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Clinical Cancer Research

Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition

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Abstract

Purpose: Using gene-disrupted allogeneic T cells as universal effector cells provides an alternative and potentially improves current chimeric antigen receptor (CAR) T-cell therapy against cancers and infectious diseases.

Experimental Design: The CRISPR/Cas9 system has recently emerged as a simple and efficient way for multiplex genome engineering. By combining lentiviral delivery of CAR and electro-transfer of Cas9 mRNA and gRNAs targeting endogenous TCR, β-2 microglobulin (B2M) and PD1 simultaneously, to generate gene-disrupted allogeneic CAR T cells deficient of TCR, HLA class I molecule and PD1.

Results: The CRISPR gene–edited CAR T cells showed potent antitumor activities, both *in vitro* and in animal models and were as potent as non-gene–edited CAR T cells. In addition, the TCR and HLA class I double deficient T cells had reduced alloreactivity and did not cause graft-versus-host disease. Finally, simultaneous triple genome editing by adding the disruption of PD1 led to enhanced *in vivo* antitumor activity of the gene-disrupted CAR T cells.

Conclusions: Gene-disrupted allogeneic CAR and TCR T cells could provide an alternative as a universal donor to autologous T cells, which carry difficulties and high production costs. Gene-disrupted CAR and TCR T cells with disabled checkpoint molecules may be potent effector cells against cancers and infectious diseases. *Clin Cancer Res; 1–12. ©2016 AACR.*

Introduction

Engineered T-cell receptor (TCR) and CAR T (CART) cell treatments of patients with cancer have shown promising results (1–6). The majority of current TCR and CAR T clinical trials utilize autologous T cells and might therefore be hampered by the poor quality and quantity of T cells as well as the time and expense of manufacturing autologous T cell products. These limitations would be circumvented by the use of allogeneic T cells. However, the endogenous TCR on allogeneic T cells may recognize the alloantigens of the recipient, leading to graft-versus-host disease (GVHD); furthermore, the expression of HLA on the surface of allogeneic T cells causes rapid rejection by the host immune system. Therefore, simple and efficient methods are needed for multiplex genomic editing of T cells.

The CRISPR/Cas9 system has recently emerged as a potentially robust alternative for inducing targeted genetic alterations and as a process for multiplex genome engineering (7–10). In the current study, by using CRISPR/Cas9 system to simultaneously disrupt multiple genomic loci, we have generated CAR T cells deficient in the expression of endogenous TCR and HLA class I (HLA-I) that can be used as gene-disrupted allogeneic CAR T and further developed as universal CAR T cells. We found that *TCR* and *B2M* genes could be disrupted with high efficiency through the co-introduction of mRNA encoding the Cas9 with gRNAs targeting these genes by RNA electroporation. We generated gene-disrupted allogeneic CART cells by combining the lentiviral (LV) delivery of CAR and CRISPR RNA electroporation to disrupt endogenous TCR and B2M genes simultaneously. In addition, we demonstrate that disruption of endogenous PD1 enhances the efficacy of gene-disrupted allogeneic CAR therapy in tumor models.

Materials and Methods

Primary human lymphocytes

Primary human CD4 and CD8 T cells were isolated from healthy volunteer donors following leukapheresis by negative selection using RosetteSep Kits (Stem Cell Technologies). All specimens were collected under a University Institutional Review Board–approved protocol, and written informed consent was obtained from each donor. Primary lymphocytes were stimulated with anti-CD3/CD28 Dynabeads (Life Technologies). T cells were cryopreserved at day 10 in a solution of 90% FCS and 10% dimethylsulfoxide (DMSO) at 1×10^8 cells per vial.

Generation of TCR or CAR constructs for mRNA electroporation and lentiviral transduction

CARs (PSCA or CD19) were synthesized and/or amplified by PCR based on sequencing information provided by the relevant publications (11–13) and subcloned into a pGEM.64A RNA–based vector or pTRPE lentiviral vectors.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Engineered CAR T cell treatments of patients with cancer have shown promising clinical results. The majority of current clinical trials utilizes autologous engineered T cells and might therefore be hampered by the poor quality and quantity of T cells as well as the time and expense of CAR T cell manufacturing. These limitations could be circumvented by the use of allogeneic T cells. The CRISPR/Cas9 system has recently emerged as a unique robust tool for multiplex genome engineering. By combining the lentiviral delivery of CAR and CRISPR RNA electroporation to cointroduce RNA encoding the Cas9 and gRNAs targeting endogenous TCR and β-2 microglobulin (B2M) simultaneously, we have developed a clinically scalable technology of generating universal CAR T cells that showed potent anti-tumor activities, both *in vitro* and in animal models and were as potent as non-gene–edited CAR T cells, which could be potentially translated into the clinic as an alternative for cancer adoptive immunotherapy.

Design and construction of CRISPRs

Cas9 and eSpCas9(1.1) DNA was synthesized by PCR based on Dr. Zhang Feng's publications (7, 14), then inserted to PGEM vector. gRNAs were selected by GN19 with an NGG PAM site, and some were selected from N20 with a NGG PAM site. gRNAs containing more than 13 base pairs of complementary sequences to non-targeting sites are excluded to minimize off-target events. gRNAs were designed, as shown in Supplementary Fig. S1A, and synthesized by overlap PCR. All gRNA PCR products were ligated into the MSGV vector. The Cas9 mRNA and gRNAs targeting constant regions of TCR α and β chains, β2M and PD1 were *in vitro* transcribed. gRNAs were designed to target either a sequence within exon 1 of the TCR α constant region, a consensus sequence common to exon 1 of both TCR β constant regions 1 and 2, exon 1 of β-2 microglobulin or PD1. Sequences encoding the gRNAs were assembled using overlap PCR and cloned into the MSGV vector containing a T7 promoter. These plasmids were linearized with *Eco*RI before conducting RNA *in vitro* transcription (IVT). The IVT RNA was stored at −80°C in nuclease-free vials for single use. The following gRNA targeting sequences were used in the study: TRAC-gRNA-1: AGAGTCTCTCAGCTGGTACA; TRAC-gRNA-2: TGTGCTAGACATGAGGTCTA; TRBC-gRNA-1: GCAGTATCTGGAGTCATTGA; TRBC-gRNA-2: GGAGAATGACGAGTGGACCC; B2M-gRNA: CGCGAGCACAGCTAAGGCCA; and PD1-gRNA: GGCCAGGATGGTTCTTAGGT.

Flow cytometry

The following mAbs and reagents were used with the indicated specificity and the appropriate isotype controls. From BD Biosciences: APC-conjugated anti-CD3 (555335), FITC-anti-CD8 (555366), PE-anti-CD8 (555635), PE-anti-CD28 (561793), PE-anti-CD107a (555801), and PE-anti-β-2 microglobulin (551337), FITC-anti-HLA-I (555552), APC-anti-CD137 (550890). From Biolegend: APC-anti-PD1 (114102), APC-anti-PDL1 (329702), FITC-anti-CD45RO (304204), APC-anti-CD62L (304814). From Beckman Coulter: PE-anti-Vb13.1 (IM2021U). Data were acquired on a FACS Accuri (BD Biosciences) using CellQuest version 3.3 (BD Biosciences) and analyzed by FCS Express version 3.00 (De Novo Software) or FlowJo version 7.6.1 (Tree Star, Inc.).

