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TITLE:

Production of human CRISPR-engineered CAR-T cells

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SUMMARY

Here, we present a protocol for gene editing in primary human T cells using CRISPR Cas Technology to modify CAR-T cells.

ABSTRACT

Adoptive cell therapies using chimeric antigen receptor T cells (CAR-T cells) have demonstrated remarkable clinical efficacy in patients with hematological malignancies and are currently being investigated for various solid tumors. CAR-T cells are generated by removing T cells from a patient's blood and engineering them to express a synthetic immune receptor that redirects the T-cells to recognize and eliminate target tumor cells. Gene editing of CAR-T cells has the potential to improve safety of current CAR-T cell therapies and further increase the efficacy of CAR-T cells. Here, we describe methods for the activation, expansion, and characterization of human CRISPR-engineered CD19 directed CAR-T cells. This comprises transduction of the CAR lentiviral vector and use of single guide RNA (sgRNA) and Cas9 endonuclease to target genes of interest in T cells. The methods described in this protocol can be universally applied to other CAR constructs and target genes beyond the ones used for this study. Furthermore, this protocol discusses strategies for gRNA design, lead gRNA selection and target gene knockout validation to reproducibly achieve high-efficiency, multiplex CRISPR-Cas9 engineering of clinical grade human T cells.

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the field of adoptive cell therapies and cancer immunotherapy. CAR-T-cells are engineered T-cells expressing a synthetic immune receptor that combines an antigen-specific single chain antibody fragment with signaling domains derived from the TCRzeta chain and costimulatory domains necessary and sufficient for T-cell activation and co-stimulation[1-4]. The manufacturing of CAR-T cells starts by extracting the patient's own T-cells, followed by ex vivo viral transduction of the CAR module and expansion of the CAR-T cell product with magnetic beads that function as artificial antigen presenting cells[5]. Expanded CAR-T cells are re-infused into the patient where they can engraft, eliminate target tumor cells and even persist for multiple years post infusion[6-8]. Although CAR-T cell therapy has resulted in remarkable response rates in B-cell malignancies, clinical success for solid tumors has been challenged by multiple factors including poor T-cell infiltration[9], an immunosuppressive tumor microenvironment[10], antigen coverage and specificity, and CAR-T cell dysfunction[11,12]. Another limitation of current CAR-T cell therapy includes the use of autologous T-cells. After multiple rounds of chemotherapy and high tumor burden, CAR-T cells can be of poor quality as compared to allogeneic CAR-T products from healthy donors in addition to the time and expense associated with manufacturing of autologous CAR-T cells. Gene-editing of the CAR-T cell product by CRISPR/Cas9 represents a new strategy to overcome current limitations of CAR-T cells[13-17].

CRISPR/Cas9 is a two component system that can be used for targeted genome editing in mammalian cells[18,19]. The CRISPR-associated endonuclease Cas9 can induce site-specific double-strand breaks guided by small RNAs through base-pairing with the target DNA sequence[20]. In the absence of a repair template, double-strand breaks are repaired by the error prone nonhomologous end joining (NHEJ) pathway, resulting in frameshift mutations or premature stop codons through insertion and deletion mutations (INDELs)[19-21]. Efficiency, ease of use, cost-effectiveness and the ability for multiplex genome editing make CRISPR/Cas9 a powerful tool to enhance the efficacy and safety of autologous and allogeneic CAR-T cells. This approach can also be used to edit TCR directed T cells by replacing the CAR construct with a TCR. Additionally, allogeneic CAR-T cells that have limited potential to cause graft versus host disease can also be generated by gene editing the TCR, b2m, and HLA locus.

In this protocol, we show how CRISPR-engineering of T cells can be combined with viral vector mediated delivery of the CAR-Transgene to generate genome-edited CAR-T cell products with enhanced efficacy and safety. A schematic diagram of the entire process is shown in **Figure 1**. Using this approach, we have demonstrated high-efficiency gene knockout in primary human CAR-T cells. **Figure 2A** describes in detail the timeline of each step for editing and manufacturing T cells. Strategies for guide RNA design and knockout validation are also discussed to apply this approach to various target genes.

PROTOCOL:

Human T cells were procured through the University of Pennsylvania Human Immunology Core, which operates under principles of Good Laboratory Practice with established standard operating procedures and/or protocols for sample receipt, processing, freezing, and analysis conform to MIATA and University of Pennsylvania ethics guidelines.

1. Lentiviral vector production

NOTE: The viral products have been made replication-defective by separation of packaging constructs (Rev, gag/pol/RRE, VSVg and transfer plasmid) into four separate plasmids, greatly reducing the likelihood of recombination events that may result in replication-competent virus.

1.1. Prepare HEK293T cells one day prior to the transfection. Plate approximately 6 x 10^6 cells in T150 culture vessels in 30 mL of standard culture media (referred to as R10:RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 1% Pen/Strep, 1% L-glutamine) and incubate overnight at 37 °C. 18-24 h later, cells should be 60-70% confluent and look healthy (dendritic projections and uniform distribution across the flask). If cells look healthy, proceed to step 1.2.

1.2. Prepare the transfection mix containing lipofection reagent (96 µL), pTRP gag/pol (Lot# RR13SEP19A) (18 µg), pTRP RSV-Rev (Lot# RR13SEP19B-3) (18 µg), pTRP VSVG (Lot# RR13SEP19C) (7 µg) packaging plasmids and 15 µg of expression plasmid (CD19bbz scFv cloned in pTRPE).

1.2.1. For each transfection reaction, prepare one tube containing 1 mL of reduced-serum minimal essential media plus the four plasmids described in step 1.2 as well as one tube containing 2 mL of reduced-serum minimal essential media plus 90 µL of lipofection reagent. Combine these two solutions by dropwise addition of the 1 mL plasmid mix to the 2 mL of lipofection reagent mix. Be sure to vigorously mix the solution by pipetting up and down several times. Incubate the solution for 15 min at room temperature.

1.2.2. In the meantime, aspirate the media from the HEK293T cell flask and gently wash cells with 10 mL of reduced-serum minimal essential media and aspirate again.

1.2.3. Gently add the transfection mix (3 mL) from step 1.2.1 to the bottom corner of cell culture flask using a 5 mL serological pipette. Gently tilt the flask to evenly distribute the transfection mix across the cell culture flask and incubate for 10 min at room temperature. Make sure to not disturb the cell monolayer. After a 10 min incubation, add 35 mL of R10 media and return flask to the incubator for 24 h.

1.3. After 24 h, collect the supernatant from the HEK293T cell culture flasks and transfer into 50 mL conical tubes. Add fresh R10 to the HEK29T cells and put the cells back into the incubator for another 24 h. Spin down the supernatant (300 x g) to remove cell debris and filter the supernatant through a 0.45 µm filter.

1.4. Concentrate the filtrate from step 1.3 by high-speed ultracentrifugation (25,000 x g for 2.5 h or 8000 x g overnight (O/N)). Store the 24 h virus pellet at 4 °C while pooling the 48 h virus.

1.5. Repeat steps 1.3-1.4 for the 48 h collection and combine 24 h and 48 h virus collections. Pellets from the 48 h collection will contain a combined harvest of the lentivirus.

