhibitors, such as programmed cell death protein 1 (PD-1) antagonists, have shown promise in treating cancer. The combined benefits of these medicines are yet not fully understood. In this work, it was discovered that human CAR T cells become hypo-functional due to the expression of programmed cell death ligand 1 (PD-L1) on tumor cells, which reduces their efficacy in a sub-cutaneous xenograft model. To solve this problem, scientists created a procedure that combines lentiviral transduction with Cas9 ribonucleoprotein (Cas9 RNP)-mediated gene editing to produce PD-1-deficient anti-CD19 CAR T cells. Disrupting the Pdcd1 (PD-1) gene led to increased killing of tumor cells in vitro and improved clearance of PDL1+ tumor xenograft in vivo. This study highlights the potential of precision genome engineering to enhance next-generation cell therapies and improve the efficacy of CAR T cell immunotherapy.

Keywords---CAR T cells, Cas9 RNP, PD-1, PD-L1, CRISPR/Cas9.

1 Introduction

Chimeric antigen receptor (CAR) T cells have shown promise in treating certain types of B cell leukemia's and lymphomas, but there are still challenges in targeting other liquid and solid tumors. The emergence of CRISPR/Cas9-based gene editing of primary human T cells presents an opportunity to enhance CAR T cell therapy through gene modification or disruption. The purpose of this study was to determine whether anti-CD19 CAR T cells could be more effective if tumor cells lacked the inhibitory receptor PD-L1. Developed a protocol for combining Cas9-based gene disruption with lentiviral transduction to create gene-modified human CAR T cells. Using a xenograft tumor model, investigated whether Cas9-
mediated disruption of PD-1 in CAR T cells could increase their anti-tumor activity in vitro and in vivo.\textsuperscript{1-5}

Chimeric antigen receptor (CAR) T cells are altered immune cells that exhibit a synthetic antigen recognition domain and intracellular domains that trigger signalling pathways akin to those activated by natural T cells. Second-generation CARs incorporate CD3ζ and costimulatory proteins such as CD28 or 4-1BB (CD137) to promote T cell proliferation and survival. While CAR T cells have shown effectiveness in treating some types of leukemia and lymphoma and did not successful in treating many solid tumors and some liquid tumors. The existence of an immunosuppressive tumor microenvironment, where tumor can decrease T cell function and efficacy by expressing inhibitory ligands like PD-L1 on tumor cells and adjacent tissues like stroma or tumor vasculature, is one explanation for this.\textsuperscript{6-10}

T cell activity and destiny are tightly regulated by the PD-1/PD-L1 axis. Even though PD-1 is momentarily up regulated on T cells after activation, it is also a sign of T cell fatigue, a condition of diminished activity that can be observed in persistent viral infections and in tumor-infiltrating lymphocytes in advanced cancer patients. The PD-1, PD-L1, and PD-L2 ligands are frequently linked to poor prognosis in a variety of tumor types. Patients with a variety of malignancies have demonstrated that blocking antibodies against PD-1 or PD-L1 produce potent anti-tumor immune responses, underscoring the crucial function of the PD-1/PD-L1 axis in anti-tumor immunity.\textsuperscript{11-15}

The goal of the study was to determine whether deleting the Pdcd1 gene in CAR T cells will result in the generation of T cells with improved tumor cell detection and eradication abilities. Although the PD-1/PD-L1 axis has a well-established function in controlling endogenous anti-tumor responses, its impact on CAR T cell activity has not been thoroughly investigated. Previous research has demonstrated that inhibiting PD-1 can improve CAR T cell performance, although it is yet unknown how much PD-1 ligation inhibits CAR T cells. Targeting Pdcd1 directly in CAR T cells may be a less dangerous way to overcome tumor immunosuppression because widespread PD-1 inhibition can result in toxicities. According to the research, combining Pdcd1 ablation in CAR T cells with TCR disruption may stop the activation of auto reactive T cells and offer cancer patients a safer and more efficient treatment option.\textsuperscript{16-18}