Generation of CD3$^-$ T cells

gRNA was *in vitro* transcribed by a T7 mScript Standard mRNA Production System (Cambio, C-MSC100625). Cas9, TCR α, and TCR β mRNA was *in vitro* transcribed using mMESSAGE mMACHINE T7 ULTRA kits (Life Technologies, AM1345). T cells were stimulated by CD3/CD28 Dynabeads for three days prior to RNA electroporation. T cells were electroporated as described previously (15, 16). Briefly, T cells were washed three times with OPTI-MEM and resuspended in OPTI-MEM (Invitrogen) at a final concentration of 1–3 × 10^8 cells/mL. Subsequently, 0.1 mL of the cells was mixed with IVT RNA and electroporated in a 2-mm cuvette. Twenty micrograms of Cas9 mRNA and 10 μg of gRNA were electroporated into the cells using a BTX830 (Harvard Apparatus BTX) at 360 V and 1 ms; this process was followed by a second electrotransfer of 5 μg of gRNA 12 to 24 hours later. Following electroporation, the cells were immediately placed in prewarmed culture media and cultured in the presence of IL2 (100 IU/mL) at 37°C and 5% CO_2.

Enrichment of CD3$^-$ T cells

Cells washed with Auto MACS buffer were incubated for 30 minutes with CD3 microbeads (Miltenyi Biotec, 130-050-101) at 4°C. After being washed twice, the cells were passed through an LD column (Miltenyi Biotec), and the flow-through fraction was collected for further use.

Proliferation capability of CD3$^-$ T-cell test

CD3$^-$ T cells were electroporated with a total of 15 μg NY-ESO-1 TCR (1G4) α and β chain mRNA (7.5 μg each), using a BTX830 (Harvard Apparatus BTX) at 500 V and 700 μs. CD3 expression was measured 24 hours later and then stimulated with CD3/CD28 Dynabeads. Proliferation was monitored every 2 to 3 days.

TCR and B2M double disruption or TCR, B2M, and PD1 triple disruption

To generate TCR, B2M double and TCR, B2M, PD1 triple knockout T cells, Cas9 mRNA was coelectroporated with different gRNAs targeting TRBC, B2M or TRBC, B2M, PD1. The Cas9 mRNA and gRNA delivery procedure was the same as generation of CD3$^-$ T cells. The total amount gRNAs of the first electroporation was half of the Cas9 mRNA, the total amount gRNAs of the second electroporation was one fourth of the Cas9 mRNA. The TCR and HLA-I double-negative cell population was sorted on day 9 to obtain gene-disrupted T cells. TCR and HLA-I molecule expression was confirmed at each step.

Generation of gene-disrupted and PD1-deficient CAR T cells

Gene-disrupted CAR T cells were generated by combing the lentiviral transduction of CD19 or PSCA CAR with the RNA electroporation of CRISPR/gRNAs. One day after anti-CD3/CD28 beads stimulation, T cells were transduced with lentiviral-CD19 or PSCA CAR. Two days later, Cas9 and gRNAs targeting TRBC, B2M or TRBC, B2M, PD1 were transferred into T cells by electroporation. On day 9 after stimulation, T cells negative for CD3, HLA-I were sorted by microbeads depletion.

Measuring allele modification frequencies using T7E1 assay, TIDE, and sequencing of PCR fragments

The level of genomic disruption of TRAC, TRBC1, and TRBC2 in T cells was determined by a T7E1 Nuclease assay (NEB). The

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percent target disruption was quantified by densitometry. PCR products were ligated to TOPO cloning vector (Invitrogen) then transformed in *E. coli*. Single clone was picked and sequenced to calculate the indels and insertions. PD1 disruption was confirmed by Sanger sequencing. The PCR primers used for the amplification of the target locus were as follows: TRAC forward, 5′-TCATGTCCTAACCCTGATCCTCTT-3′; TRAC reverse, 5′-TTGGACTTTTCCCAGCTGACAGA-3′; TRBC total forward, 5′- TACCAGGACCAGACAGCTCTTAGA-3′; TRBC total reverse, 5′- TCTCACCTAATCTCCTCCAGGCAT-3′; PD1 forward, 5′-GTAATAAAATGCTCAGCACAGAATA-3′; PD1 reverse, 5′-GAGAAAAATATCACCAGCTCATCT-3′. For analyzing allele modification frequencies using TIDE (Tracking of Indels by Decomposition; ref. 17), the purified PCR products were Sanger-sequenced using both PCR primers and each sequence chromatogram was analyzed with the online TIDE software available at http://tide.nki.nl. Analyses were performed using a reference sequence from a Cas9 mock-transfected sample. Parameters were set to the default maximum indel size of 10 nucleotides and the decomposition window to cover the largest possible window with high quality traces. All TIDE analyses below the detection sensitivity of 1.5% were set to 0%. Primers used for TIDE off-target measurement are listed in Supplementary Table S1.

ELISA assays

Target cells were washed and suspended at 1×10^6 cells/mL in R10 medium. Next, 100 μL of each target cell type was added in triplicate to a 96-well round-bottom plate (Corning). Effector T cells were washed and resuspended at 1×10^6 cells/mL in R10 medium, and then 100 μL of T cells was combined with the target cells in the indicated wells. The plates were incubated at 37°C for 18 to 24 hours. After the incubation, the supernatant was harvested and subjected to an ELISA (eBioscience).

IFNγ ELISpot

CRISPR-edited T cells were plated in ELISpot plates (R&D Systems) at the concentration of 2×10^4 cells per well with irradiated allogenic PBMCs. Another experiment was performed by co-culturing of allogenic PBMCs with irradiated CRISPR-edited T cells. Cells were incubated for 18 hours at a stimulator-to-responder ratio of 1:1. Experiments were performed according to the manufacturer's instructions. The spots were automatically quantified using an ELISpot plate reader for scanning and analyzing.

In vivo reactivity of allogeneic T cells against HLA-I$^-$ T cells

A total of 1×10^7 TCR$^-$ or TCR/HLA-I$^-$ T cells and 2×10^6 allogeneic effector T cells were mixed and infused into NSG mice (i.v.). The presence of CD45$^+$CD3$^+$ allogeneic T cells and CD45$^+$ CD3$^-$ gene-edited T cells were measured by Trucount assay at day 2, day 9, and day 16 after T-cell infusion.

CD107a staining

Cells were plated at an E:T of 1:1 (1×10^5 effectors: 1×10^5 targets) in 160 μL of R10 medium in a 96-well plate. Next, 20 μL of phycoerythrin-labeled anti-CD107a Ab was added, and the plate was incubated at 37°C for 1 hour before the addition of Golgi Stop (2 μL of Golgi Stop in 3 mL of R10 medium, 20 μL/well; BD Biosciences, 51–2092KZ) and incubation for another 2.5 hours. Then, 5 μL of FITC-anti-CD8 and 5 μL of APC-anti-CD3 were added for incubation at 37°C for 30 minutes. After the incubation, the samples were washed with FACS buffer and analyzed by flow cytometry.

Luciferase-based CTL assay

Nalm6-CBG tumor cells were generated and employed in a modified version of a luciferase-based CTL assay (18). Briefly, click beetle green luciferase (CBG)-T2A-eGFP was lentivirally transduced into Nalm6 tumor cells and sorted for GFP expression. The resulting Nalm6-CBG cells were resuspended at 1×10^5 cells/mL in R10 medium and incubated with different ratios of T cells (e.g., 30:1, 15:1, etc.) overnight at 37°C. Then, 100 μL of the mixture was transferred to a 96-well white luminometer plate. Next, 100 μL of substrate was added, and the luminescence was immediately determined. The results are reported as percent killing based on the luciferase activity in the wells with tumor cells, but no T cells [% killing = 100 − ((RLU from well with effector and target cell coculture)/(RLU from well with target cells) × 100)].