1.6. Resuspend viral pellet in approximately 1 mL of cold R10 and aliquot into 100 µL vials. Immediately snap freeze the aliquots using dry ice and store at -80 °C.

1.7. Calculate functional viral titer in transducing units/mL by transducing a fixed amount of CD3/CD28 bead-activated primary human T-cells with serial dilutions of the lentiviral supernatant. Measure CAR-Transduction after 72 h by flow-cytometry.

1.7.1. Stain for the CD19-directed CAR with an anti-FCM63 scFv antibody. For other chimeric antigen receptors, stain using fluorophore-labeled recombinant protein that is specific to the CAR scFv. Virus dilution that generates under 20% CAR positive cells by flow-cytometry is the most accurate dilution to calculate viral titer from. Above 20% CAR-positive cells, the chance for each positive cell to be transduced twice increases, resulting in an underestimation of the number of transducing particles. Calculate the transducing units per mL(TU/mL) = (number of cells transduced x percent CAR positive cells x dilution factor)/(transduction volume in mL).

2. Designing of sgRNAs and gene disruption in primary human T cells

2.1. Designing CRISPR sgRNA's

NOTE: Several servers and programs facilitate the design of target-specific sgRNA's. In this protocol, CRISPR sgRNAs were designed using CHOPCHOP (https://chopchop.cbu.uib.no), and the gRNA design portal from the Broad institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).

2.1.1. For each target gene, design six to ten sgRNA sequences to target early coding exon sequences.

NOTE: sgRNA should have high on-target efficacy and low off target efficacy. Each machine learning algorithm for determining sgRNA efficacy works slightly differently. Therefore, comparing multiple sgRNA design tools and curating a list of six to ten sgRNA for screening is recommended.

2.2. Gene disruption in primary human T-cells

2.2.1. Obtain autologous peripheral blood mononuclear cells (PBMC's) from healthy volunteer donors. A schematic timeline of the protocol is described in **Figure 2A** and described below.

2.2.2. Isolate $CD4^+$ and $CD8^+$ T-cells using commercially available CD4 and CD8 selection kits.

2.2.3. Combine $CD4^+$ and $CD8^+$ T-cells at a 1:1 ratio and incubate overnight at $3x10^6$ cells/mL in R10 supplemented with 5 ng/mL huIL-7 and huIL-15 each. Addition of IL-7 and IL-15 is recommended as they are known to promote a central memory phenotype and CAR-T cells expanded with IL-7 and IL-15 have superior anti-tumor efficacy compared to IL-2 expanded CAR-

T cells[22-24].

2.2.4. The next day, count T-cells and centrifuge 5-10 x 10^6 cells at 300 x g for 5 min. Discard all the supernatant and wash the cell pellet in reduced-serum minimal essential media. Resuspend the pellet in 100 µL of nucleofection solution according to the manufacturer's instructions (**Table of Materials**).

2.2.5. While washing the cells, prepare ribonucleoprotein (RNP) complex with Cas9 and gRNA by incubating 10 µg of Cas9 nuclease with 5 µg of sgRNA for 10 min at room temperature (RT). The molar ratio of Cas9 to sgRNA is 1:2.4. A mock control that contains Cas9 and electroporation enhancer, but no sgRNA, is recommended.

NOTE: Multiplex gene editing can be performed at this step by making RNP complexes for each target separately and combining them with cells at the time nucleofection as described in the next step. Choosing the reagents to assemble the RNP complex are key to getting high KO efficiency. For example, Cas9 nucleases from different vendors have varying off-target effects and chemically modified gRNA decrease toxicity to T cells, thereby increasing KO efficiency.

2.2.6. Combine the resuspended cells from step 2.2.4 with the RNP complex from step 2.2.5 and add 4.2 µL of 4 µM electroporation enhancer (ssDNA oligonucleotide that is non-homologous to human genome). Mix well and transfer into electroporation cuvettes. Avoid bubbles as they impair electroporation efficiency.

2.2.7. Electroporate cells using pulse code EH111. After electroporation, incubate cells in R10 supplemented with 5 ng/mL huIL-7 and huIL-15 at 5x10^6 cells/mL at 30 °C for 48 h in 12-well plates. After 48 h, proceed with T-cell activation and expansion.

3. T cell activation, lentiviral transduction and expansion

NOTE: For screening sgRNA's, lentiviral transduction (Step 3.2) of the CAR construct is not necessary.

3.1. Count electroporated T-cells and dilute to a concentration of 1 x 10^6 cells/mL using warm R10 supplemented with 5 ng/mL huIL-7 and huIL-15. Activate cells by adding anti-CD3/anti-CD28 monoclonal antibody coated magnetic beads at a ratio of 3 beads per live T-cell.

NOTE: Electroporation causes significant cell death resulting in roughly 60% ± 15% viable cells compared to non-electroporated cells. Use a live/dead cell count to determine the appropriate amount of beads. Transfer the cells to 37 °C and incubate overnight.

3.2. After overnight stimulation, transduce CRISPR-engineered T-cells with lentiviral supernatant from step 1. Add the appropriate volume based on the viral titer calculated in step 1.7 to achieve a multiplicity of infection (MOI) of 3 and incubate cells for 72 h at 37 °C.

NOTE: The virus pellet resuspended in R10 is simply added on top of the cells according to the cell concentration, viral titer, and desired MOI.

3.3. After 72 h, feed cells with 50% of the current culture volume R10 containing 10 ng/mL huIL-7 and huIL-15 and return them to the incubator for another 48 h. Do not disturb the clusters that have formed between the T-cells and the beads.

3.4. After 48 h, remove the beads from the cells by gently resuspending the cell-bead mixture followed by magnetic separation. Count cells after bead removal and bring to a concentration of 0.8 x 10^6 cells/mL using R10 supplemented with 5 ng/mL huIL-7 and huIL-15. After de-beading, cell numbers should be similar to the cell count on Day 1 prior to electroporation (**Figure 2B**).

3.5. After 24 h, count cells and feed to a concentration of 1 x 10^6 cells/mL with R10 + 5 ng/mL huIL-7 and huIL-15. Repeat this every 24 h until growth kinetics and cell size demonstrate cells have rested from stimulation.

NOTE: From this point, T-cells double approximately every 24h. T-cell usually undergo 5-7 population doublings and have a cell volume of approximately 300 ± 50 fL when rested.

3.6. Once rested, spin down CRISPR-engineered CAR-T cells and resuspend in freeze media (1:1 X-Vivo and FBS plus 10% DMSO) for cryopreservation. CAR-T cells used should be thawed and rested at 37 °C for 16 hours before an experiment.

NOTE: Keep approximately 10x10^6 cells reserved for measuring knockout efficiency and CAR integration. Expected population doublings and volume changes during the expansion have been shown in **Figure 2B,2C**. Additionally, **Figure 2D** shows levels of CAR expression on both Mock and gene edited CAR-T cells.

4. CRISPR Efficiency

4.1. Genomic DNA extraction and target gene amplification

4.1.1. From each screening culture, spin down 3-5 x 10^6 T-cells. Cells can be either frozen down as a dry pellet or one can proceed with genomic DNA extraction. At time of DNA extraction, resuspend pellets in 200 µL of Phosphate Buffer Saline (PBS) and extract genomic DNA from electroporated cells using a standard DNA extraction kit according to the manufacturer instruction.

