CRISPR/Cas9-mediated PD-1 Knock-out in Human Primary T Cells Shows Enhanced Cytotoxicity Against Cancer Cells

Application Note

CORNING

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Introduction

The existence of an adaptive immune system in single-cell organisms was debated for a long time until the CRISPR/Cas9 mechanism had been identified[1,2]. In prokaryotes, invading DNA (i.e., from bacteriophages) is processed and inserted into a specific locus called Clusters of Regularly Interspaced Short Palindromic Repeats (CRISPR). From this locus, specific RNAs are transcribed which assemble into a complex with CRISPR-associated protein 9 (Cas9). Cas9 is an RNA-guided endonuclease that cleaves DNA which is complementary to the associated RNA[3,4].

The elegant mechanisms of the CRISPR/Cas9 mediated immune response has been proven to be as efficient in editing other genomes, including in human cells[3,5]. As such, the CRISPR technology revolutionizes genomic editing by enabling RNA-guided, site-specific DNA cleavage with high efficiency, which holds a great potential for curing disease. The simplicity of this approach, in contrast to other genome editing techniques lies in the nature of the guide RNA (gRNA). The gRNA is designed in such a way, that it is complementary to the DNA sequence of the target gene, constrained only by the requirement of the PAM (protospacer-adjacent motif) sequence. Genome editing (knock-in, knock-out, or the insertion of mutations) in cells can be accomplished by an active Cas9 complex loaded with the designed gRNA. This guides the CRISPR/Cas9 complex to the target sequence in the DNA of the host, which is subsequently incised by the Cas9 protein. The resulting DNA double-strand break initiates DNA repair (non-homologous end joining (NHEJ) and/or homology-directed repair (HDR)) which can be manipulated such that the genome is edited (insertion, deletion, or change of a sequence). This highly versatile method can, in principle, target any genomic site and this facile, but robust, and extremely specific editing tool facilitates not only research, but could also be implemented for gene and cell therapies.