Mouse xenograft studies

Studies were performed as previously described with certain modifications (19, 20). Briefly, for the Nalm6 tumor model, 6- to 10-week-old NSG mice were injected with 1×10^6 Nalm6 or Nalm6-PDL1 tumors cells through the tail vein on day 0. The T-cell treatment began on day 7 after the tumor inoculation. For the PC3-PDL1 solid tumor model, 6- to 10-week-old NOD/SCID gamma (NSG) mice were injected subcutaneously with 1×10^6 PC3-PDL1-CBG or PC3-CBG tumors cells in the right flank on day 0. The mice were treated with T cells via the tail vein at day 22 post PC3-PDL1-CBG tumor inoculation, when the tumors were approximately 200 mm^3 in volume. T cells were given at 2×10^6 cells/mouse.

GVHD studies

For the *in vivo* GVHD studies, we resuspended the cells in FBS and infused them intravenously into the mice after sublethal irradiation (175 cGy). We monitored the mice for clinical GVHD 2 to 3 times per week. The following signs were included in the clinical index: weight loss, hunching, activity, fur texture, and skin integrity. We euthanized moribund mice for ethical reasons.

Results

Multiple deliveries of gRNAs disrupt genes in human primary T cells with high efficiency without impairing effector function

Efficient multiplex genomic editing is required to generate gene-disrupted T cells that are deficient in TCR, HLA, and other genes. We optimized CRISPR/gRNA RNA electroporation to achieve high gene disruption efficiency in T cells. First, Cas9 and gRNAs were generated using an *in vitro* transcription system (Supplementary Fig. S1A), and a "hit-and-run" delivery strategy was developed to transiently deliver the Cas9 mRNA and gRNAs to T cells by electroporation (Supplementary Fig. S1B). An initial experiment targeting the TCR α constant region (TRAC) or β constant region (TRBC) with single electroporation resulted in 7.51% and 12% CD3$^-$ (CD3^{neg}) T cells, respectively (Fig. 1A and Supplementary Fig. S2A).The optimal molecular ratio of Cas9:gRNA for maximum disruption efficiency was 1:1 to 2:1, and the gene disruption efficiency was correlated with the amount of electrotransferred RNA (Supplementary Fig. S2A).

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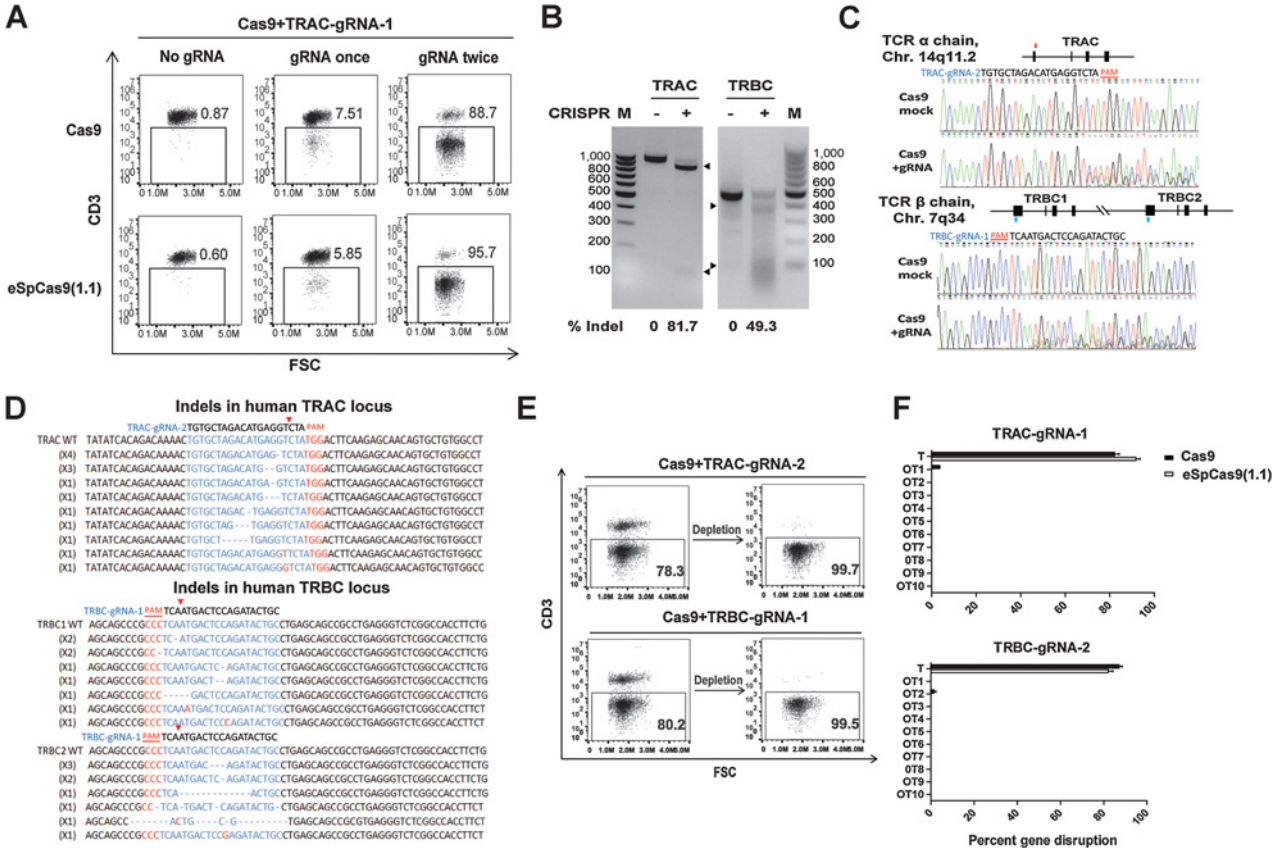

Figure 1.

CRISPR/Cas9 mediates efficient TCR disruption in T cells. **A,** CD3 expression of T cells after sequential CRISPR RNA electroporation with Cas9 and eSpCas9(1.1) (healthy donors, $n = 5$). **B,** Amount of TCR-targeted gene disruption measured by a mismatch-selective T7E1 nuclease assay on DNA amplified from the cells shown. The calculated amount of targeted gene disruption in TRAC and TRBC is shown at the bottom. Arrows indicate expected bands. **C,** A diagram of the human locus encoding the TCR α and β CRISPR gRNA targeting sites within the genomic locus of the TCR α and β constant region. Multiple peaks in the Sanger sequencing results show the CRISPR-mediated events of NHEJ at the TRAC and TRBC genomic loci. Each exon is shown by a block. Red arrow, sense strand gRNA-targeting site; blue arrow, antisense strand gRNA–targeting site. **D,** Indels and insertions observed by clonal sequence analysis of PCR amplicons after CRISPR-mediated recombination of the TCR α and β locus. Red arrow indicates putative cleavage site. **E,** CD3 expression on purified TCR$^-$ population ($n = 3$). **F,** Off-target mutagenesis measurement of TRAC and TRBC. Indel frequencies were measured by TIDE analysis ($n = 3$). All TIDE analyses below the detection sensitivity of 1.5% were set to 0%. Bars, SE, $n = 3$; T, target; OT, off-target.