4.1.1.1. Briefly, lyse cells with proteinase K and load lysate onto a DNA binding column. During centrifugation, DNA is binding to the membrane while contaminants will pass through. After two washing steps to remove remaining contaminants and enzyme inhibitors, elute DNA in water.

4.1.2. Amplify 200-300 ng of genomic DNA using standard PCR mix containing DNA polymerase,

accessory proteins, salts, and dNTPs and 10 µM forward and reverse primers flanking the region of the intended double-strand break.

NOTE: For PCR primer design, the reference genomic sequence (can be found using http://www.ensemble.org) flanking the gRNA cut site are entered into the NCBI primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). PCR primers should be designed such that the amplicon has a target size of 600-700bp. This length allows to design sequencing primers that bind within the amplicon with sufficient distance from the gRNA cut site (at least 150 bp) to ensure good sequencing quality around the Cas9 induced indels (see 4.2.1). Each gRNA requires a unique primer pair, unless the gRNA cut sites are in close proximity.

4.1.3. Run the entire PCR product on a 1% agarose gel and purify the amplicon using a standard agarose gel purification kit.

NOTE: The process of determining the optimal Tm for PCR can take multiple rounds of troubleshooting. This is because the KO sequence has indels and the target gene should be primed for sequencing where there would be no indels usually 100 bp upstream or downstream of the cut site. This can vary widely due to indels resulting from non-homologous end joining. Designing nested sequencing primers to the PCR primers and increasing the concentration of product sequenced can eliminate problems with sequencing.

4.2. Sequencing and indel detection

4.2.1. Design sequencing primers that bind to the gel purified amplicon and send mock and knockout amplicons for Sanger sequencing. Once sequencing is complete, upload the trace files to the Desktop Genetics software (tide.deskgen.com, Desktop Genetics) for TIDE analysis.

4.2.1.1. For sequencing primer design, enter the sequence of the PCR amplicon into a standard primer design software (NCBI, Eurofins or other publicly available tools). The design software will suggest multiple primer sequences that are suitable to sanger sequencing.

4.2.1.2. Choose forward and reverse primers that bind within the amplicon at least 150 bp upstream or downstream of the gRNA cut site to ensure good sequencing quality around the indels. The sequencing chromatogram will be used in 4.2 for TIDE analysis, therefore it is important that sequencing quality is good for accurate indel decomposition (see Hultquist et al.)[25].

4.2.2. Use TIDE (Tracking of Indels by DEcomposition) analysis to detect knock out (KO) efficiency at the genomic level[26]. The algorithm accurately reconstructs the spectrum of indels from the sequence traces and calculates R^2 values, reflecting goodness of fit after non-negative linear modeling by the TIDE software.

4.3. Target protein detection

4.3.1. For target genes whose gene product is expressed on the surface of T-cells, stain approximately 1 x 10^5 cells from each screening culture with a fluorochrome-tagged antibody specific for the target protein. Compare expression levels of the target protein between the mock control and knockout groups by flow cytometry as shown in **Figure 3A,3B**.

4.3.2. For target genes whose gene product is expressed intracellularly, lyse approximately 3 x 10^6 million cells per screening group in lysis buffer and follow standard protocols for SDS-PAGE and western blotting. Alternatively, surface or intracellular flow-cytometry can be used to probe for the target protein.

5. Monitoring Off-target effects using iGUIDE - Library preparation, DNA sequencing, and analysis

NOTE: iGUIDE technique allows for detection of locations of Cas9 guided cleavage and quantify the distributions of those DNA double-stranded breaks.

5.1. Perform iGUIDE as described by Nobles et al.[21]. The off-target effects of sgRNA targeting TRAC locus using the iGUIDE technique have been shown in Stadtmauer et al, 2020[13].

REPRESENTATIVE RESULTS:

We describe here a protocol to genetically engineer T cells, that can be used to generate both autologous and allogeneic CAR-T cells, as well as TCR redirected T cells.

Figure 1 provides a detailed description of the stages involved in the process of manufacturing CRISPR edited T cells. The process begins by designing sgRNA to the gene of interest. Once the sgRNA are designed and synthesized they are then used to make RNP complexes with the appropriate Cas protein. T cells are isolated from either a healthy donor or a patient apheresis and RNP complexes are delivered either by electroporation or nucleofection. Post editing, the T cells are activated and transduced with the lentiviral vector coding for the CAR or the TCR construct. After activation, T cells are expanded in culture and cryopreserved for future studies. The detailed protocol followed in the laboratory is described in **Figure 2**. During the expansion, the population doubling and volume changes are tracked throughout the protocol and an example is shown in Figure 2B and C for both mock and edited CAR-T cells. **Figure 2B,C** show that the KO of the gene of interest did not cause any significant changes in the proliferation and activation during the expansion. These results, however, depend on the target gene being edited and hence may or may not lead to changes in proliferation and expansion.

Once the cells are cryopreserved, levels of CAR expression are also determined for further functional studies. In this case, as shown in Figure 2D we checked CD19 CAR expression on both the Mock edited and KO CAR-T cells and did not see any significant changes. This will again depend on the gene of interest being edited. Finally, the KO efficiency can be determined using multiple techniques such as flow cytometry and western blot for protein level detection and also Sanger sequencing for gene level detection of the KO. **Figure 3A** and **3B** show representative flow cytometry plots where PDCD1 and TRAC locus is targeted using sgRNA, showing an efficiency of

90% for the PDCD1 sgRNA and 98% for the TRAC sgRNA across multiple healthy donors. Thus, this protocol can achieve high efficiency knockout with minimal loss in viability.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic diagram showing T cell editing using CRISPR Cas9 Technology and manufacturing of primary human CAR-T cells.

Figure 2. Expansion of edited CAR-T cells and their population doublings. (**A**) Timeline of CRISPR editing and manufacturing in primary human CART cells**.** (**B**) Population Doublings in Mock and CRISPR edited CD19 CAR-T cells measured using a Coulter Counter during the expansion of the CAR-T cells (n=3 healthy donors; KO=knockout) (**C**) Cell size (μm^3) measured using a Coulter Counter during the expansion of the CAR-T cells (n=3 healthy donors). (**D**) Representative flow cytometry plots showing CAR staining and average in multiple donors showing CAR expression in both mock and edited CAR-T cells. CAR expression was detected using an anti-idiotype antibody conjugated to a fluorophore and gated on Lymphocytes/Singlets/Live Cells (n=3 healthy donors, UTD=untransduced,).

Figure 3. Characterizing KO efficiency in edited CAR-T Cells using flow cytometry. (**A**) Representative flow cytometry plots showing PD-1 staining and average in multiple donors showing PD-1 KO efficiency using a gRNA targeting the TRAC locus in mock and edited CAR-T cells. PD-1 expression was detected using PD-1 antibody (Clone EH12.2H7) conjugated to fluorophore and gated on Lymphocytes/Singlets/Live Cells (n=3 healthy donors). Error bars indicate mean±standard error of the mean (SEM). **** $p<0.0001$, *** $p=0.0005$, ** $p=0.001$ by Welch's t test. (**B**) Representative flow cytometry plots showing CD3 staining and average in multiple donors showing CD3 KO efficiency using a gRNA targeting the TRAC locus in mock and edited CAR-T cells. CD3 expression was detected using CD3 antibody (Clone OKT3) conjugated to fluorophore and gated on Lymphocytes/Singlets/Live Cells (n=3 healthy donors). Error bars indicate mean±standard error of the mean (SEM). **** $p<0.0001$, *** $p=0.0005$, ** $p=0.001$ by Welch's t test.