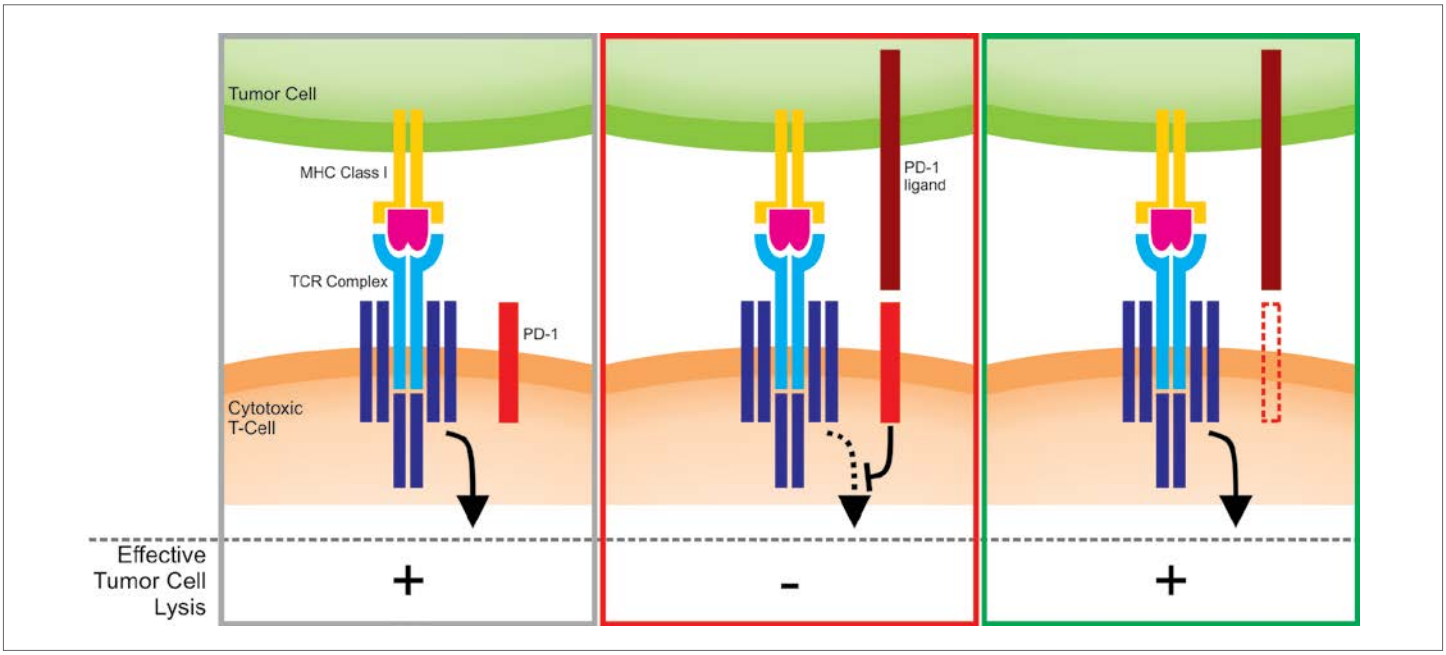

Figure 1. The PD-1 ligand interaction with the PD-1 receptor is commonly exploited by tumor cells to inhibit T cell regulated immune responses. The presentation of a target antigen by antigen-presenting cells to the T-cell through the TCR, in combination with co-stimulatory signaling, leads to the activation of the T cells. Activated T cells survey for their target antigen and upon recognition become cytotoxic resulting in cell lysis of the target antigen-presenting cell (left, grey panel). However, this signaling cascade can be inhibited, when the PD-1 receptor is stimulated. Tumor cells utilize this mechanism to suppress an immune response by expressing PD-1 ligands on the surface. The interaction of the PD-1 ligands with the PD-1 receptor on T cells inhibits the signaling cascade that is triggered upon the recognition of the T cells target-antigen (middle, red panel). Regulating the expression of the PD-1 receptor by T cells therefore serves as a tool to manipulate T cells effector functions: The down regulation of the PD-1 receptor on T cells is favorable in a cancer-cell environment as the T-cell effector signaling (cytotoxicity and cytokine production) remain activated which results in effective tumor cell lysis (right, green panel). TCR: T-cell receptor; PD-1: Programmed cell death protein 1; PD-1 ligands: programmed cell death protein ligand (PD-1L and PD-2L). Adapted from Reference 9.

As part of the adaptive immune system T cells play a crucial role in identifying and eliminating harmful pathogens and tumor cells. The activation of T cells occurs through the presentation of an antigen to the T-cell receptor (TCR) by an antigen-presenting cell (APC), in combination with costimulatory signaling (such as CD28)[6]. The induced TCR signaling results in the activation of effector functions (such as cytotoxicity and cytokine production) and upregulates proliferation. Those activated T cells then infiltrate tissues in search for their target antigen. Recognition of the target antigen induces signaling in the T cell resulting in the cell lysis of the targeted cell (for example a tumor cell) (Figure 1, grey panel). However, several checkpoints exist in order to regulate and balance T-cell activity, one of which is mediated by the programmed cell death protein 1 (PD-1) receptor in T cells. The PD-1 receptor plays a key role in tolerance to self-antigens and modulates the T cell response: overaction of T cells (PD-1 receptor unstimulated or blocked) can result in auto-immunity and conversely, suppressed activity of T cells (active PD-1 signaling) is commonly detected in chronic infections and favors tumor evasion[7,8].

Tumor cells exploit the activation of the PD-1 receptor in T cells to evade the immune system. The ligands of the PD-1 receptor, programmed cell death protein ligand 1 or 2 (PD-1L or PD-2L; combined to PD-1 ligand, PD-1L) are commonly expressed on tumor cells[7,9] and their interaction results in the inhibition of the TCR-mediated signaling and blocks the cytotoxic activity of T cells (Figure 1, middle, red panel). Several clinical agents have been developed to block the signaling of PD-1 receptor in order to induce an anti-tumor response in T cells[8].