Compared with mRNA, gRNAs are more prone to rapid degradation, which potentially limits the targeting efficiency. Thus, sequential electroporations of gRNA were tested after the initial Cas9/gRNA electroporation. There was a marked increase in disruption frequency at the protein level, as 88.7% (85.7 ± 6.7, $n = 5$) of cells were CD3$^-$ after the second gRNA electroporation targeting TRAC (Fig. 1A). To improve gene-targeting specificity, high-fidelity Cas9 mutant eSpCas9(1.1) was tested. As high as 95.7% (93.6% ± 6.2, $n = 3$) CD3 disruption was achieved after a second delivery of gRNA. Clonal sequencing showed that the genomic targeting efficiency reached 89.4% (Supplementary Fig. S2B). A surveyor assay confirmed a cleavage rate of 81.7% and 49.3% at the genomic loci of TRAC and TRBC, respectively (Fig. 1B). Multiple peaks in the Sanger sequencing data flanking the TRAC and TRBC target sites confirmed that the genomic reading frame shifted downstream of the target sites (Fig. 1C). The occurrence of insertions or deletions (indels) caused by NHEJ repair was confirmed by clonal sequencing (Fig. 1D). The TCR-disrupted TCR/CD3$^-$ population was enriched to over 99% (99.70 ± 0.20%) by a single step of CD3$^-$ selection (Fig. 1E). The CRISPR-treated T cells could be expanded 12-folds (12 ± 1.1, $n = 3$), which is one thirds of the non-genome edited T cells (40 ± 2.6) in a standard 9 days T expansion period due to the toxicity of the second electroporation. Around 8-folds (8.4 ± 0.7, $n = 3$) TCR/CD3$^-$ T cells can be obtained from the starting T cells (Supplementary Fig. S3A). Recent studies showed a low incidence of off-target mutagenesis in T cells using lentivirus and adenovirus-delivered CRISPR/Cas9 to knock out CCR5 (21, 22). Another report showed no detectable off-target mutations in the CXCR4-knockout CD4 T cells (23). Consistent with these studies, we observed very rare off-target events when targeting TRAC and TRBC with Cas9 and no detectable of off-target mutagenesis with eSpCas9(1.1), indicating CRISPR/Cas9 gene editing is more specific in T cells than other cell types (Fig. 1F).

To test whether CRISPR/Cas9 gene editing would affect the proliferating capability of the T cells, CD3 expression was restored by electroporation of 1G4 TCR mRNA into the TCR/CD3$^-$ T cells, and then simulated with CD3/CD28 dynabeads. No expansion

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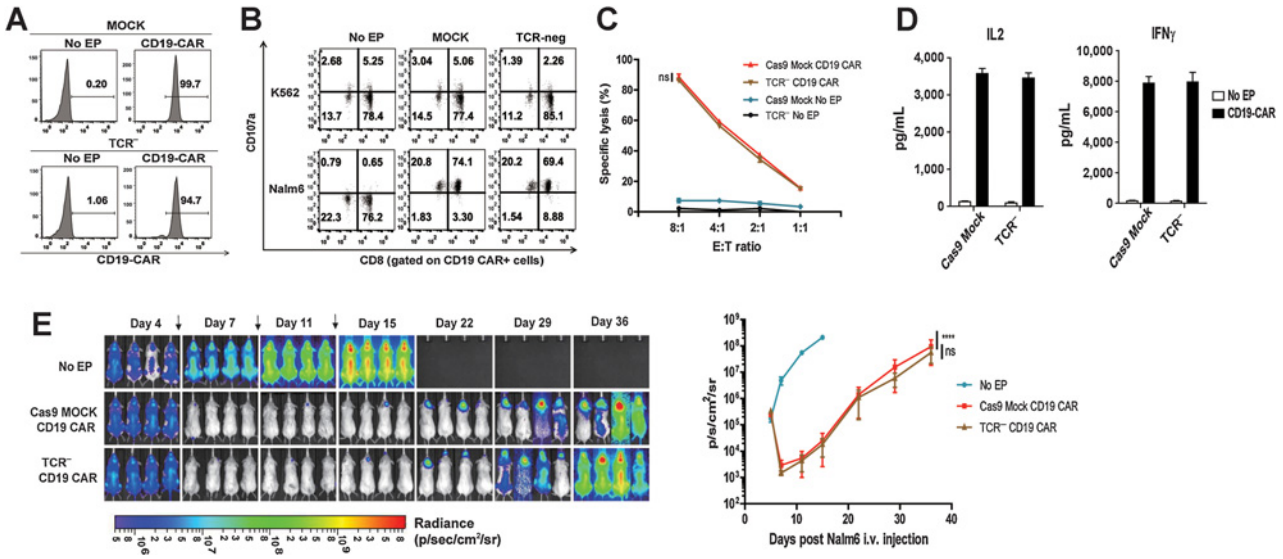

Figure 2.

CRISPR/Cas9 editing does not impair antitumor efficacy of primary T cells. **A,** Relative CD19-CAR expression after the electrotransfer of CD19-CAR RNA into Cas9 MOCK and TCR/CD3$^-$ cells. No significant functional difference was observed between CD19-CAR–redirected Cas9 MOCK and TCR/CD3$^-$ cells as confirmed by CD107 release assay (**B**), (cytotoxicity assay; **C**), and IL2 and IFNγ secretion (**D**) when incubated with the Nalm6 target cell line. Representative data from three independent experiments are shown. Bars, SE. **E,** NSG mice ($n = 12$) were injected with 1×10^6 Nalm6 tumor cells (i.v.), and the mice were randomly sorted into three groups. Cas9 MOCK and TCR/CD3$^-$ T cells (1×10^7) expressing the CD19-CAR after electroporation were injected intravenously every 4 days for a total of three injections (arrows); mice treated with no RNA-electroporated T cells from the same donor served as the control. Images were obtained from the surviving animals as indicated. Imaging commenced 1 day before the start of T-cell treatment. Bars, SE; E:T, effector-to-tumor ratio; arrow, time point of T-cell infusion; ns, not significant. ****, $P < 0.001$, ns, by comparison of the slopes with linear regression.

difference was observed between wild-type and TCR-restored CD3$^-$ T cells (Supplementary Fig. S3).

To test whether CRISPR/Cas9 gene editing would affect the effector function of the T cells, the anti-tumor activity was tested after electroporation of CD19 CAR mRNA into TCR/CD3$^-$ T cells. The surface CAR expression of the TCR/CD3$^-$ T cells was equal to that of the control group (Fig. 2A). When the TCR/CD3$^-$ CD19-CART cells were stimulated with CD19$^+$ Nalm6 leukemia cells, the CD107a upregulation, cytokine secretion and killing activity of CD19-CAR TCR/CD3- T cells was equivalent to those of the wild-type control cells (Fig. 2B–D). The CD19-CAR TCR/CD3$^-$ T cells were infused into Nalm6-bearing NOD/scid/γc(−/−) mice (NSG) mice to test their *in vivo* anti-tumor activity. Tumor regression was evident with an efficacy equivalent to that for the CD19-CAR wild-type counterpart cells that were produced using tissue culture conditions used in ongoing clinical trials (Fig. 2E). The results indicate that CRISPR/Cas9 editing of the endogenous TCR does not adversely affect the function of primary T cells for adoptive immunotherapy.