DISCUSSION:

Here we describe approaches to gene edit CAR-T cells using CRISPR Cas9 technology and manufacture products to further test for function and efficacy. The above protocol has been optimized for performing CRIPSR gene editing in primary human T cells combined with engineering T cells with chimeric antigen receptors. This protocol allows high knockout efficiency with minimal donor-to-donor variability. Modification using CRISPR can improve both the efficacy and safety of CAR-T cells by eliminating receptors that inhibit T cells function and manufacturing allogeneic CAR-T cells.

For small scale expansion protocols, starting with 10^6 cells per group leads to around 500^6 CAR-T cells with an average of 6 population doublings in a healthy normal donor. However, this can vary depending upon if the gene of interest affects T cell activation, transduction and proliferation. Five hundred million cells are sufficient for confirming KO efficiency, in vitro assays and in vivo

assays. There are different variations to the protocol wherein CRISPR editing could be performed before or after activation and CAR-Transduction. Ren et al described one such variation where cells are edited after bead stimulation and CAR-Transduction[15]. The advantage of CRISPR editing before bead stimulation is lower cell quantities need to be edited since the T cells have not proliferated yet, making the procedure less time consuming and more cost-efficient. Additionally, performing editing upfront is directly translatable to the clinic. In fact, many steps in this protocol have been informed by what can be adopted in the clinic which increases the consistency of the KO efficiency when moving from bench to bedside.

There are multiple variables that can be chosen at each step of the protocol. For example, T cells can be electroporated or nucleofected. While both achieve comparable KO efficiencies, in our experience using the nucleofector has higher viability post editing and hence is preferred. However, using the electroporator may prove to be more cost-effective in the long run. Both of these equipments are used for small scale T cell expansions. For large scale and clinical scale expansions, protocols must be re-optimized to perform editing and requires different equipment for nucleofection. There are multiple technological platforms that can be used for both small scale and large-scale editing and expansion depending on the needs of the user.

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DISCLOSURES:

The authors have no disclosures.

REFERENCES:

1 Kowolik, C. M. et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Research.* **66** (22), 10995-11004 (2006).

2 Krause, A. et al. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *The Journal of Experimental Medicine.* **188** (4), 619-626 (1998).

3 Kuwana, Y. et al. Expression of chimeric receptor composed of immunoglobulin-derived V resions and T-cell receptor-derived C regions. *Biochemical and biophysical research Communications.* **149** (3), 960-968 (1987).

4 Maher, J., Brentjens, R. J., Gunset, G., Rivière, I., Sadelain, M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ/CD28 receptor. *Nature Biotechnology.* **20** (1), 70-75 (2002).

5 Levine, B. L., Miskin, J., Wonnacott, K., Keir, C. Global manufacturing of CAR-T cell therapy. *Molecular Therapy-Methods & Clinical Development.* **4** 92-101 (2017).

6 Neelapu, S. S. et al. Axicabtagene ciloleucel CAR-T-cell therapy in refractory large B-cell lymphoma. *New England Journal of Medicine.* **377** (26), 2531-2544 (2017).

7 Porter, D. L. et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Science Translational Medicine.*

7 (303), 303ra139-303ra139 (2015).
8 Maude, S. L. et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *New England Journal of Medicine.* **371** (16), 1507-1517 (2014).
9 Melero, I., Rouzaut, A., Motz, G. T., Coukos, G. T-cell and NK-cell infiltration into solid tumors: a key limiting factor for efficacious cancer immunotherapy. *Cancer Discovery.* **4** (5), 522-526 (2014).
10 Mohammed, S. et al. Improving chimeric antigen receptor-modified T cell function by reversing the immunosuppressive tumor microenvironment of pancreatic cancer. *Molecular Therapy.* **25** (1), 249-258 (2017).
11 Moon, E. K. et al. Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor–transduced human T cells in solid tumors. *Clinical Cancer Research.* **20** (16), 4262-4273 (2014).
12 Beatty, G. L., O'Hara, M. Chimeric antigen receptor-modified T cells for the treatment of solid tumors: defining the challenges and next steps. *Pharmacology & Therapeutics.* **166** 30-39 (2016).
13 Stadtmauer, E. A. et al. CRISPR-engineered T cells in patients with refractory cancer. *Science.* **367** (6481) (2020).
14 MacLeod, D. T. et al. Integration of a CD19 CAR into the TCR alpha chain locus streamlines production of allogeneic gene-edited CAR-T cells. *Molecular Therapy.* **25** (4), 949-961 (2017).
15 Ren, J. et al. Multiplex genome editing to generate universal CAR-T cells resistant to PD1 inhibition. *Clinical Cancer Research.* **23** (9), 2255-2266 (2017).
16 Ureña-Bailén, G. et al. CRISPR/Cas9 technology: towards a new generation of improved CAR-T cells for anticancer therapies. *Briefings in Functional Genomics.* **19** (3), 191-200 (2020).
17 Li, C., Mei, H., Hu, Y. Applications and explorations of CRISPR/Cas9 in CAR-T-cell therapy. *Briefings in Functional Genomics.* **19** (3), 175-182 (2020).
18 Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols.* **8** (11), 2281-2308 (2013).
19 Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* **339** (6121), 819-823 (2013).
20 Jinek, M. et al. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science.* **337** (6096), 816-821 (2012).
21 Jinek, M. et al. RNA-programmed genome editing in human cells. *Elife.* **2** e00471 (2013).
22 Schluns, K. S., Kieper, W. C., Jameson, S. C., Lefrançois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature Immunology.* **1** (5), 426-432 (2000).
23 Prlic, M., Lefrancois, L., Jameson, S. C. Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. *The Journal of Experimental Medicine.* **195** (12), F49-F52 (2002).
24 Zhou, J. et al. Chimeric antigen receptor T (CAR-T) cells expanded with IL-7/IL-15 mediate superior antitumor effects. *Protein & Cell.* **10** (10), 764-769 (2019).
25 Hultquist, J. F. et al. CRISPR–Cas9 genome engineering of primary CD4+ T cells for the interrogation of HIV–host factor interactions. *Nature Protocols.* **14** (1), 1-27 (2019).
26 Brinkman, E. K., Chen, T., Amendola, M., van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Research.* **42** (22), e168-e168 (2014).

