SUPPLEMENTARY INFORMATION, Data S1 Materials and Methods

Supplemental data S1

Comparison of gene edited T cells unique Indels and sgRNA sequences. 100bp windows of genomic sequences surrounding called genetic variations were extracted. All 20mer sequences followed by NGG PAM from both strands were enumerated and aligned to the sgRNA spacers. The best alignment (with minimal number of mismatches) of each sgRNA spacer against each 100bp window was reported with the number of mismatches.

Materials and Methods

Isolation and expansion of T cells from UCB units

Fresh umbilical cord blood (UCB) units were obtained from healthy volunteer donors who had provided informed consent from the Beijing Cord Blood Bank (Beijing, China), and mononuclear cells were separated using density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich). T cells were isolated using the EasySep human T cell enrichment kit (Stemcell Technologies), activated and expanded with anti-CD3/anti-CD28 Dynabeads (ThermoFisher Scientific) at a bead to T cell ratio of 1:1 according to the manufacturer's instructions. UCB-derived T cells were cultured in X-vivo15 medium (Lonza) supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate in the presence of 300 IU/mL recombinant human IL-2 (all from ThermoFisher Scientific). Viable cells were enumerated using Trypan blue (ThermoFisher Scientific) exclusion. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

Cell lines

The following CD19-expressing immortalized cell lines were used: Raji (Burkitt's lymphoma cell line, ATCC-CCL86), Daudi (B lymphoblast cell line, ATCC-CCL213), and K562-CD19. Raji-ffluc cells for bioluminescent imaging were generated by transfection of Raji cells with an expression cassette for firefly luciferase lentiviral particles from Genechem (Shanghai, China). To isolate a stable line expressing firefly luciferase (Raji-ffluc), transfected cells underwent puromycine selection and single cell cloning. K562-CD19 cells expressing CD19 were prepared by lentiviral transfection of K562 cells (human erythroleukemic cell line, ATCC-CCL243) with an expression cassette for CD19. The cDNA for full-length CD19 derived from Daudi cells was cloned into the FUW lentiviral backbone. Lentivirus-containing supernatant was prepared and K562 cells were transduced with this supernatant and then CD19-expressing cells were sorted by flow cytometry (MoFlo XDP, Beckman Coulter Inc) and underwent single cell cloning to obtain a population of K562 cells that uniformly expressed high levels of CD19. All above cell lines

were maintained in RPMI1640 medium (ThermoFisher Scientific). Lentiviral producer cell lines 293T (ATCC-CRL3216) were maintained in DMEM (ThermoFisher Scientific). All media were supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. All cell lines were grown at 37 °C in a 5% CO_2 atmosphere.

CAR-T generation

Second-generation CD19 CARs were constructed using single-chain variable fragment (scFv) derived from antibody clones FMC63, hinge and transmembrane regions from CD8α, intracellular domain from CD137, and intracellular domain from CD247 (CD3-zeta) [1]. The CAR sequence is followed in frame by the 2A ribosomal skipping sequence and eGFP sequence. The final CD19CAR-2A-eGFP was cloned into the FUW lentiviral vector backbone downstream from an EF1α promoter (Fuw-EF1α-CD19CAR-2A-eGFP). Lentiviruses were produced by co-transfecting 293T cells with Fuw-EF1α-CD19CAR-2A-eGFP and packaging plasmids pMD2.G, psPAX2 (addgen) using Lipofectamine3000 (ThermoFish Scientific). Virus supernatants were harvested on days 2 and 3 and concentrated by ultracentrifugation (Merck Millipore). UCB-derived T cells were transduced with the supernatant and CAR+ T cells were identified by eGFP expression.

Generating sgRNAs using *in vitro* transcription

We used oligonucleotides containing T7 promoter and 20bp targeting sequences as forward primer, and an sgRNA backbone reverse primer to amplify sgRNA-coding fragment using pX330 plasmid (Addgene plasmid #4223) as template. The T7-sgRNA PCR products were gel-purified and used as the template for IVT using MEGAshortscript T7 kit (ThermoFisher Scientific). RNAs were purified with MEGAclear columns (ThermoFisher Scientific) and eluted in RNase-free water.