Reduced alloreactivity of TCR and B2M double-disrupted T cells

As disrupting either TCR α or β is sufficient to ablate TCR/CD3 expression and B2M is essential for the assembly and expression of HLA-I complex (24), TCR and B2M double disruption was developed to generate gene-disrupted T cells. First, the ability to eliminate HLA-I expression on the T cells by disrupting B2M was tested. T cells were electroporated with B2M-targeting Cas9/gRNA RNA; this process resulted in a B2M and HLA-I double-negative population of 79.9%. The HLA-I$^-$ population could be further enriched by negative selection (Fig. 3A). To generate double-knockout T cells lacking the TCR and B2M, Cas9 mRNA was co-electroporated with two different gRNAs targeting TRBC and B2M. As a result, the CD3 and HLA-I double-negative cell population was 65.4% (Fig. 3B). After enrichment of the double knockout cells, we found that the TCR and B2M double-knockout T cells abrogated the allogeneic killing of HLA-unmatched tumor cell lines (Supplementary Fig. S4A). We did not observe any response when these cells were challenged by allogeneic whole-blood irradiated PBMCs in an IFNγ ELISpot assay (Fig. 3C, top). The ablation of HLA-I molecules also sharply reduced the alloreactivity, as confirmed by coculture of allogenic PBMCs with irradiated B2M-disrupted cells (Fig. 3C, bottom). Although the ability of HLA-I$^-$ T cells to stimulate an allogenic PBMC response caused by T cell was markedly reduced, it was not completely diminished, probably due to the activation of NK cells within the PBMCs, which was supported by the finding that allogeneic T cell activation was completely abrogated as long as the HLA-I of stimulating gene-disrupted T cell was ablated when the purified CD4 and CD8 T cells, instead of PBMCs, were used as allogeneic effectors (Supplementary Fig. S4C). It was further confirmed that B2M-disrupted, HLA-I$^-$ T cells had reduced target recognition by co-injecting TCR$^-$ or TCR, HLA-I double deficient (TCR/HLA-I$^-$) T cells with allogeneic effector T cells into NSG mice. As shown in Fig. 3F, significantly reduced number of allogeneic effector T cells (CD45$^+$ CD3$^+$) was found when TCR/HLA-I$^-$ T cells were coinfused (Effector+ TCR/HLA-I$^-$), compared with coinfusing of TCR$^-$ (Effector+ TCR$^-$)T cells. However, without disruption of HLA-I, the TCR single gene-edited T cells were eliminated when co-infused with allogeneic T cells, while TCR and HLA-I double gene-disrupted T cells remain unchanged, suggesting the rejection of the B2M-disrupted T cells by the allogeneic effector T cells was reduced. T cells express high levels of HLA class II after being

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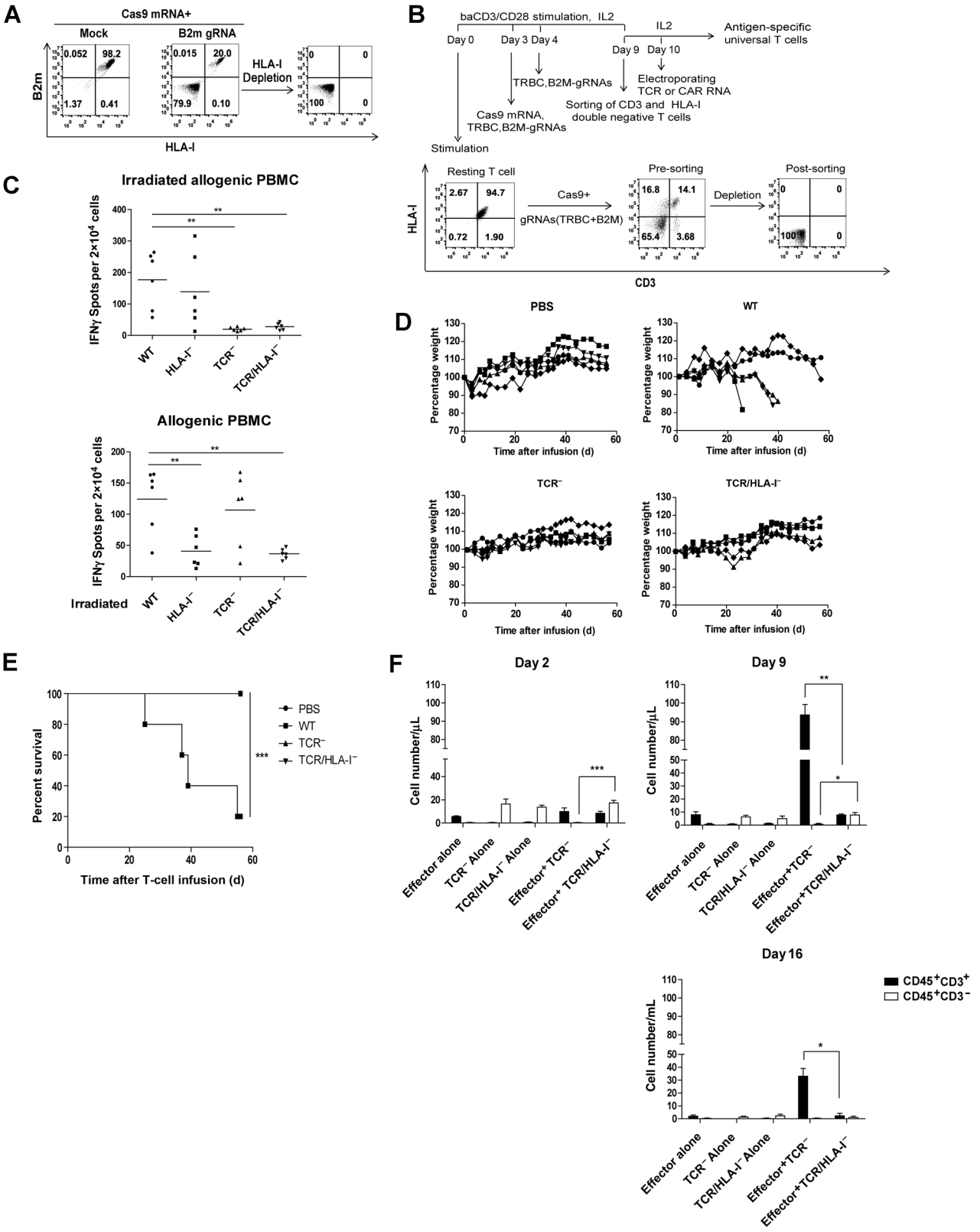

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activated, which could potentially lead to the accelerated rejection of infused allogeneic T cells. Although high levels of HLA class II expression on TCR/HLA-I$^-$ T cells could be by detected after activation (Supplementary Fig. S4B), we were unable to detect primed CD4 T cell activation (Supplementary Fig. S4C), suggesting this *in vitro* assay was not sensitive enough to detect CD4 alloreactivity, even for dendritic cell–primed CD4 T cells. To confirm that the TCR and HLA-I editing process abrogates the GVHD reactivity of the gene-modified cells *in vivo*, we infused TCR ablated, TCR/HLA-I double ablated or non-manipulated cells into 8- to 10-week-old NSG mice that had been conditioned with 175 cGy irradiation. Four of the 5 mice infused with non-manipulated lymphocytes developed lethal xenogeneic graft-versus-host disease (GVHD) within 2 months after the infusion. GVHD was also evidenced by elevated number of CD8$^+$ cells infiltrating in different organs, as well as elevated CD45$^+$ cell counts in the peripheral blood. (Supplementary Fig. S5A–S5C), whereas none of the mice receiving TCR single or TCR/HLA-I double ablated cells ($n = 5$, per group) developed GVHD (Fig. 3D and E).

Gene-disrupted CAR T cells retain antitumor efficacy

Gene-disrupted CD19 CAR T cells were generated by combing CD19 CAR lentiviral transduction with the RNA electroporation of Cas9/gRNAs (Fig. 4A). The cells were then sorted, and were negative for CD3 with high levels of CD19 CAR expression (Fig. 4B). The majority of the gene-disrupted CD19 CAR T cells expressed high level of CD62L and a medium level of CD28, consistent with central memory status for the major T-cell population (Fig. 4C). The TCR/HLA-I double-negative CD19 CAR T showed robust *in vitro* antitumor activities, such as lytic capacity, cytokine secretion, and proliferation, which were as potent as those of the wild-type CD19 CAR T cells (Fig. 4D–G). The T cells were infused into NSG mice bearing disseminated Nalm6 leukemia. Mice treated with CAR T cells with a disrupted endogenous TCR (LV-CD19 CAR TCR$^-$) or with a simultaneous disruption of TCR and HLA-I (LV-CD19 CAR TCR/HLA-I$^-$) exhibited similar survival rates to that of mice treated with wild-type CD19 CAR T cells (LV-CD19 CAR; Fig. 4H). The numbers of human T cells in the peripheral blood of the Nalm6-bearing mice treated with TCR$^-$ or TCR/HLA-I$^-$ were comparable with mice treated with wild-type CD19 CAR T cells (Fig. 4I), suggesting that the disruption of TCR alone or together with B2M does not affect CAR T-cell engraftment, *in vivo* proliferation, and antitumor activity.