This study sought to determine whether disrupting the endogenous Pdcd1 gene in primary human CAR T cells could enhance their anti-tumor effectiveness. The scientists discovered that the inhibitory receptor PD-1 expressed on CAR T cells can reduce those cells' capacity to destroy tumor cells both in vitro and in vivo. However, this inhibition could be overridden by Cas9-mediated Pdcd1 breakdown within the CAR T cells. The researchers also discovered that lentiviral transduction of CAR T cells combined with CRISPR-mediated gene editing was highly effective. This finding may allow for future modification of cell therapy products to increase their safety and anti-tumor efficacy.\textsuperscript{17-20}
CAR-T cell therapy is a kind of treatment in which a patients T cells are genetically engineered in lab and bind with a specific proteins (antigens) on cancer cells and kill them. (1) A patients T cells are removed from their blood, then (2) reprogram the T cells with Chimeric Antigen Receptors (CAR) in the lab. The (3) millions of CAR T cells are grown in the lab, then (4) CAR T cells are given to the patients with intravenous infusion. (5) The CAR T cells bind to antigen on the cancer cells and kill them.

2 Materials and Methods

2.1 Experimental methodology:
Unless stated otherwise, all experiments were repeated a minimum of three times using at least two distinct T cell donors.

2.2 Animal experiments:
All animal research was authorized by the Institutional Animal Care and conducted following institutional protocols.

2.3 T cell isolation and culture condition:
For this study, human T cells were obtained from donors with verbal consent. CD4+ or CD8+ T cells were isolated using negative selection (EasySep) and cryopreserved in RPMI supplemented with 20% human AB serum and 10% DMSO. After thawing, the cells were rested overnight in T cell medium (TCM) supplemented with IL-2 (30U/mL) before activation. TCM was composed of X-Vivo 15 (Lonza), 5% human AB serum (Valley Biomedical), 55μM β-mercaptoethanol, and 10mM N-acetyl L-cysteine.
2.4 Lentiviral production:
The production of lentiviral vector was carried out according to the protocol. The anti-CD19 4-1BBζ CAR construct, provided by the University of Pennsylvania, was modified to contain a C-terminal GFP fusion to enable the direct identification of transduced cells. For the generation of the PD-L1 lentiviral vector, a codon-optimized human PD-L1 was synthesized by IDT and inserted into the BamHI site of the lentiviral vector pHR'SIN: CSW using In-Fusion cloning from Clontech.

2.5 Culture of K562 cells and generation of CD19+ PD-L1+ K562 cell line:
The cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 5% FBS and maintained according to ATCC guidelines. In order to generate the CD19+ PD-L1+ cell line, a quantity of 1×10^5 CD19+ K562 cells were transduced with lentiviral supernatant for 24 hours. After transduction, flow cytometry was used to confirm PD-L1 expression. Subsequently, bulk PD-L1+ cells were sorted to enhance the uniformity of the PD-L1+ population.

2.6 Antibodies and flow cytometry staining employed in the study:
To prepare for surface staining, Fixable Live/Dead stains from Invitrogen were used following the manufacturer’s instructions. Once quenched and washed, surface staining was carried out in RPMI media supplemented with 1% FBS and conducted at 4°C unless otherwise stated. Antibody clones from various manufacturers were used, including RPA-T8 from BD for anti-CD8, H4A3 from BD for anti-CD107a, EH12.2H7 from eBioscience for anti-PD-1, MIH1 from BD for anti-PD-L1, FN50 from BD for anti-PD-1, FN50 from BD for anti-β2M, and HIB19 from BD for anti-CD19. Data were collected using an LSR II from BD and analyzed using FlowJo.

2.7 Assessment of T cell degranulation using CD107a staining:
In this assay, CD8+ anti-CD19 CAR T cells that had been rested were co-cultured with CD19+ or CD19+ PD-L1+ K562-s at a ratio of 1:2 in T cell medium supplemented with anti-CD107a. 1×10^5 CAR+ T cells were seeded per well in a 96-well plate. After 5 hours of co-culture, the cells were stained with a Live/Dead stain and anti-CD8 antibody, and then analyzed by flow cytometry.