In this study, the CRISPR/Cas9 system was used to knock-out PD-1 gene expression in human primary T cells (Figure 1, right, green panel). The efficient lentiviral transduction of Cas9 nuclease and human PD-1 sgRNA in human primary T cells has been monitored. Cytotoxicity mediated by the engineered T cells was tested by co-culture with a leukemia cell line (K562 cells, PD-1L expressing). The results demonstrate that the CRISPR/Cas9 mediated knock-out of PD-1 in primary T cells increases their cytotoxicity towards PD-L1 expressing cancer cells. This shows that in addition to clinical agents blocking PD-1 receptor signaling, genome-engineered T cells in which the PD-1 receptor is down-regulated or knocked-out can serve as a potent cell therapy.

Materials and Methods

Plasmid construction

Human PD-1 full sequence and coding sequence were obtained from the NCBI GENE database (www.ncbi.nlm.nih.gov/gene). According to the sequence, 3 gRNAs targeting human PD-1 gene (Table 1) were designed according to principle of existence of PAM NGG and G for U6 promoter-driven transcription initiation. The gRNAs were aligned in Guide Design Resources of Feng Zhang's lab (zlab.bio/guide-design-resources) to check for specificity and were synthesized by Sangon Biotech.

Table 1. PD-1 gRNA sequences

gRNA number	Sequence (5' to 3')
gRNA-1	TGGCCAGTCGTCTGGGCGGTC
gRNA-2	GGATGGTTCTTAGGTAGGTGC
gRNA-3	GCGCCTTCTCCACTGCTCAGG

Lentiviral system carrying Cas9 and gRNA were bought from Hanbio Biotechnology: psPAX2, pMD2.G, and pHBLV-U6-gRNA-EF1-CAS9-PURO. The pHBLV-U6-gRNA-EF1-CAS9-PURO plasmid carries an U6 promoter to drive gRNA expression and an EF1 promoter to drive Cas9 expression.

For the generation of the plasmid, the Axygen® AxyPrep Plasmid Miniprep Kit (Corning AP-MN-P-50), Axygen AxyPrep PCR Clean-up Kit (Corning AP-PCR-50), and Axygen AxyPrep DNA Gel Extraction Kit (Corning AP-GX-50) were used according to the manufacturer's instructions. Competent E.coli DH5α were purchased (Biomed BC116-01). Restriction enzymes used were EcoRI (NEB R0101S) and BsmBI (NEB R0580S). The correct insertion of gRNAs into the pHBLV-U6-gRNA-EF1-CAS9-PURO plasmid was confirmed by Sanger sequencing.

Determination of gRNAs editing efficiency in HEK-293T cells

HEK-293T cells (ATCC® CRL-3216) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning 10-013-CV) supplemented with 10% Fetal Bovine Serum (FBS, 35-081-CV) and incubated in a 37°C, 5% CO_2 incubator. DNA of the pHBLV-U6-gRNA-EF1-CAS9-PURO (carrying PD-1 gRNA1, gRNA2, or gRNA3) plasmid was extracted using a standard Maxiprep protocol) in line with manufacturer's protocol and was transfected into HEK-293T cells separately using LipoFiter™ (Hanbio Biotechnology HB-TRLF-200) according to the manufacturer's instructions. Medium was refreshed after 6 hours of transfection, and genomic DNA was extracted 48 hours post-transfection using a standard genomic DNA extraction protocol. In order to amplify a ~1 kb DNA region of the PD-1 gene that contains the target site, PCR was conducted on the genomic DNA (Forward 5'-3': AGTTTCCCTTCCGCTCACC; Reverse 5'-3': GGGGCTCATCCCATCCTTAG). Purified PCR fragments were sent for Sanger Sequencing to verify the PD-1 gene editing. T7 Endonuclease I (T7EI) assay was performed to determine the specificity and efficacy of the gRNAs used by employing EnGen® Mutation Detection Kit (NEB Cat. No E3321) according to the manufacturer's instructions. Primers used for the T7EI assay were: Forward 5'-3': AGTTTCCCTTCCGCTCACCT; Reverse 5'-3': ACCTTGGCTTTACGACGTCAA.