Disruption of PD1 in CAR T cells leads to enhanced antitumor efficacy

Given the robust anti-tumor efficacy of PD1 antagonists in multiple clinical trials, and that combination therapy with CAR T cells and PD1 antagonists have enhanced antitumor activity in preclinical models (25), we next tested whether disruption of PD1 in CAR T cells would enhance anti-tumor activity. A CAR specific for prostate stem cell antigen (ref. 26; PSCA) was expressed in T cells using lentiviral vector gene transfer. gRNAs for PD1 were developed, and RNA electroporation of Cas9/gRNAs using the strategy shown in Fig. 5A was done to generate a population of PSCA CAR T cells that no longer expressed PD1 upon stimulation. PD1 upregulation were abolished on CRISPR-edited PSCA CAR T cells after coculture with PC3 tumor cells transfected with PDL1 (PC3-PDL1). Enhanced T-cell activation was confirmed by the upregulated expression of CD137 on PD1-ablated CAR T cells (Fig. 5B). The function of PD1-deficient CAR T cells were tested *in vivo* in NSG mice bearing established large PC3-PDL1 tumors (Fig. 5C and D). The PSCA PD1$^-$ CAR T cells showed significantly enhanced anti-tumor activity compared with the conventional PSCA CAR T cells. Similar results were observed in the setting of adaptive resistance when a native PC3 tumor without forced expression of PDL1 was treated with PSCA-CAR T cells. Over 90% PC3 tumor gained PDL1 expression after encountering PSCA-CAR T cells *in vitro* (Supplementary Fig. S6C). When tested *in vivo*, the PSCA PD1$^-$ CAR T cells also showed significantly enhanced antitumor activity compared with wild-type PSCA CAR T cells (Supplementary Figs. S6D and S5E). To test whether PD1 disruption might improve the function of gene-disrupted CART cells, TCR, B2M, and PD1 triple ablated gene-disrupted CD19 CAR T cells were generated. Enhanced anti-tumor activity of PD1 disrupted gene-disrupted CD19 CART cells were observed in a Nalm6-PDL1 leukemia model, evidenced by more quick and robust anti-tumor response in PD1 ablated gene-disrupted CAR T-cell treatment group, which led to complete elimination of leukemia cells in this aggressive mouse model (Fig. 5E–G).

Discussion

Multiplex genome editing is one of most attractive applications of the CRISPR/Cas9 system and holds great promise for advancing T cell–based adoptive immunotherapy. However, the low targeting efficiency of DNA transfection limits the use of multiplex genome engineering in primary T cells (27). A "hit-and-run" delivery strategy was developed to introduce CRISPRs to T cells via the co-electroporation of Cas9 mRNA and gRNA. After two rounds of gRNA electroporation, a targeting efficiency >90% at the protein level was routinely achieved for a single gene disruption. More encouragingly, the triple gene disruption of TRBC, B2M, and PD1 yielded double-negative CD3 and HLA-I at 65% without any purification or selection. Our results also demonstrate that enrichment to >99% purity of gene-disrupted T cells could be easily

Figure 3.

Multiple gene ablation by CRISPR/Cas9 to generate universal effector cells. **A,** HLA-I disruption with gRNA-targeting B2M. **B,** Flow chart of the protocol to generate universal effector cells as described in Materials and Methods. **C,** The alloreactivity of TCR and TCR/HLA disrupted was tested with an IFNγ ELISpot assay by challenging the gene-ablated T cells with irradiated allogenic PBMCs (left) or coculturing allogenic PBMCs with irradiated gene-ablated T cells. Specific spots are shown on the *y*-axis as the spots produced in the presence of stimulators minus the spots produced by the effectors alone. **, $P < 0.01$ by Mann–Whitney test. **D,** Survival without severe GVHD and (**E**) weight loss in mice after infusion of PBS ($n = 5$), Cas9 Mock wild-type (Cas9 Mock) T-cell ($n = 5$), TCR-ablated (TCR^{neg}) cells ($n = 5$) or TCR/HLA-I double ablated (TCR/HLA-I$^-$; $n = 5$). ***, $P < 0.005$ by the log-rank Mantel–Cox test. **F,** Abolishment of target recognition of allogeneic T cells by disrupting MHC-I on target T cells. Allogeneic T cells were primed by dendritic cells of the same donor with gene-disrupted T cells and infused into NSG mice with TCR^{neg} or TCR/HLA-I^{neg} target T cells. Significant prolonged survival of HLA-I–ablated T cells was observed by the presence of CD3^{neg} T cells, which is also confirmed by the failed expansion of allogeneic effector T cells ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, by Mann–Whitney test.

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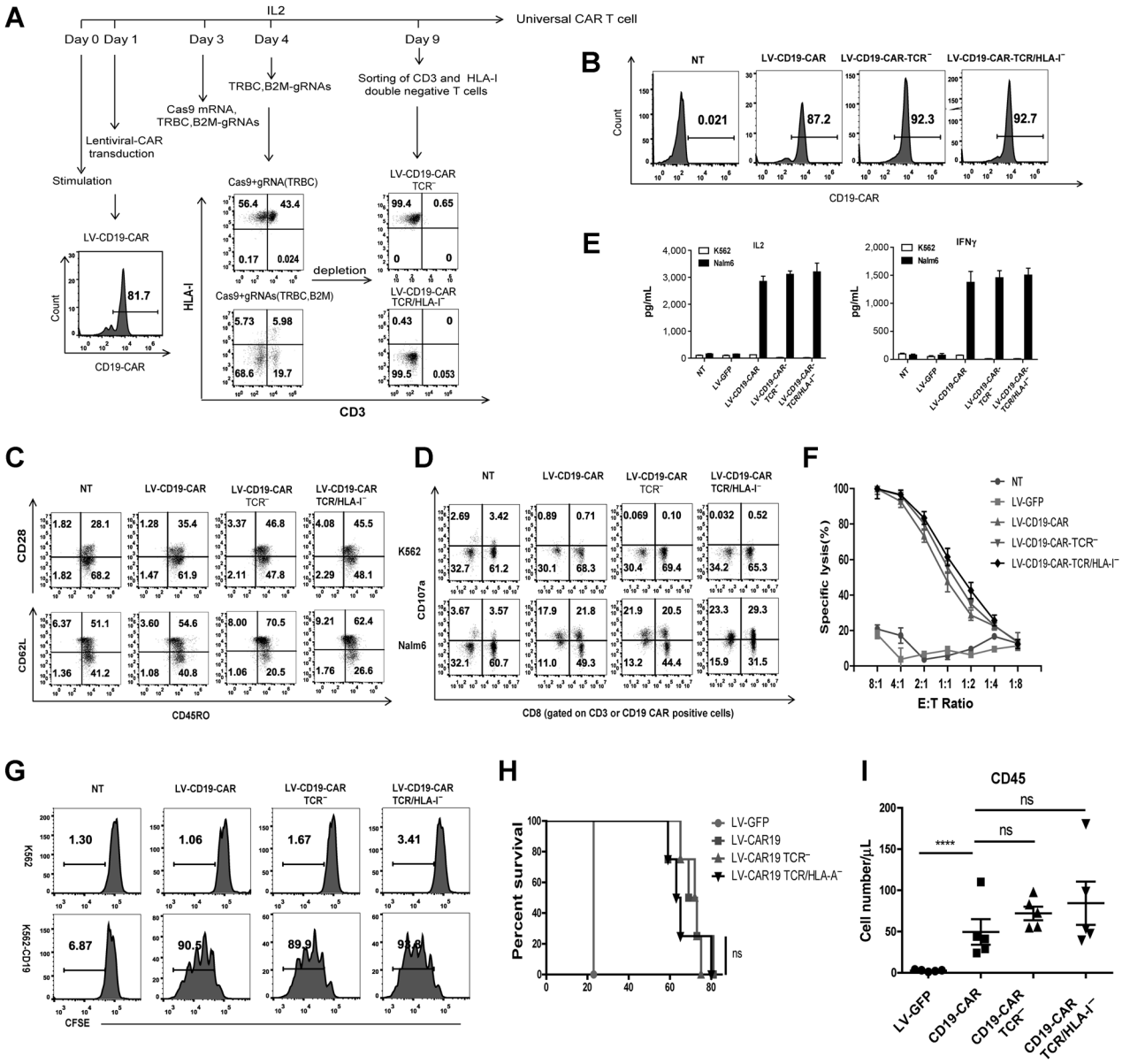