2.8 In vitro killing assay:
CD19+ (Ag+) or antigen irrelevant (Ag−) K562 cells were labeled with CellTrace Violet and CellTrace Far Red, respectively. Then, CD19+ or CD19+ PD-L1+ (Ag+) targets were mixed with Ag− targets at a 1:1 ratio and 1×10^5 total K562 cells were seeded per well in 96-well plates. Rested CD8+ anti-CD19 CAR T cells were added to the target cells at the indicated effector: target ratio. After 18 hours of co-culture, cells were harvested and stained with Live/Dead, and analyzed by flow cytometry. Specific lysis was calculated by normalizing to Ag irrelevant targets and control wells containing no T cells, after excluding non-K562 cells from the gating scheme. The formula used for calculating specific lysis was: Specific Lysis= [(%CD19+ cells in control wells – %CD19+ cells T cell wells)/%CD19+ cells control wells] × 100.
2.9 Primary human T cells edited using Cas9 RNP and lentiviral transduction:

Primary human CD4+ or CD8+ T cells were subjected to Cas9 RNP-mediated editing, with modifications to the previously described protocol. The T cells were first thawed and rested for 24 hours in TCM, followed by stimulation with anti-CD3 and anti-CD28 for 48 hours in T cell medium supplemented with IL-2. Electroporation was carried out using the Amaxa P3 primary cell kit and 4D-Nucleofector, with recombinant S. Pyogenes Cas9 protein that expresses a C-terminal HA tag and two nuclear localization signal (NLS) peptides to aid in transport across the nuclear membrane. The Cas9 protein was stored at -80°C in 20 mM HEPES at pH 7.5, 150 mM KCl, 10% (v/v) glycerol, 1 mM TCEP. Cas9 RNPs were freshly prepared for each experiment by mixing chemically synthesized tracrRNA and crRNA targeting Pdcd1 exon 1 in a 1:1 ratio and incubating them at 37°C for 30 minutes to generate 40 µM crRNA: tracrRNA duplexes. Then, 40 µM S. pyogenes Cas9-NLS was slowly added to the duplexes and incubated at 37°C for 15 minutes to generate 20 µM Cas9 RNPs. Stimulated T cells were nucleofected with 3 µl of 20 µM Cas9 RNP mix and seeded at 1×10⁶ cells/mL in TCM containing Dynabeads human T activator anti-CD3/anti-CD28 at a bead: cell ratio of 1:1. Lentiviral supernatant was added to the wells for 24-36 hours, and the beads were removed 4-6 days later.

2.10 Quantification of Pdcd1 disruption:

To amplify the genomic regions surrounding the Cas9 target site for the PD-1 target region, PCR was performed with specific primers. The PCR reaction was carried out with 150 ng genomic DNA from both the edited and control samples using Kapa Hot start high-fidelity polymerase as per the manufacturer’s protocol. The reaction conditions involved cycles of annealing temperatures gradually decreasing by 0.5 °C per cycle. The resulting PCR product was purified, and Nextera Index primers were added in a second PCR step. The resulting amplicon was purified and quantified using Qubit 2.0 Fluorometer and size-analyzed by BioAnalyzer. The libraries were sequenced using the Illumina MiSeq desktop sequencer. To analyze the data, trimmed reads were aligned to hg19, and reads spanning a 100 bp window flanking the cut site were extracted. Samtools mpileup was used to quantify the extracted reads, and insertions/deletions (INDELs) were quantified as a percentage of total trimmed and filtered reads.

**Primers:**

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5′- TCGTCGCCAGCGTCAGATGTGTATAAGAGACAG(N/NN/NNN)CCCACCTACCTAACCATCC-3′
and
5′- GTCTCGTGGGCTGGAGATGTGTATAAGAGACAG(N/NN/NNN)CACCCTCCCTTCAACC-3′
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2.11 Tumor model in immunodeficient mice:

Six to ten-week-old female NOD-scid-IL-2Rγ−/− (NSG) mice were obtained from USA and used in the study. To initiate tumor growth, mice were subcutaneously injected with 5×10⁶ logarithmic growth phase CD19+ or CD19+ PD-L1+ K562 cells. Once tumors reached a volume of 100–1000 mm³, either 2–7.2×10⁶ CAR+ CD4+ and 2–4.1×10⁶ CAR+ CD8+ control CAR T cells or PD-1 edited CAR T cells were intravenously injected as indicated in the figures. Tumor size was monitored longitudinally using an electronic caliper.
3 Results