Figure 1

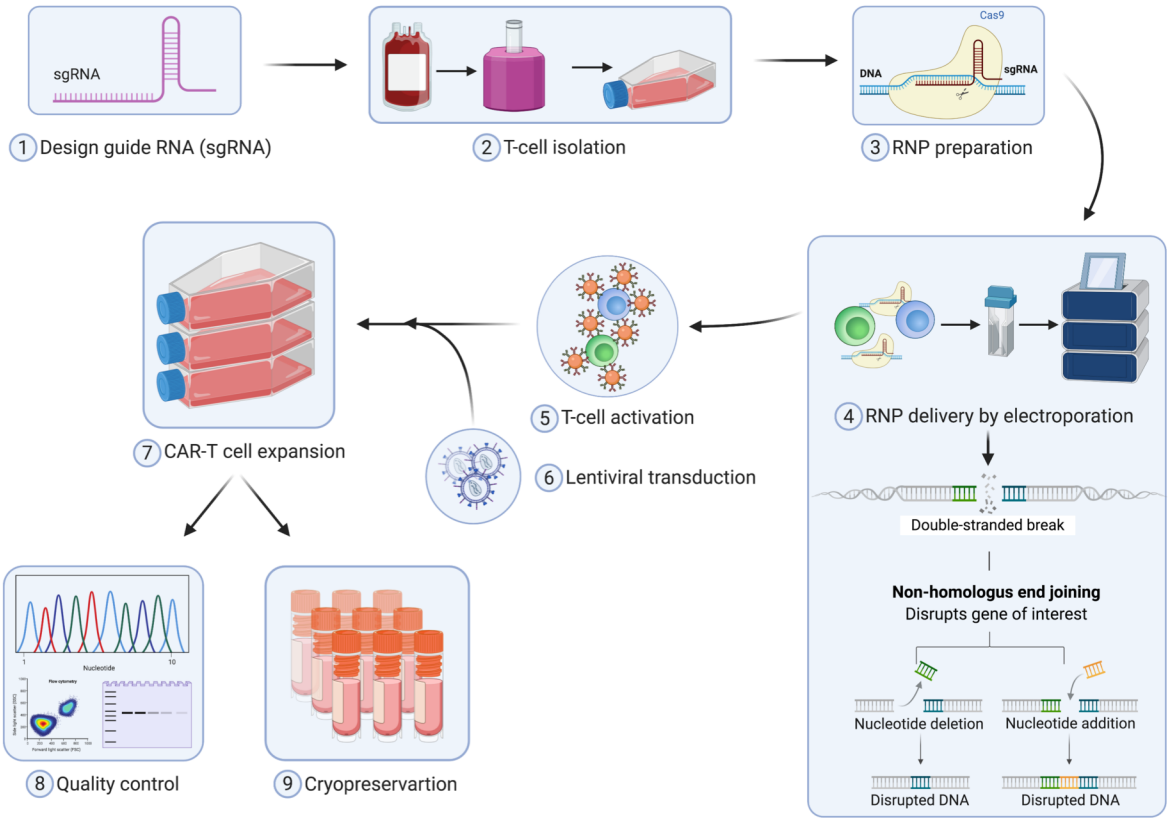

Figure 2

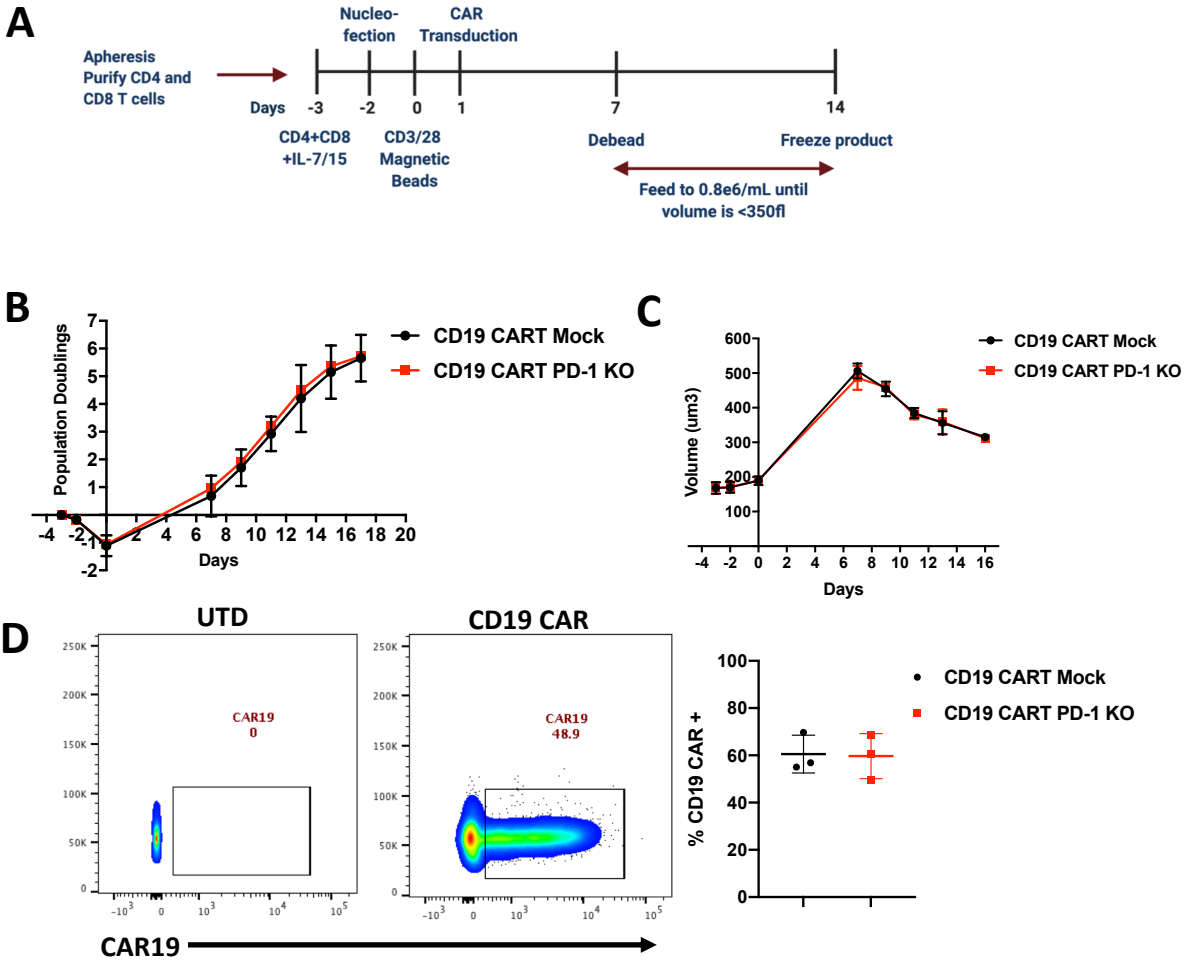

Figure 3

Click here to access/download;Figure;Figures 3.pdf

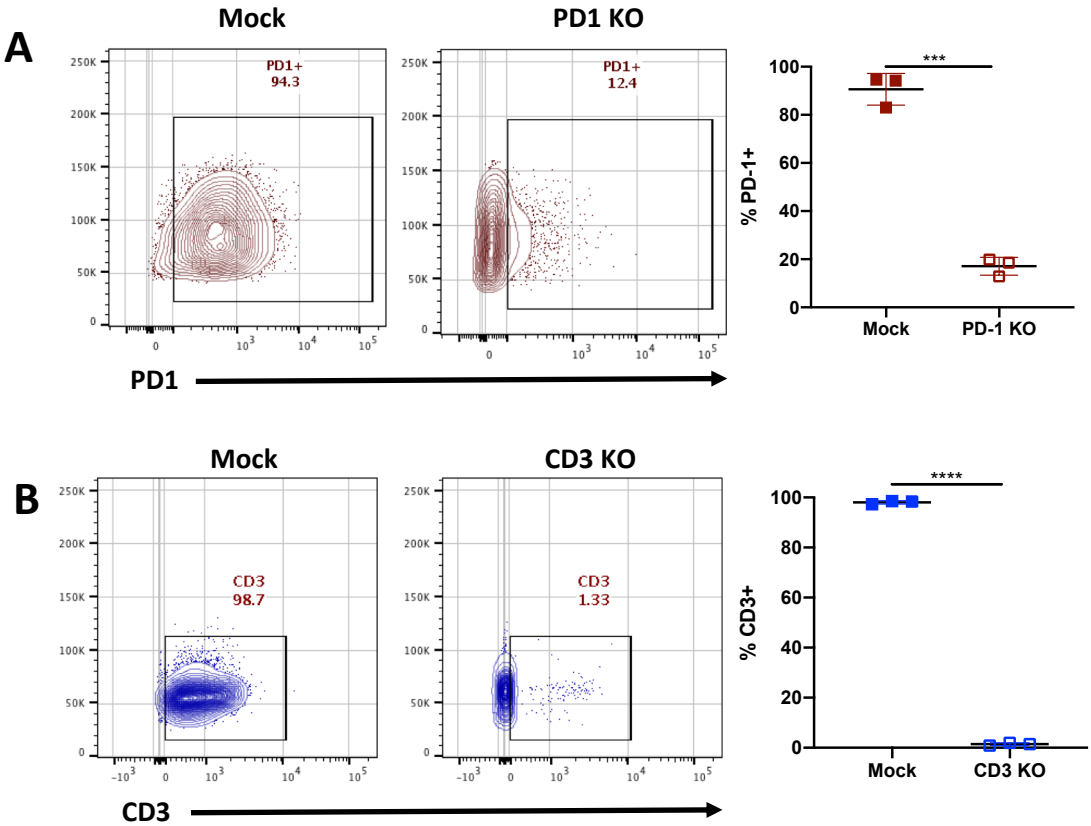

Table of Materials

Name	Company	Catalog Number	Comments
4D-Nucleofactor Core Unit	Lonza	AAF-1002B	
4D-Nucleofactor X-Unit	Lonza	AAF-1002X	
Accuprime Pfx Supermix	ThermoFisher	12344040	
Beckman Optima XPN ultracentrifuge	Beckman Coulter		
Brilliant Violet 605 anti-human CD3	Biolegend	317322	Clone OKT3
BV711 Anti-human PD1	Biolegend		Clone EH12.2H7
Cas9-Electroporation enhancers	IDT	1075915	
CD3/CD28 Dynabeads	ThermoFisher	40203D	
CD4+ T cell isolation Kit	StemCell technologies	15062	
CD8+ T cell isolation Kit	StemCell technologies	15063	
Corning 0.45 micron vacuum filter/bottle	Corning	430768	
Corning T150 cell culture flask	Millipore Sigma	CLS430825	
DMSO	Millipore Sigma	D2650	
DNAeasy Blood and Tissue Kit	Qiagen	69504	
DynaMag Magnet	ThermoFisher	12321D	
Glutamax supplement	ThermoFisher	35050061	
HEK293T cells	ATCC	CRL-3216	
HEPES (1 M)	ThermoFisher	15630080	
huIL-15	PeproTech	200-15	
huIL-7	PeproTech	200-07	
Lipofectamine 2000	ThermoFisher	11668019	
Nucleospin Gel and PCR cleanup	Takara	740609.25	
Opti-MEM	ThermoFisher	31985062	

P3 Primary cell 4D-nucleofactor X Kit L	Lonza	V4XP-3024
Penicilin-Streptomycin-Glutamine	ThermoFisher	10378016
pTRPE expression Plasmid	in house	
Rabbit Anti-Mouse FMC63 scFv Mo	CytoArt	200105
RPMI1640	ThermoFisher	12633012
sgRNA	IDT	
Spy Fi Cas9	Aldevron	9214
	Beckman	
Ultracentrifuge tubes	Coulter	326823
Viral packaging mix	in house	
X-Vivo-15 Media	Lonza	BE02-060F

Dear Editor's and Reviewers,

Thank you for taking your time to review our recent submission (JoVE62299-Production of human CRISPR-engineered CAR-T cells). We appreciate all the constructive feedback and criticism raised by the editors and reviewers to make this a stronger manuscript. Please find the comments on how we addressed each comment/concern below:

Editor comments:

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The authors have proofread the manuscript to the best of their ability to ensure that there are no spelling or grammar issues.

2. Please provide an institutional email address for each author.

Email addresses for each author have been added to the title page of the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Lipofectamine 2000, Opti-MEM, Benchling, Miltenyi, SPY Fi Cas9, P3 primary Cell 4-D Kit, Desktop Genetics, Maxcyte, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

Commercial language has been removed from the manuscript and replaced by generic terms.

4. Line 104/151: Please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

Line 104/151: "minutes" has been replaced by "min".

5. Line 105: Please include the details of volume of the media used for washing.