Figure 4.

Generation of universal CART cells with a combination of lentiviral gene transfer and CRISPR/Cas9 electroporation. **A,** Flow chart of the generation of universal CD19-CART cells. T cells were transduced with lentiviral CD19-CAR on day 1 after stimulation, and Cas9 mRNA and gRNAs targeting the TCR β chain and B2M were electroporated in the T cells on day 3 followed by a second delivery of gRNAs on day 4. The TCR and HLA-I double-negative cell population was enriched by negative selection using microbeads on day9. **B,** CD19-CAR expression of gene-modified lenti-CD19-CAR T cells. **C,** Phenotype of universal CD19-CAR T cells. Function of TCR$^-$ and TCR/HLA-I double-negative CD19-CAR T cells tested by CD107a release (**D**), cytokine secretion (**E**), and tumor lytic capability (**F**). Representative data from three independent experiments are shown. Bars, SE. **G,** CFSE-labeled CD19-CAR and nontransduced T cells were incubated with K562 and target K562-CD19 tumor cells at the indicated E:T ratio for 72 hours. **H,** Survival curve of mice receiving gene-edited CD19-CART cells. Tumors were established in NSG mice ($n = 5$ per group) by intravenous injection of 1×10^6 Nalm6 cells. Beginning on day 7, T cells (5×10^6) expressing lentiviral (LV) transduced CD19-CAR were infused with a single injection. T cells expressing LV GFP protein were injected as controls. ns, no difference by the log-rank Mantel–Cox test. **I,** Peripheral blood from Nalm6-bearing NSG mice treated with CD19 CART cells was obtained on day 21 and quantified for the presence of CD45 T cells by a FACS Trucount assay. Results are expressed as the mean absolute count per microliter of peripheral blood ± SD with $n = 5$ for all groups. ns, ****, $P < 0.001$ by Mann–Whitney test.

achieved using clinically approved paramagnetic beads. The disrupted T cells did not cause GVHD, suggesting that they may be used as allogeneic CAR T cells. Importantly, the gene-edited T cells showed anti-tumor activities both *in vitro* and in different tumor mouse models that were as potent as non-gene–edited T cells.

In this report, we demonstrate that CRISPR/Cas9 is a powerful multiplex genome editing tool in primary human T cells with high efficiency. This report is the first to describe the simultaneous editing of three different genetic loci, thereby creating checkpoint-resistant T cells that have the potential to be off-the-shelf. Previous

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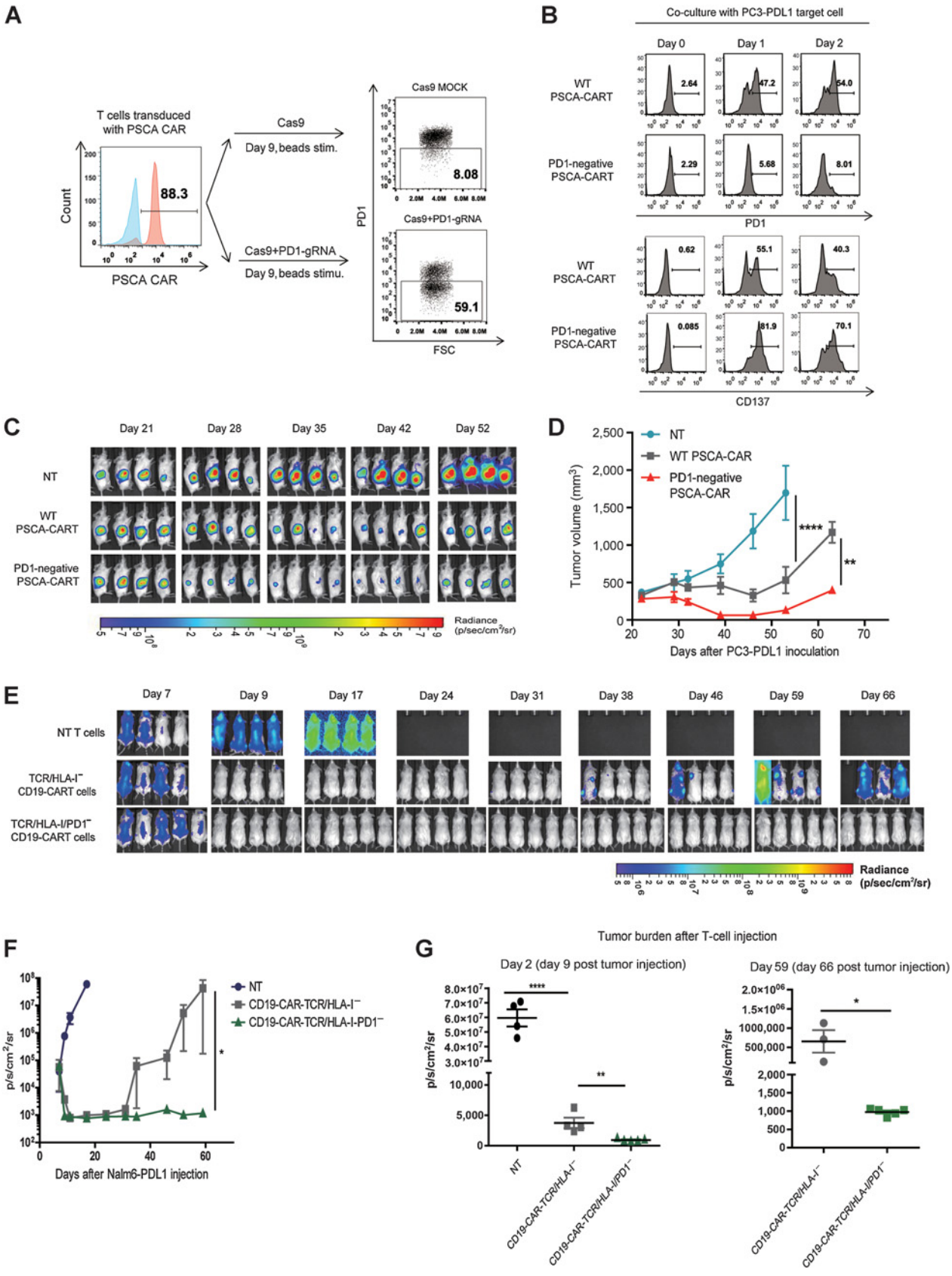

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reports showed that T cells can be genetically edited by zinc finger nucleases (ZFN) or TAL effector nuclease (TALEN) to eliminate the expression of the endogenous TCR α and β chains to avoid GVHD (28, 29) or PD-1 in tumor-infiltrating lymphocytes for the treatment of metastatic melanoma (30). However, due to the complexity in constructing and difficulty in targeting multiple genes with ZFN and TALEN in T cells (31–33), previous studies have not simultaneously targeted 3 gene loci. The advantage of the efficient multiplex genome editing of CRISPR over TALEN is demonstrated by the superior function of PD1-deficient gene-disrupted CAR T cells that are resistant to an inhibitory pathway other than "off-the-shelf."