Generation of *TCR*/*B2M* double knockout (DKO) and *TCR*/*B2M*/*PD-1* triple knockout (TKO) CAR-T cells

Freshly purified primary T cells were activated for 3 days according to the procedure described above, and then transduced with lentiviral vectors harboring the CD19 CARs. Two days after transduction, CAR-T cells were electroporated with Cas9 protein (ThermoFisher Scientific) and the intended sgRNAs targeting the TCRα constant chain (*TRAC*), β2-microglobulin (*B2M*) exon1, and*PD-1* exon1 by 4D-Nucleofector System N (Lonza) using the P3 Primary Cell 4D-

Nucleofector X Kit, V4XP-3024 (Lonza). Cas9:single-guide RNA ribonucleoproteins (Cas9RNPs) were prepared immediately before experiments by incubating Cas9 protein with sgRNA at a 1:1 ratio at room temperature for 10 min. 3×10^6 cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, ThermoFisher Scientific) by centrifuging at 200g for 5 minutes and resuspended in 100 μL transfection buffer containing Cas9 RNP and then transferred into the electroporation cuvette. Program EO-115 was selected for high efficiency. After electroporation, cells were resuspended in 2 mL pre-warmed T cell medium and transferred into a 12-well cell plate and incubated at 37 °C in 5% CO_2. The transfection efficiency was evaluated 3 and 7 days after electroporation. Cell culture medium was half replaced by fresh complete medium every 2 ~ 3 days.

Flow cytometry

CytoFLEX (Beckman Coulter Inc) was used to perform fluorescent expression analysis. Cells were harvested on the following days after transfection and stained with mouse anti-human antibody labeled by fluorescence for 10 minutes at room temperature in the dark as follows: TCR α/β-PE/Cy7 (IP26, Biolegend), TCRα/β-PE (IP26, Biolegend), β2-microglobulin (B2M)-PE (2M2, Biolegend), β2-microglobulin (B2M)-FITC (2M2, Biolegend), CD279(PD-1)-APC (EH12.2H7, Biolegend), CD279(PD-1)-PE (EH12.2H7, Biolegend), HLA-A2-FITC (BB7.2, BD Pharmingen).

Surveyor nuclease assay and sequencing

The levels of genomic disruption of *TRAC*, *B2M*, *PD-1* in T cells or CAR-T cells were determined by surveyor nuclease assay using surveyor mutation detection kit (Integrated DNA Technologies, Inc). The percentage target disruption was quantified by densitometry and calculated as described [2]. The PCR products were also sequenced for TIDE (Tracking of Indels by Decomposition) analyses using specially designed software provided as a simple web tool (available at http://tide.nki.nl). The PCR primers used for the amplification of target loci and sequencing are listed in Supplementary Table 1. The purified PCR products were ligated with pEASY blunt cloning vector using pEASY Blunt Cloning Kit (Transgen Biotech) to detect mutant alleles. Ligation products were used for transformation and about 20-30 colonies per sample are sequenced using universal primer M13F.

Enrichment of DKO and TKO CAR-T cells

DKO and TKO CAR-T Cells were enriched using EasySep PE selection kit (Stemcell Technologies) according to the manufacturer's instructions. Briefly, the gene modified CAR-T cells were labeled with PE-conjugated antibody (TCRα/β-PE, β2-microglobulin-PE, PD-1-PE) and anti-PE MicroBeads, and then the labeled cells were put into a magnetic field. Using this procedure, the magnetically labeled PE-positive cells were retained in the tube while the unlabeled DKO and TKO CAR-T cells could be recovered in the supernatant.

Cytokine enzyme-linked immunosorbent assay (ELISA)

Cytokine production by effector (CAR-T, DKO CAR-T, TKO CAR-T, T) cells was evaluated by co-incubation with target tumor cells (Daudi, Raji, K562-CD19, K562) at a 1:1 ratio (10^4 cells each) for 24 hours. Supernatants were harvested and IL-2 and IFN-γ levels were analyzed by ELISA (Biolegend).