Lentivirus Production

5 x 10^6 HEK-293T cells were seeded in a Corning CellBIND® surface T-75 flask (Corning 3290) to obtain 50% to 70% confluency of cells for the transfection. DMEM medium and transfection reagent LipoFiter™ were brought to room temperature. A total of 30 µg of plasmids were used for each transfection. Plasmids were used in a 1:1:1 ratio (pHBLV-U6-gRNA-EF1-CAS9-PURO plasmid, PMD2.G, and psPAX2, 10 µg each) in accordance to the manufacturer's instructions of the LipoFiter. After 6 hours, medium was changed with fresh DMEM containing 10% FBS. 48 hours post-transfection, supernatant containing lentiviral particles was collected, and fresh medium was added to the cells. This step was repeated 72 hours post-transfection.

Transduction of primary human T cells

Fresh peripheral blood mononuclear cells (PBMCs) from a healthy donor were purchased (AllCells PB006-C). T cells were isolated using EasySep™ Human T Cell Isolation Kit (StemCell Technologies 17951). The EasySep™ procedure involves the labelling of unwanted cells with antibody complexes and magnetic particles. KBM581 medium (Corning 88-581-CM) was supplemented with

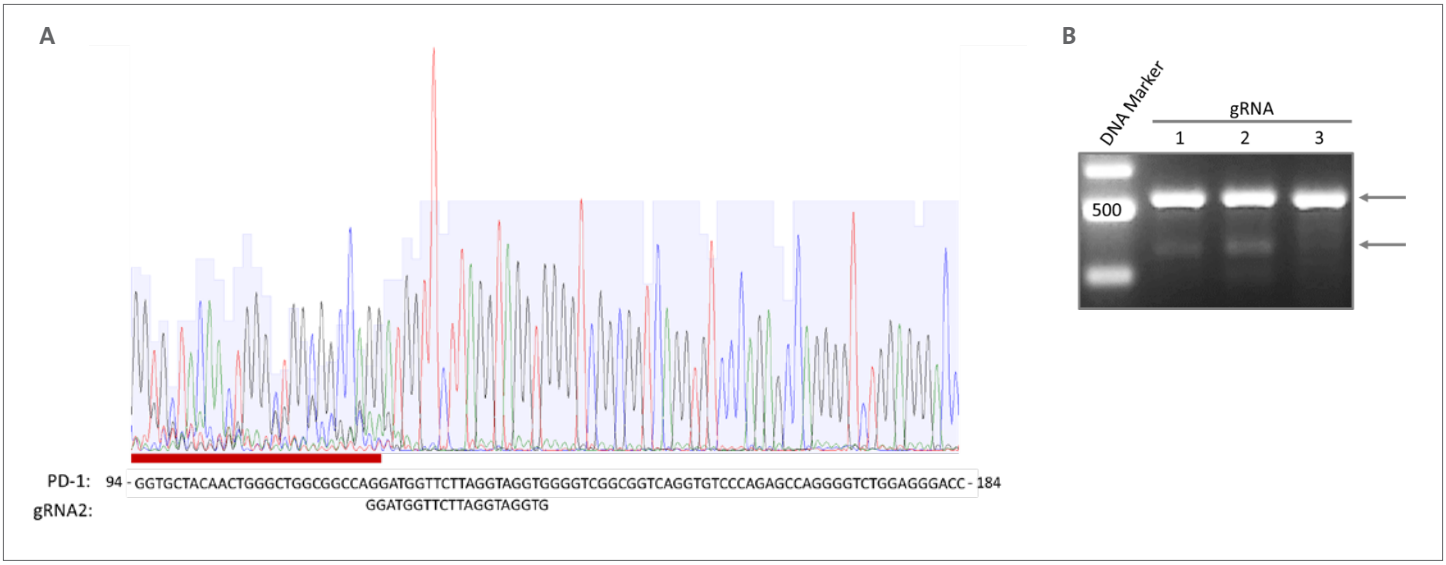

Figure 2. Determination of genome editing in HEK-293T cells. A) Sequencing confirmed genome editing. An extract of the sequencing reaction is shown on top and the analyzed sequence of the PD-1 gene is shown. The sequence of the chosen gRNA (gRNA 2) is represented. The induced genome-engineered sequence upstream of the gRNA is underlined in red. B) Isolated genomic DNA of HEK-293T cells was subjected to PCR reactions and the T7EI assay. The PCR reaction generated DNA fragments of 650 base pairs. The presence of multiple bands (650 and 310 base pairs, indicated by arrows) after the T7EI assay indicates that genome engineering has occurred.