Gene disruption in T cells is not so efficient with lentiviral and Adenoviral CRISPR (21, 22). Although Mandal and colleagues reported better gene ablation in CD4 T cells with DNA nucleofection of CRISPR reagents, DNA nucleofection–associated high toxicity to T cells was a major difficulty for its application (27). By modifying our protocol to clinical settings, we developed a process to generate synthetic cells that could be easily translated into current GMP-compliant manufacturing procedures. We are currently conducting trials to test non-gene–edited CAR T cells that have been manufactured with RNA electroporation (34). Our current protocols generate sufficient cells for a clinical trial to test the safety and feasibility of gene-disrupted CAR T cells. Our data indicate that HLA class I ablation can significantly reduce the surveillance of allogeneic T cells and potentially prevent being rejected rapidly after infusion to the allogeneic recipients. However, testing human T cells in immunodeficient mice for the GVHD and host versus graft reaction may not be correlated well to the allogenic setting in humans. Further rigorous testing in non-human primates and further modification of the T cells could establish the safety of these cells as well as their ability to evade the immune system.

CRISPR/Cas9 gene editing has been shown to generate off-target mutations depending upon the experimental setting and cell type (35, 36). However, Pankaj and colleagues reported an extremely low incidence of off-target mutagenesis of CRISPR in hematopoietic stem cells (27). Recent studies also showed a low incidence of off-target mutagenesis in T cells using lentivirus and adenovirus-delivered CRISPR/Cas9 to knock out CCR5 (21, 22). Another report showed no detectable off-target mutations in the CXCR4 knockout CD4 T cells (23). We observed very rare off-target mutagenesis targeting TRAC or TRBC with Cas9. Although no detectable mutagenesis observed with eSpCas9(1.1) reduces worries regarding off-target effects in applied and therapeutic settings, deep sequencing of off-target gene disruption should be carefully characterized before conducting human trials. The careful selection of targeting sequences could also be considered to minimize the potential of off-target incidence. Researchers from the Joung laboratory improved the on-target specificity of the Cas9 nuclease with another independently discovered CRISPR variant SpCas9-HF1 (37) that can be used for more precise genomic edits in mammalian cells. Further comparison of these two Cas9 variants can be carried out for safer clinical use.

The genomic integrity of gene-edited T cells should be considered prior to conducting clinical trials. Unlike the UCAR T cells generated by TALEN (with substantial chromosome translocation (38) from universal CAR T cells derived from three different donors), no chromosome translocation has been identified (unpublished data).

The therapeutic value of blocking the PD1 inhibitory pathway is confirmed in our system by the ablation PD1 of CAR T cells in a solid tumor model and by the triple ablation of TCR, B2M, and PD1 of CAR T cells in a leukemia tumor model. One potential limitation of our triple gene–disrupted CAR or TCR T cells is that they may trigger NK cell activation, leading to the eventual rejection of the edited T cells. We showed that although the ability of HLA-I− T cells' allo-stimulation was markedly reduced (Fig. 3C), it was not completely diminished due to the activation of NK cells in the absence of HLA-I. Lymphodepletion via chemotherapy, or using NK cell–specific antibody (39, 40) to deplete most of NK cells could potentially avoid or reduce NK mediated rejection of transferred HLA-I^{-} T cells. Furthermore, NK-mediated host versus graft reaction could also be minimized via the ablation of stimulatory NK ligands by CRISPR/Cas9 or by the expression of non-classical HLA class I molecules on the T cells, such as HLA-E (32). T cells express high levels of HLA class II after activation, which can potentially lead to the accelerated rejection of infused allogeneic T cells. Therefore, abrogated HLA class II should be considered to incorporate into next generation of gene-edited allogeneic CAR T cells.

In summary, clinical-scale gene-disrupted CAR T cells with potent antitumor activity and reduced alloreactivity can be efficiently generated using multiplex CRISPR technology and potentially could be used as off-the-self universal T cells. This approach can be incorporated into current GMP-compliant manufacturing procedures and has a high potential for translation due to the successful translation of adoptive transfer therapy with ZFNs for HIV/AIDS (41, 42). Although they may be compromised due to shorter persistence, gene-disrupted

Figure 5.

PD1 ablation enhances the therapeutic effect of CART cells. **A,** Generation of PD1-negative PSCA-CAR T cells. T-cell PD1 ablation was confirmed by flow cytometry after stimulation. PD1-deficient CART cells were sorted. **B,** Co-culture of PD1-disrupted CAR T cells with PC3-PDL1 tumor cells. PD1 and CD137 expression were measured on the CRISPR/Cas9–edited CART cells. **C,** PC3-PSCA-PDL1 tumors were established in the flank of NSG mice by inoculating 1×10^6 tumor cells/mouse (s.c. with Matrigel, $n = 4$). After 3 weeks, the mice were treated with 2×10^6 PSCA CAR-transduced WT (PSCA CAR) or PD1^{-} (PSCA CAR PD1^{-}) T cells (i.v.); mice treated with nontransduced T cells (NT) served as the control. BLI conducted before (day 21) and after the mice treated with a single T-cell injection. **D,** Tumor volume of mice. Results are expressed as the mean tumor volume ($mm^3 \pm$ SE) with $n = 4$ for all groups. **, $P < 0.01$; ****, $P < 0.001$ by comparison of the slopes with linear regression. **E,** PD1 ablated universal CD19-CART cells were generated by codelivery of Cas9 mRNA and gRNAs targeting TRBC, B2M, and PD1 after transduction with lenti-CD19-CAR. TCR and HLA-I disruption was confirmed by flow cytometry, PD1 disruption was confirmed by Sanger sequencing (Supplementary Fig. S6A and S6B). TCR and HLA-I double-negative cells were sorted at day 9. Nalm6-PDL1 tumor were established in NSG mice ($n = 4$ or 5 per group) by intravenous injection of 1×10^6 cells. Beginning on day 7, T cells (5×10^6) expressing lentivirus-transduced CD19-CAR were infused with a single injection. T cells expressing LV GFP protein were injected as controls. **F,** Bioluminescence of mice receiving different treatment. Imaging commenced 1 day before the start of T cell treatment. $P < 0.05$, by comparison of the slopes with liner regression. **G,** Tumor burden of different groups were compared at day 2 and day 59 after T cell treatment ($n = 4$ or 5). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$ by Mann–Whitney test.

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allogeneic CAR and TCR T cells could provide an alternative to autologous T cells, which carry difficulties and high production costs. Gene-disrupted allogeneic CAR and TCR T cells with disabled checkpoint molecules may be potent effector cells against cancers and infectious diseases.

Disclosure of Potential Conflicts of Interest

C.H. June reports receiving commercial research grants from and holds ownership interest (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J. Ren, C.H. June, Y. Zhao
Development of methodology: J. Ren, X. Liu, Y. Zhao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Ren, C. Fang, S. Jiang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Ren, X. Liu, C. Fang, Y. Zhao
Writing, review, and/or revision of the manuscript: J. Ren, C.H. June, Y. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Jiang
Study supervision: Y. Zhao

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Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition

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