Volume for wash has been added to the manuscript.

6. Line 107-109: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Please mention how much of the transfection reagent is added. What is the composition of R10 media? If it is commercial product, please provide the details of it in the Table of Materials and use generic terms to describe the product ion the protocol.

Composition of R10 media has been described in 1.1 and name reference has been added in parentheses. More details have been added to this step of the protocol and the volume of the transfection mix has been specified.

7. Line 117: Please define the abbreviations before use (O/N).

Definition has been added.

8. Line 125-126: Please elaborate on the calculation of viral titer.

Formula for calculation of viral titer and more elaborate description has been added to the protocol.

9. Line 144-146: Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Ethics statement has been added.

10. Line 149: Please use the standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

Abbreviations have been corrected.

11. Line 206: Please include more details of genomic DNA extraction.

A brief description regarding genomic DNA extraction has been added. Detailed step-to-step instructions depend on the vendor used for the extraction columns.

12. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

One line space between protocol step has been added and highlights for video production have been added.

13. Figure 2: Please define what the numbers (-3, -2, 0, 1, 7, 14) represent (Figure 2A). Please revise "ml" to "mL". Please remove the commercial label "Dyna Beads" (Figure 2A). Please define the terms used in the figure in the Figure Legends (Figure 2B, 2C).

The numbers represent days and have been added to the figure, other details have also been edited.

14. Figure 3: Please mention what the numbers 48.9 (Figure 3A) and 98.7, 1.33 (Figure 3B) represent. Do these numbers indicate the percentage of expression?

Yes, the numbers represent CAR and CD3 expression and has been added in the figure legend.

Reviewer #1:

The manuscript is scientific, interesting and well written

Thank you for the kind words. We are glad you find the manuscript interesting and well written.

Reviewer #2:

Manuscript Summary:

Authors provide the protocols for CRISPR edited CAR T cells. It will be of interest for other researches in the field.

Minor Concerns:

1. In 1.1, Please indicate the total volume of medium in T150 culture vessels

The total volume of medium (30 mL) has been added to step 1.1

2. In 1.2.2 and 1.3, is R10 medium the standard media in 1.1?

Yes, R10 is the standard medium used for most steps in the entire protocol. We apologize if that was not clear. To clarify that, it has now been specified in 1.1 that the standard media is called R10 throughout the manuscript.