100U/mL rIL-2 (Corning 354043) and 2% autologous plasma (donated by AllCells). Dynabeads® Human T-Activator CD3/CD28 (Thermo Fisher 11131D) was added to isolated T cells to activate T cells following the manufacturer's instructions (Day 0). At Day 2, Lentivirus-pHBLV-U6-PD-1 sgRNA-EF1-CAS9-PURO and 5 µg/mL polybrene was added to T cells at MOI = 100. Twelve hours after transduction, the lentivirus was removed by refreshing medium (KBM581 media supplemented with 100U/mL rIL-2 and 2% autologous plasma). At Day 5, flow cytometry analysis was performed using BD Accuri™ C6 flow cytometry system (BD Accuri C6) to test T cell markers and PD-1 gene expression.

Cytotoxicity analysis of PD-1 engineered T cells

The cytotoxicity of PD-1 knock-down T cells against PD-1L expressing tumor cells (K562, human immortalized myelogenous leukemia cell line) was measured in a co-culture experiment by CCK8 reagent (Dojindo CK04). Briefly, K562 cell suspension was seeded into a 96-well microplate (Corning 3599) at a concentration of 1 x 10^4 cells/well. T cell density was adjusted to 1 x 10^6 cells/mL and seeded in ratios of 2.5:1, 5:1, and 10:1 to the K562 cells. The total volume/well was adjusted to 200 µL. Each condition was performed in triplet and wells containing only medium were used for blank measurements. Wells containing T cells or K562 cells only were set as control. The plate was incubated at 37°C for 24 hours and 20 µL of CCK8 reagent was added to each well to test cell viability according to the manufacturer's instructions. After 4 hours, the optical density of each well was read at 450 nm with a MD4 plate reader. The cytotoxic ability of the T cells was calculated using the following formula: Cytotoxicity = [1-(co-culture cells OD450 – T cells OD450)/K562 cells OD450] x 100%. Statistical analysis was conducted by calculating the average of the different measurements/condition using a *t*-test. Standard deviation is shown.

Results and Discussion

The efficiency of the gRNAs was assessed in HEK-293T cells by extracting genomic DNA and subsequently confirmed with the T7EI assay. Sequencing of the target region confirmed genome editing at the expected area (Figure 2A) and with the T7EI assay the expected cleavage products of 310 base pairs could be detected (Figure 2B). The combination of sequencing and T7EI assay results confirmed the efficacy of gRNA2 which was chosen for genome editing of the PD-1 receptor in human primary T cells.