The study aimed to investigate whether PD-L1 expression on tumor cells can impair the function of CAR T cells in vitro. To achieve this, the researchers created a PD-L1 expressing tumor cell line by introducing human PD-L1 into CD19+ K562 myelogenous leukemia cells, resulting in CD19+ PD-L1+ K562 cells. Since K562 cells lack MHC I expression and do not induce TCR-mediated signaling in CD8+ CAR T cells, eliminating any confounding effects. The researchers used a second generation anti-CD19 4-1BBζ CAR, known for its potent anti-tumor activity, to test the hypothesis that it presents a stringent challenge for PD-L1 mediated suppression of CAR T cell function.

In this study, primary human CD8+ T cells were activated using anti-CD3/anti-CD28 beads and transduced with a lentiviral vector encoding the anti-CD19 CAR. The CD8+ T cells expressing the anti-CD19 CAR were then stimulated with either CD19+ or CD19+ PD-L1+ K562 cells to determine the effect of PD-L1 expression on CAR T cell function. The results showed that CD19+ PD-L1+ targets exhibited an ~15% reduction in degranulation and were 10–40% less susceptible to anti-CD19 CAR T cell mediated lysis in an overnight killing assay. These findings suggest that PD-L1 expression on tumor cells directly impairs the function and lytic capacity of primary human CD8+ T cells expressing the anti-CD19 4-1BBζ CAR.

3.1 Anti-CD19 CAR T cells show reduced ability to clear subcutaneous PD-L1+ tumor xenograft:

To investigate whether the impaired function of CAR T cells in the presence of PD-L1 had an impact on tumor clearance in vivo. To do so, first established tumors in NOD-scid-IL-2Rγ−/− (NSG) mice by injecting them subcutaneously with 5×10^6 CD19+ or CD19+ PD-L1+ K562 cells, allowing tumors to grow to ~100–250mm, and then transferred 2×10^6 CD4+ and 2×10^6 CD8+ anti-CD19 CAR T cells into the mice. Both CD4+ and CD8+ CAR T cells were used to mimic current clinical protocols for CAR therapy.

At the specified tumor burden and dose, the study observed that 80% of mice treated with CD19+ tumors were cleared by day 28, while 80% of animals with CD19+ PD-L1+ tumors had to be euthanized due to disease progression. The difference was significant (*p=0.016, Gehan-Breslow-Wilcoxon test). Similar trends were observed in other experiments conducted with different T cell doses and initial tumor burdens. These findings suggest that PD-L1 expression on tumor cells can negatively impact the clearance of tumors by CAR T cells in vivo.
Figure 2: Depicts the effect of PD-L1 expression in K562 myelogenous leukemia cells on anti-CD19 CAR T cell function both in vitro and in vivo.

Panel (a) shows a schematic representation of CAR T cell interaction with CD19+ or CD19+ PD-L1 K562 tumor cells. Panel (b) presents data showing that CD8+ anti-CD19 CAR T cells exhibit reduced degranulation (CD107a staining) upon re-stimulation with CD19+ PD-L1+ K562 cells. This reduction in degranulation was observed in ~15% of cases over three independent experiments. Panel (c) demonstrates that CD19+ PD-L1+ K562 cells are resistant to anti-CD19 CAR-mediated lysis in an in vitro killing assay. The experiment was performed three times and a ~40% reduction in specific lysis was observed relative to CD19+ K562 cells. Panel (d) illustrates the experimental design for the subcutaneous xenograft model. Panel (e) shows that CD19+ PD-L1+ subcutaneous xenograft impair anti-CD19 CAR mediated tumor clearance. Mice with established tumors (100–250mm) were injected with CD4+ and CD8+ anti-CD19 CAR T cells, and tumor burden was measured longitudinally. Panel (f) presents a Kaplan-Meier curve for the experiment described in panel (e), indicating a statistically significant difference in survival between animals bearing CD19+ vs. CD19+ PD-L1+ tumors (*p=0.016, Gehan-Breslow-Wilcoxon test).
3.2 Cas9 RNPs enable efficient generation of PD-1 deficient human CAR T cells:

It was suggested that the diminished anti-tumor activity against PD-L1+ cancer cells could be recovered by deleting the Pdcd1 gene in CAR T cells and created a procedure that combines lentiviral transduction of primary human T cells with Cas9 ribonucleoprotein (RNP) gene editing in order to accomplish this. Previous research has demonstrated that Cas9 RNP electroporation was efficient for gene editing in primary human T cells. The modified procedure involves activating T cells with anti-CD3 and anti-CD28, nucleofecting Pdcd1 exon 13 with Cas9 and crRNA: tracrRNA duplex, and transducing anti-CD19 CAR using lentiviral vector. The cells were then expanded by culturing with anti-CD3/anti-CD28 beads.

By analyzing the cells using flow cytometry 2-4 days after nucleofection, the efficacy of PD-1 elimination and CAR transduction through lentiviral transduction was assessed. PD-1 elimination was successful in CD4+ and CD8+ cells, and results consistently demonstrated a drop of over 50% in CAR+ PD-1+ cells 48 hours after editing. Deep sequencing was done on both the Cas9 control and PD-1 edited samples to verify the editing at the molecular level, and the results revealed high levels of insertions/deletions (INDELs) at the target locus in the PD-1 edited samples. According to earlier research, deletions were the most common type of alteration.

The frequency of PD-1 ablation between CAR+ and CAR- T cells was therefore not significantly different, indicating that the two processes are independent. So restimulated bulk PD-1 edited CD8+ CAR T cells with CD19+ K562s and tracked T cell activation by CD69 and PD-1 expression to determine the effect of PD-1 deletion on CAR T cell activation. Our findings show that PD-1 deletion had no impact on CAR-mediated activation, as seen by the uniform up-regulation of CD69 after restimulation in both edited and control CD8+ CAR T cells. Further evidence that PD-1 deficient cells were persistent during anti-CD3/anti-CD28 induced expansion after nucleofection comes from the fact that the decrease in PD-1+ cells in altered cultures 48 hours after restimulation was comparable to that seen 48 hours after nucleofection.

The investigated impact of PD-1 targeting on the development, survival, or proliferation of CAR T cells following electroporation. PD-1 edited cells did exhibit lower expansion in some trials, but generally, there was no statistically significant difference in expansion between control and PD-1 targeted CAR T cells. These findings imply that primary T cells can be successfully modified and transduced to produce CAR T cells defective in PD-1 utilizing CRISPR technology.

3.3 CAR T cells with PD-1 edited genes demonstrate increased effectiveness against tumors

The researchers used PD-1 edited anti-CD19 CAR T cells in in vitro tests to look into the effects of PD-1 loss on CAR T cell function. The tests were carried out on large populations of cells, both edited and unedited. Contrary to control Cas9 nucleofected cells, the findings showed that Pdcd1 disruption (PD-1) was able to correct the deficiency in degranulation seen in CD8+ CAR T cells after stimulation.
with CD19+ PD-L1+ tumor cells. The researchers also evaluated the cytolytic ability of PD-1 edited anti-CD19 CAR T cells against both CD19+ and CD19+ PD-L1+ targets. The findings revealed that PD-1 edited CD8+ CAR T cells demonstrated a higher efficiency in killing PD-L1+ tumor cells compared to control Cas9 nucleofected cells (*p=0.03 at E:T ratio of 2:1). Overall, these results suggest that the disruption of Pdcd1 can rescue functional defects caused by PD-L1+ tumor cells in CD8+ anti-CD19 4-1BBζ CAR T cells.