3. For 2.2.1, what is the T cell culture medium? Is there IL-2 added in the medium in T cell culture medium?

The T-cell culture medium is also R10. This has now been specified in in step 2.2.1. For this protocol, we only add IL7 and IL15, not IL-2.

4. In 4.2, please describe the detailed design procedures for the sequencing primers. It is important that the readers can understand and follow.

The procedure of designing sequencing primers has been edited to contain more detail. We hope that this will make the design procedure more understandable.

Reviewer #3:

Manuscript Summary:

Wellhausen et al. describe a technique for CRISPR/Cas9 editing of T cells followed by transduction and expansion of the edited CAR-T cells. This is an important methodology as it can be used in the development of allogeneic CAR-T cells with improved characteristics, such as reducing the capacity for graft versus host disease to produce allogeneic CAR-T cells.

Major Concerns:

In general the manuscript seems somewhat incomplete and is lacking in experimental detail throughout. Many of these lacking details are alluded to in minor concerns below. Although each of these individually are quite minor, in the aggregate they are a major concern.

I think it is perfectly acceptable for the scope of this manuscript to illustrate the data with a single donor. However given effect of donor-to-donor variability on the outcomes of every step of the

methodology (editing efficiency, transduction efficiency, expansion capacity, etc.) it would be hugely beneficial to the reader if the authors were to provide a range and average of these outcomes based on their experiences with multiple donors.
I believe that the discussion needs to be rewritten to better summarize the expected outcomes (ie on average, how many edited CART cells can one expect at the end of this process?) and place the methodology into context.

Experimental details have been carefully added in the methodology and results section. Additionally, data from 3 donors has been added to reflect the variability in expansion capacity, transduction efficiency and also editing efficiency. Moreover, editing efficiency for two different targets has been included to show the consistency in the knockout with the described protocol. The discussion has been edited to give an idea of the number of cells that should be expected and also includes other expected outcomes.

Minor Concerns:
Line 55 - Can the authors please clarify what is meant by "poor quality"?
Poor quality here refers to the fact that given patients have been through multiple rounds of chemotherapy and radiotherapy, and also have high tumor burden, they are immunosuppressive and hence the quality of CAR T cells will be better when manufactured with healthy donors, which can be done using CRISPR technology to manufacture off-the-shelf CAR-T cells.
Lines 37-38 in the Abstract the authors mention the demonstration of multiplex editing, however no mention is made in the protocol of how multiple loci could be targeted. Either this should be removed from the abstract or an explanation of how one might multiplex this procedure should be included.
A note at step 2.2.3 has been added to the manuscript to perform multiplex gene editing.
Line 109 - R10 was not defined within the text up to now (assume it is RPMI with FBS etc.).
R10 has now been defined in step 1.1 according to a previous reviewer comment.
Line 111 In step 1.3 - Please include how fast you centrifuge to pellet out cellular debris.
Centrifuge speed has been added. Thank you for pointing this out.
Line 116 - Steps 1.4-1.5. At what temperature is the 24 hour virus pellet stored until pooling with the 48 hour virus?
The 24 h virus pellet should be stored at 4C while pooling the 48 h virus. This has been added to the manuscript.
Line 125 - Step 1.7 needs to be described in more detail or needs to make reference to a paper that describes this step in more detail.
Step 1.7 has been described in more detail according to a previous reviewer comment. We have added the procedure for titering the virus and how to stain for the CAR after transduction. A formula to calculate transducing units/mL has also been added.
Line 168 - It would be useful to have the "Note: For screening sgRNA's, lentiviral transduction of the CAR construct is not necessary" immediately following the title of this section rather than repeating the note throughout the previous steps and subsequent.
The note has been removed from line 168 and added at the beginning of section 3 with reference to the specific step that can be left out for screening gRNA's.
Line 170 - Step 3.1 the authors indicate that a number of dead cells will be observed, an estimate of viability (average and range) based on the authors' experiences with multiple donors would be useful for the reader.
Thank you very much for pointing this out. An estimate and range of viability post-electroporation has been added to step 3.1.
Step 3.2 - a more detailed explanation of the transduction procedure should be provided (spinoculation?

Polybrene or protamine sulfate?, etc.) or reference to a paper with a more detailed protocol should be provided.

The virus pellet resuspended in R10 is simply added on top of the cells according to the cell concentration, viral titer, and desired MOI.

Step 3.3 - I am confused - is 3 mL always added regardless of the cell number/culture volume?

We add 50% of the current culture volume R10 with IL7/15 (i.e. if the culture volume is 6 mL, we add 3mL). This has now been corrected in the manuscript. Thank you for pointing that out.

Step 3.4 and 3.5 - an indication of relative cell numbers based on the authors' experiences at these stages would be useful for the reader (average and range).

Thank you for bringing that to our attention. Relative cell numbers have been added to step 3.4 and 3.5 and expected doubling time has been added.

Step 3.6 - Again, for the CAR expression and TCR KO alluded to in Figure 3, an average of %CAR positive and range across multiple donors would be useful for the reader.

Figure 3A shows %CAR positive for three different healthy donors and using an MOI of 3 CAR expression averages at 60% as shown in the figure. Data from three donors for PD1 and TCR KO has been included in figure 3.

For Steps 4.1, 4.2, etc. - more detailed explanation of TIDE primer design should be included OR the authors should make reference to a published protocol (for example as described in Hultquist JF et al. Nat Protoc. 2019 Jan;14(1):1-27).

A more detailed explanation for both PCR and sequencing primer design in steps 4.1 and 4.2 has been provided and the suggested published protocol has been referenced.

Moreover - the authors bring up in the discussion the difficulties in optimization of primers, sequencing, etc. I believe that this does not belong in the discussion, but instead should be fleshed out more fully in the body of the methods.

Great point. We have mentioned the troubleshooting for each step in the notes section in the body of the methods.

Section 5 appears incomplete - or the sentence contained within it is difficult to understand, so should be reworded

The application of the technique has been described briefly for clarity and the link to the pipeline and publication has been added for reference.

Figure 2A - discrepancy between the figure and the text - the text says 1e6 cells/mL, the figure says 0.8e6 cells/mL.

During expansion, the cells are fed to a concentration of 0.8e6/mL and this has been corrected in Step 3.4.

Figure 2B Legend - more explanatory details please. What does each panel represent, what is UTD, etc.

Details have been added and UTD represents untransduced cells.

Figure 3A Legend - more details needed. How is the CAR detected? How is CD3 detected? Do the plots on the bottom represent CAR+ cells only or all cells? Etc.

The CAR is detected using anti-idiotype antibody conjugated to a fluorophore and CD3 expression is detected using a CD3 antibody conjugated to a fluorophore. The plots for CD3 expression are gated on lymphocytes/Singlets/Live cells. This has been added to the figure legends.

Representative results section lacks detail and a lot of context - For example, what is the T7E1 assay? (was never mentioned until this point). Why is CD3 detection used to detect TRAC knockout?, etc.