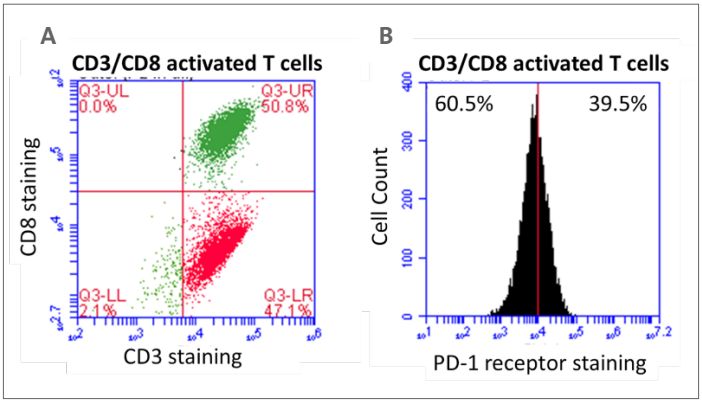

Figure 3. CD3/CD8 and PD-1 expression profiles in T cells 2 days after T cell activation. A) Representative FACS scatter plots of activated T cells. The X-axis represents CD3 staining, the Y-axis represents CD8 staining. The percentage of cells expressing the CD3 and CD8 receptor is shown in the scatter plot. B) Histogram shows the number of cells expressing the PD-1 receptor on the Y-axis against fluorescence (Accuri C6) for the PD-1 receptor on the X-axis.

Isolated T cells were activated using Dynabeads® Human T-Activator CD3/CD28. After 2 days of activation, T cells were analysed by flow cytometry for human CD3/CD8 and PD-1 expression. At day 2, 50.8% of T cells expressed CD3/CD28 (Figure 3A) and 39.5% of T cells expressed PD-1 (Figure 3B).

The expression of CD3/CD8 was used as control for T cell activation. The levels of CD3/CD8 and PD-1 were used as a baseline and this pool of T cells was divided into 3 groups. The first group was transduced with the Lentivirus carrying Cas9 and PD-1 gRNA2. This group will be referred to as PD-1 Knock-out group (short: KO, MOI=100). The other two groups served as controls, either as negative control (NC, Lentivirus with Cas9 only) or as blank (BL, no virus transduction). All T cells were cultured for 12 hours before the medium was refreshed with KBM581 medium supplemented with 100U/mL rIL-2 and 2% autologous plasma.

At day 5, flow cytometry analysis was performed to analyze the knock-out of the PD-1 receptor. The expression levels of CD3/CD8 and PD-1 were compared to the expression levels of T cells on day 2 (Figure 3). To assess whether the CRISPR-Cas9 and gRNA transfection had a general effect on the expression of surface receptors, the expression levels of CD3/CD8 were analysed (Figure 4A). Under all conditions (KO, NC, and BL) the CD3/CD8 expression levels showed comparable levels. In addition, CD3/CD8 expression levels, as expected, seem to increase over time (compare with Figure 3A, 50.8%). This confirms that the transduction of the CRISPR/Cas9 machinery does not have a general effect on the functionality of the T cells. We then analysed the expression of the PD-1 receptor which showed a decrease from 39.5% at day 2 to 7.9% at day 5 in PD-1 KO group (Figure 4, Top panel). This 5-fold reduction of the PD-1 receptor confirms the approach for receptor knock-out, especially, as the PD-1 receptor expression in the NC group and BL group remained at similar levels (36.2% and 38.8%, respectively) compared to day 2 (39.5%). In conclusion, the knock-out of the PD-1 receptor on activated T cells is efficient and specific.

Although the knock-out of the PD-1 receptor on activated T cells has been confirmed, its functional impact on cancer cells has not been assessed. It is expected that the PD-1 knock-out in T cells would enhance their cytotoxicity. Therefore, the three subgroups, KO, NC, and BL were co-cultured with tumour cells (K562). K562 cells express the ligand for the PD-1 receptor (PD-1L) and the binding of PD-1L to PD-1 results in the inhibition of the cytotoxic signal in the T cell (Figure 1). The PD-1L expression in K562 cells was analysed by flow cytometry and 72.2% of the K562 cells indeed expressed PD-1L (Figure 5).

The effect of the PD-1 knock-out in T cells was assessed by using the three genome-engineered T cells (KO, NC, and BL) in co-culture with K562. The viability of the K562 cells as a measure of T cell cytotoxicity was assessed by the CCK8 assay (Figure 6). The co-culture experiment was conducted with different ratios of T cells to K562 cells: 2.5:1, 5:1, and 10:1. Regardless of the ratios of K562 and T cells used, K562 cells were less viable when co-cultured with T cells in which the PD-1 receptor was knocked out (Figure 6, KO-group). The PD-1 KO T cells exhibited higher cytotoxicity (82.8%) compared to the control conditions (NC: 75.7% and BL: 73.4%) (Figure 6). Statistical analysis confirmed a significant increase of cytotoxicity of PD-1 KO T cells in comparison to NC and BL T cells.