Flow-based cytotoxicity assay

The cytolytic activity and specificity of CAR-T cells were assessed according to the flow cytometry-based cytotoxicity assay described in [3]. Lytic activities of effector cells were tested by Violet/AnnexinV and 7-AAD labeling cytotoxicity assay. Target tumor cells were labeled with 1 μM Celltrace Violet (ThermoFisher Scientific) for 25 min at 37°C in PBS. Labeling was stopped by adding 10 mL complete culture medium and incubated at 37 °C for 5 minutes and extensively washed in complete culture medium before seeding into the 48-well plates (Corning). Violet-labeled target cells were then incubated with effector cells by different effector to target ratio for 4 hours. FITC-AnnexinV and 7-AAD (Biolegend) were added to determine the ratio of dead target cells. Samples were analyzed by flow cytometry. Target cells were selected by gating on the Violet-positive cell population and further analyzed for different subpopulations. The percentages of cytotoxic activity was calculated using the following equation: %specific cell death={[%($Violet^+AnnexinV^+$+$Violet^+$ $AnnexinV^-7\text{-}AAD^+$)-%spontaneous ($Violet^+AnnexinV^+$+$Violet^+$ $AnnexinV^-7\text{-}AAD^+$)]/[100%-% spontaneous ($Violet^+$Annexin V^++$Violet^+$ $AnnexinV^-7\text{-}AAD^+$)]}×100%

***In vivo* studies**

6-12-week-old NOD-$Prkdc^{scid}$ $Il2rg^{null}$ (NPG) mice (VITALSTAR, Beijing, China) were injected with 2×10^5 Raji-ffluc cells via intraperitoneal injection in a volume of 50μL DPBS and 50μL matrigel matrix (Corning). Two days after injection, tumor engraftment was evaluated by serial biophotonicimaging using the Xenogen IVIS Imaging System (Perkin Elmer Life Sciences). Mice

were injected intraperitoneally with 3 mg d-luciferin (Perkin Elmer Life Sciences), and then imaged 4 minutes later with an exposure time of 30 seconds. Luminescence images were analyzed using Living Image software (Perkin Elmer Life Sciences). The bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons/s/cm^2/steradian (p/s/cm^2/sr). Mice with progressively growing tumors were segregated into treatment groups bearing comparable tumor loads and received 200 uL DPBS /mouse, 5x10^6 T cells /mouse, 5x10^{6}CAR-T cells /mouse, 5x10^6 DKO CAR-T cells /mouse intraperitoneally one day later. The tumor loads were evaluated 7 days after treatment.

Off target analysis

The potential off target of each sgRNA is predicted using Benchling software using algorithm described in [4]. The top five targets for each sgRNA were amplified by PCR and subjected to Sanger sequencing. Sequencing results were analyzed using the TIDE method [5].

In exome sequencing experiments, we used NimbleGen SeqCap_EZ_Exome_v3+UTR exonic target sequences to capture and enrich human exonic region following the manufacture's instruction. Briefly, we first built the DNA library and randomly fragmented DNA. The DNA fragments were then hybridized with the exome lipid chip forming complex. After hybridization, the library was purified, evaluated for its quality, and applied to sequencing. We used genomic alignment software (BWA [6]) to map the clean reads to the reference genome UCSChg19 and samtools [7] to sort the BAM file for mutation detection with high accuracy. Potential sequence variations were called using mutational analysis software GATK [8] against the hg19 genome and were then filtered by quality value, depth and reproducibility using default parameters. Indels called in the different samples were overlapped and presented as Venn diagrams. 100 bp window of genomic sequences surrounding called indels were extracted. All 20 mer sequences followed by NGG PAM from both strands were enumerated and aligned to the sgRNA spacers. The best alignment (with minimal number of mismatches) of each sgRNA spacer against each 100 bp window was reported with the number of mismatches.

Statistical analysis

Graphpad Prism 5.0 (Graphpad software, San Diego, CA) was used for all statistical analysis. The mean ± S.E.M. was determined for each treatment group in the individual experiments. The one-tailed Student t-test was used to determine the significances between treatment and control groups. P-values < 0.05 were significant.

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