T7E1 Assay is similar to surveyor assay where a nuclease cleaves at a mismatch between the parent and the daughter strand in the dsDNA and hence allows detection and quantification of frequency of the indels in target of interest. This has been removed to avoid any confusion since we do not show any data related to the assay.

TCRa and TCR b chains pair together to be expressed on the surface. While the gRNA targets only the TCRa chain but its knockout disables TCR pairing on the surface. Hence, CD3 antibody can be used for detection.

Reviewer #4:

Manuscript Summary:

This scientific article written by Wellhausen et al. proposes a clear methodology for CAR-T cell improvement using CRISPR/Cas9 technology. The protocol is detailed and provides good information to reproduce it as well as valuable advice to design a valid strategy for CRISPR/CAR T-cell manufacturing. The schemes are clear and self-explicative. However, more effort should have been made in showing suitable data supporting the efficiency of the proposed methodology. Several important issues (see major comments) have to be amended to accurately justify the effectiveness of the protocol and therefore my general recommendation would be to thoroughly improve the manuscript before publication.

Major Concerns:

1. The following experimental order is proposed in Figure 2A: first nucleofection (CRISPR editing), next CD3/CD28 stimulation, and then CAR transduction. In some references mentioned in the text, such as Ren et al, 2017 (PMID: 27815355), the protocol starts with CD3/CD28 stimulation, then CAR transduction and finally CRISPR editing. The authors should discuss the different chronological sequences. Why to do first CRISPR editing and afterwards CAR transduction or the other way around?
There are different variation to the protocol wherein CRISPR editing could be performed before or after CAR transduction. Ren et al described one such variation where cells are edited after bead stimulation and CAR transduction. The advantage of CRISPR editing before bead stimulation is lower cell quantities need to be edited since the T cells haven't proliferated yet, making the procedure more cost-efficient. Additionally, performing the editing upfront is directly translatable to the clinic. In fact, many steps in this protocol have been informed by what can be adopted in the clinic which increases the consistency of the KO efficiency when moving from bench to bedside. This has been added to the discussion.

2. More data supporting the high efficiency of the proposed protocol has to be provided. Flow cytometry data is available for TRAC knockout but no replicates are shown. Moreover, the 6-10 different in silico designed sgRNAs (section 2.1.2) are not shown nor compared in terms of efficiency or specificity (a similar structure to Kararoudi et al., 2018, PMID: 29985369 can be followed)
The data from multiple donors for TRAC knockout has been added to Figure 3B and another target PD-1 has also been added to Figure 3A. PD-1 and CD3 lead sgRNA were obtained from Ren et al, 2017 where they screened for sgRNA with highest efficiency.

3. It is mentioned in the introduction that the proposed protocol leads to low off-target effects (line 76). There is no data or any reference supporting this; moreover, the off-target effects will vary from one sgRNA to another and from one donor to another donor, even when using the same protocol or the same sgRNA.
We edited line 76 since the off-target effects can vary from sgRNA to another as you mention.
The off-target effects for the sgRNA targeting the TRAC locus have been shown in Stadtmauer et al, 2020. The off-target effects vary from one sgRNA to another but are investigated in detail for sgRNA showing highest on-target effects. The off-target effects can be significantly reduced by using the Aldeveron Spy-fi Cas9 which is what was used here and is known to show high fidelity. The reference has been added to the paper.

4. In the abstract, it is mentioned that strategies to achieve highly efficient multiplex editing will be discussed (line 37), which are actually not shown nor discussed in this article.
Protocol to perform multiplex editing has been added to step 2.2.3 and has been shown in Stadtmauer et al, 2020.
5. In Figure 2B/C, it has to be specified which gene is being targeted in the analysis (TRAC?). Moreover, it is needed to specify if the replicates presented in the graphs are biological (different donors) or technical replicates (same donor). Since a "general" protocol for the production of CRISPR/CAR T-cells is proposed by the authors, biological replicates have to be presented to see the variation between different donors.
In Figure 2, PD-1 KO is being performed. Each data point is a different donor and hence the error bars depict the variation between different donors. This has been added to the figure legends. Thank you for pointing this out.
6. Fig. 2 B/C: how were population doublings and cell size determined? Why is "cell size" data needed for the manufacturing?
Both cell numbers and size were determined using Coulter Counter. Cell size is important to keep track if the cells were properly activated during CD3/28 beads stimulation and also to track when they have rested down i.e. cell size below 350um^3 to complete the expansion and freeze the product for future use.
7. In Figure 3A, a flow chart showing T cells with no CAR transduction (control) could be presented as done in Fig. 3B. Moreover, it has to be mentioned which gene is targeted in 3A (graph on the right).
The graph for control of CAR transduction has been added to Figure 3A.
8. In Figure 3B, a graph summarizing the efficiency of CRISPR knock-outs should be presented, similarly as shown in 3A with the percentages of CAR expression.
This figure has been added to show CD3 KO efficiency in different donors.
9. Line 57: there are other interesting reviews summarizing CRISPR improvements for CAR T-cell production (Ureña-Bailén et al., 2019, PMID: 31844895 and Chenggong et al. 2020, PMID: 31950135) that can be included as additional references.
References have been added.

Minor Concerns:
1. CD4 and CD8 selection kits are from StemCell Technologies (table) whereas in the text (line 147) Miltenyi was indicated.
Thank you for pointing out the discrepancy. We removed the brand name from the text. We have used both StemCell technologies and Miltenyi.
2. Line 116: a longer centrifugation time and higher speed of ultracentrifugation increases the yield of lentiviral particles.
We perform ultracentrifugation at 25,000 x g for 2.5 h or 8000 x g overnight (O/N).
3. In line 150, please add a reference supporting the promotion of T-cells with central memory phenotype.
References have been added.
4. In line 156, the amounts of Cas9 and sgRNA can be expressed as a molecular or molar ratio.
The molar ratio of Cas9 to sgRNA is 1:2.4. This has been added to the manuscript.
5. In the part of monitoring off-targets, in line 239, the sentence is incomplete.
This has been edited and more details have been added.
6. In line 297, Aldevron in capital letter.
Commercial language has been removed from manuscript.
7. In line 301, Neon system (ThermoFisher) can be added to the discussion as it is also extensively

employed in small-scale CRISPR experiments. Similarly, CliniMACS Prodigy (Miltenyi Biotec) can be added in line 306 as it offers GMP-compatible large-scale productions for clinical applications.
Great points, but according to the editors commercial language should not be used in the manuscript and has been removed from elsewhere as well.
8. In the legend of Figure 1 and Figure 2A, it's needed to mention "CAR".
Thank you. This has been added to the figure legends.
9. In Figure 1, there is a spelling mistake in "cryopreservation".
This has been edited, thank you for pointing that out.
10. In Figure 2B/C legend, the abbreviations in the graph have to be specified for a better understanding, e.g. UTD (untransduced?)
Thank you. Abbreviations have now been defined in figure legends.
11. The antibody for CD19 CAR detection and CD3 flow cytometry analysis should be specified in the table summarizing materials.
These details have been added to the table.
12. The table of materials has one empty column ("comments (clone?)") without giving additional information.
This has been removed from the table of materials. It was a remnant from the template provided by the journal.

We hope that the revised manuscript sufficiently addresses the concerns raised by the editors and reviewers .

Best,