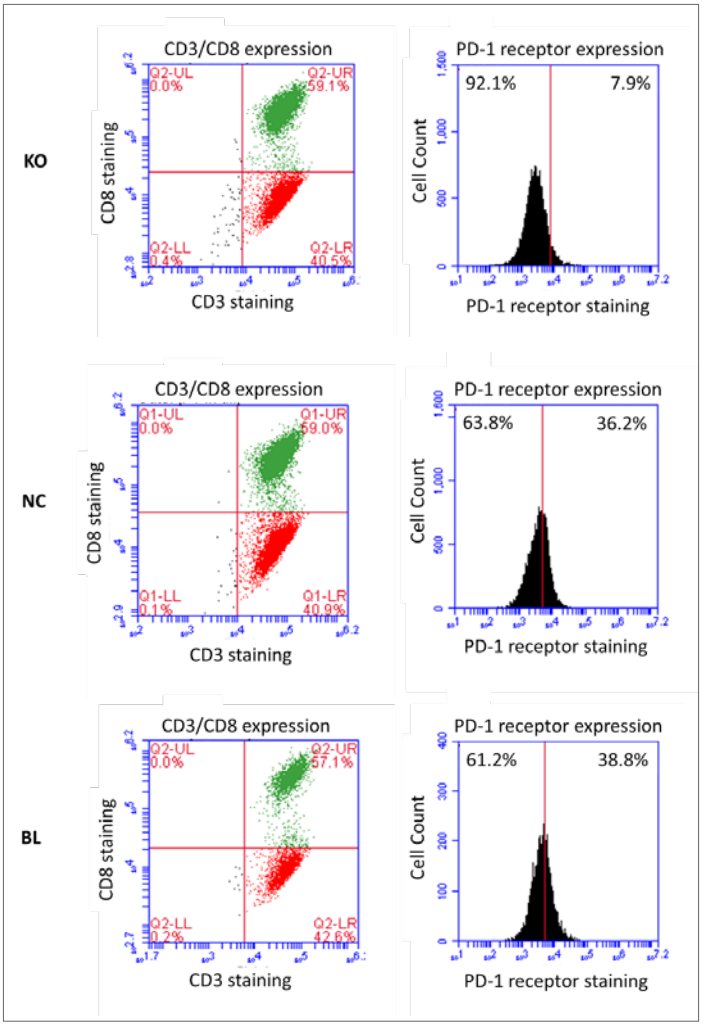

Figure 4. CD3/CD8 and PD-1 expression of T cells 3 days after Cas9/PD-1 sgRNA2 transduction. Left panels: Representative FACS scatter plots of activated T cells. The X-axis represents CD3 staining, the Y-axis represents CD8 staining. The percentage of cells expressing the CD3 and CD8 receptor is shown in the scatter plot. Right panel: Histograms show the fluorescence (Accuri C6) for the PD-1 receptor on the X-axis and the number of cells on the Y-axis. Top panel: T cells were subjected to genome engineering by CRISPR/Cas9, which induces a knock-out of the PD-1 receptor (KO). Only 7.9 % of the T cells expressed the PD-1 receptor. Middle panel: negative control (NC, Lentivirus with Cas9 only); bottom panel: blank (BL, no virus transductions).