According to the study, animals with initial tumor burdens of between 100 and 250 mm demonstrated the highest disparity in clearance between PD-1 modified CAR T cells and control anti-CD19 CAR T cells. One of six mice treated with control anti-CD19 CAR T cells at 28 days after tumor implantation showed tumor eradication, but none of the animals receiving PD-1 modified CAR T cells were able to do so. The outcome of disease depended on the number of transferred CAR T cells, with no clearance observed in mice receiving either control or PD-1 edited cells when treated with 2×10⁶ CD4+ CAR+ and 2×10⁶ CD8+ CAR+ cells. Median survival of animals treated with PD-1 edited cells was 35 days compared to 21 days for animals receiving control cells, although the difference was not statistically significant. When animals with smaller initial tumors (50-100 mm) were treated with a larger number of CAR T cells (5×10⁶ CD4+ CAR+ and 5×10⁶ CD8+ CAR+ cells), accelerated tumor clearance was observed by PD-1 edited CAR T cells relative to controls, although both un-edited and edited cells were able to clear CD19+ PD-L1+ tumors at this low tumor burden and high T cell dose. These findings suggest that targeted disruption of the Pdcd1 locus using CRISPR can improve the in vivo anti-tumor efficacy of human CAR T cells.
Figure 3: Illustrates the successful disruption of the Pdcd1 gene in CAR T cells using Cas9 ribonucleoprotein (Cas9 RNPs)

In (a), a schematic of the protocol for combined CRISPR gene editing and lentiviral transduction of human primary T cells is provided. In (b), efficient PD-1 deletion and CAR transduction in primary human T cells is shown, where more than 50% reduction in PD-1+ cells and over 70% CAR transduction was routinely observed. Individual dots in the right panel represent independent editing experiments. In (c), the stability of PD-1 edited CAR T cells in culture is demonstrated, where resting PD-1 edited CD8+ anti-CD19 CAR T cells were restimulated with CD19+ K562 cells, and activation was measured by CD69 induction. The percent reduction in PD-1+ cells was similar to that observed 48 hours after editing, indicating the stability of PD-1 edited CAR T cells in culture.

4 Discussion

This study reveals that the presence of PD-L1 expression on tumors can hinder the function of anti-CD19 4-1BBζ CAR T cells in vitro and hinder their ability to clear tumors in a subcutaneous tumor xenograft model. The researchers developed a new method that combines Cas9 RNP editing and lentiviral transduction to generate Pdcd1 deficient anti-CD19 CAR T cells. This method
helped to rescue many of the in vitro defects observed when CAR T cells were co-cultured with PD-L1+ tumors and eventually enabled them to clear PD-L1+ tumors in vivo. These findings demonstrate the inhibitory role of the PD-1/PD-L1 axis on CAR T cell anti-tumor function and provide evidence of the potential of Cas9-based gene editing to enhance CAR T cell efficacy.

Figure 4: To demonstrate that CRISPR-mediated PD-1 editing rescues anti-CD19 CAR T cell function in vitro and enhances tumor clearance in vivo

Panel (a) shows a diagram of PD-1 edited CAR T cell: K562 interactions. Panel (b) demonstrates that PD-1 edited CD8+ anti-CD19 CAR T cells exhibit greater degranulation upon co-culture with CD19+ PD-L1+ K562 cells as compared to control CD8+ CAR T cells. Panel (c) shows that PD-1 edited CAR T cells are partially resistant to CD19+ PD-L1+ mediated inhibition of cytolysis, with reduced PD-L1 dependent inhibition of killing in PD-1 edited CD8+ CAR T cells at effector: target ratio of 2:1. Panel (d) demonstrates that PD-1 deficient anti-CD19 CAR T cells exhibit enhanced anti-tumor efficacy and clear subcutaneous CD19+ PD-L1+
tumor xenograft. The experiment was performed three independent times, and a statistically significant decrease in tumor burden of mice receiving PD-1 edited CAR T cells was observed at multiple points. These results provide evidence for the inhibitory role of the PD-1/PD-L1 axis on CAR T cell anti-tumor function and demonstrate the potential of Cas9-based gene editing to enhance CAR T cell efficacy.

Future studies will need to address a number of questions raised by this work. Studies on TCR signalling have demonstrated that PD-1 ligation can reduce Akt pathway signalling and have other consequences, although the precise mechanisms by which PD-1/PD-L1 ligation inhibits CAR T cell activity are yet unknown. The development of CARs with extra or alternative costimulatory domains that resist PD-L1 mediated immunosuppression could be made easier by determining the precise level at which PD-1 ligation impairs CAR T cell signalling or function. It will be crucial to establish whether Pdcd1 loss has various effects depending on the particular CAR employed, as CAR T cells with different costimulatory domains demonstrate varied activity, phenotypes, and PD-1 expression.

The link between PD-1/PD-L1 and CD8+ T cell exhaustion is widely recognized, hence our work largely focused on CD8+ T cells in vitro. However, since this is typical procedure in the CAR T cell therapies currently being explored in clinical trials, employed both CD4+ and CD8+ Pdcd1 altered T cells for our in vivo studies. Pdcd1 deletion in CD4+ T cells has the ability to stop this reprogramming and enhance tumor clearance. It is probable that decreased tumor clearance in PD-L1+ tumor is caused by the product ion of immunosuppressive Tregs. Future research should investigate the effects of Pdcd1 disruption in CD4+ and CD8+ CAR T cells both alone and in combination.

The percentage of transplanted Pdcd1-deficient CAR T cells should also be taken into account. Recent findings have shown that full deletion of Pdcd1 in naive CD8+ T cells can surprisingly result in greater exhaustion and decreased CD8 T cell survival and function in the mouse lymphocytic choriomeningitis virus (LCMV) model of chronic infection. As a result, it's likely that robust deletion of Pdcd1 could improve CAR T cells’ short-term abilities while ultimately making them more vulnerable to depletion and poor performance, especially if the tumor is not quickly removed. The interaction between tumor burden, T cell number, and editing frequency in different tumor models has to be further investigated.

Engineered human xenograft enable the isolated study of particular biological processes. It could be possible to better understand how inhibitory receptor signalling affects CAR T cell function by conducting additional studies on T cells in models with several inhibitory receptors. Primary human T cells can be genetically altered in a powerful and effective way, opening up new research possibilities and improving adoptive immunotherapy. For lymphoma patients in particular, Pdcd1 altered CAR T cells may improve anti-tumor functionality and require fewer cells to be effective. Pdcd1-deficient CAR T cells, however, might express TCRs that are auto-reactive, leading to autoimmune adverse effects comparable to those seen with systemic PD-1 antibody blocking. In order to create highly effective, tumor-specific CAR T cells devoid of any ability to recognize
non-CAR antigens, guide RNAs targeting the endogenous TCR may be inserted and TCR+ cells could be selectively depleted. This would prevent graft-versus-host disease. Although Pdcd1 deletion may make cytokine release syndrome worse in some circumstances, genetic disruption of inhibitory checkpoints may make CAR T cell function more effective in other circumstances. Prior to the use of CRISPR/Cas9-based therapies in humans, it is crucial to investigate the off-target cleavage and genotoxicity of the provided guide RNAs strong Cas9 RNP-based editing of T cells could enable research into the fundamental biology of human T cells, which could help identify new targets that improve anti-tumor T cell performance. Last but not least, knock-in genetic modifications could be used to investigate gain of function mutations or rewire endogenous circuits to create improved cellular therapeutics for a variety of disorders. The next generation of immunotherapies for cancer should advance as a result of the capability to execute targeted genome engineering of human tumor-specific T cells and examine the functional effects in vitro and in vivo.

5 Conclusion

This study investigated the potential for primary human CAR T cells to become more potent anti-tumor agents by altering the endogenous Pdcd1 gene. The researchers found that the inhibitory receptor PD-1 expressed on CAR T cells can diminish those cells’ capacity to eradicate tumour cells both in vitro and in vivo. The Cas9-mediated Pdcd1 cleavage within the CAR T cells, however, might overcome this inhibition. The researchers also found that CAR T cell transduction with lentiviral vectors and gene editing with CRISPR were very successful together. This discovery may enable the future enhancement of the safety and anti-tumor efficacy of cell therapy products. The potential of precision genome engineering to augment current cell therapies and boost the effectiveness of CAR T cell immunotherapy is highlighted in this study.

Declaration of interest: Author declare no competing interest.

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