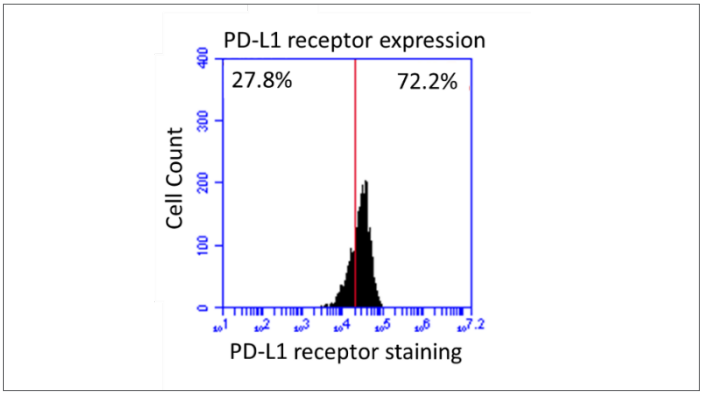

Figure 5. PD-1L expression of K562 cells. Histogram shows the number of cells expressing the PD-1L receptor on the Y-axis against fluorescence (Accuri C6) for the PD-1L receptor on the X-axis.

The discovery that parts of the bacterial immune system can be employed to edit genomes efficiently and precisely has revolutionized the field of gene editing. The CRISPR/Cas technique provides an efficient, specific, versatile and easy-to-use tool for researchers. Current advances in immunotherapy for curing cancer have proven that genome-editing is a potent approach. The PD-1 receptor interaction with its ligand, PD-1L, is one of the most studied checkpoints of the immune system and has become a frequent target for anti-tumor immunotherapy[8].

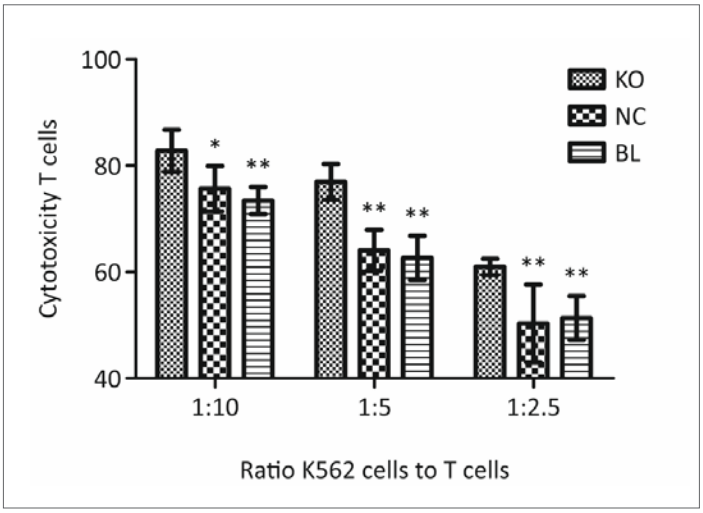

Figure 6. Cytotoxicity of differently genome-engineered T cells against K562 cells. K562 and genome-edited T cells were co-cultured in the indicated ratios on the x-axis. Three groups of T cells with differently engineered genomes were used: PD-1 knock-out group (KO), negative control (NC, Lentivirus with Cas9 only), or blank (BL, no transduced virus). The values on the Y-axis represent the calculated cytotoxicity of T cells as a measure of the viability of the K562 cells. Error bars represent the standard deviation (StDev). A *t*-test was conducted to compare the mean values of the KO response with those of the NC and BL controls.* indicates a *p*-value of <0.05; ** indicates a *p*-value of <0.01.

Conclusions

In this study, an approach for knocking out the PD-1 gene in activated primary human T cells, with the use of CRISPR/Cas genome engineering is described. This approach resulted in PD-1 knock-out in T cells and furthermore, in enhanced cytotoxic activities of those T cells.

The combination of immunotherapy with CRISPR-mediated gene editing is a promising approach for clinical treatments.

- PD-1 gene can be edited by employing the CRISPR/Cas9 system in human primary T cells.
- Components of CRISPR/Cas9 can be delivered into human primary T cells by lentivirus.
- PD-1 receptor knock-down in T cells enhances cytotoxicity to PD-1L expressing tumor cells (e.g., K562 cells).
- PD-1/PD-1L are promising targets for improving cancer therapy.

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