

Supplemental Materials

This Supplemental Materials is provided by the authors for additional information about their work.

Supplements to Dan Li, *et al.* **Anti-BCMA CAR-T cells therapy to a patient with extremely high membrane-BCMA expression: a case report**

CONTENTS:**Supplemental Methods**

Whole exome sequencing

Detection of surface BCMA and sBCMA

Cell lines

CRISPR/Cas9 gene editing

Lentiviral gene transfer

Western blotting

Cell proliferation and apoptosis assays

CAR-T-cell manufacturing for in vitro functional studies

Cytotoxicity assay

Degranulation assay

Intracellular cytokine analysis

Statistical analyses

Supplemental references**Supplemental Figures**

Figure S1. Construction of KO cells and alteration in phenotype.

Figure S2. Electrophoresis and expression levels of PSENEN mRNA.

Table S1. MFI value and sBCMA (ng/ml) levels of the patients receiving CT103A therapy

Supplemental Methods

Whole exome sequencing

Genomic DNA was extracted from the samples using a QIAamp Blood DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA (200 ng) from each sample was sheared by Biorupter (Diagenode, Belgium), end-repaired, ligated with barcoded Illumina sequencing adapters, amplified, size selected, and hybrid captured with the AIExome Enrichment Kit v1 (iGeneTech, China). Sequencing libraries were then quantified by qPCR, pooled, and sequenced with 150 base paired-end reads using Illumina NovaSeq 6000 sequencers (Illumina, USA). Raw sequencing reads were processed through the previously described pipeline^[1]. For each sample, reads were aligned to the Human Genome Reference Consortium build 37 (GRCh37) using BWA (<http://bio-bwa.sourceforge.net/>). After removing duplications and low-quality reads, variants were called and annotated using the Genome Analysis Toolkit (GATK). Afterwards, we focused on exonic nonsynonymous or splice donor/acceptor site variants. Variants found in regions with poor coverage, with quality less than 30, with read depth less than 20, and present in an in-house curated blacklist were excluded. In addition, variants with population frequency > 0.0001 in the gnomAD database (<http://gnomad.broadinstitute.org>) were also excluded. As for the copy number analysis, exome-wide copy number profiles were initially estimated using ReCapSeg based on a panel of in-house normal samples, and the allele-specific copy number was determined using Allelic Capseg as previously described^[2-3].

Detection of surface BCMA and sBCMA

The MFI of the surface expression of human BCMA was determined by flow cytometry using

an LSRFortessa (BD Biosciences, USA) and a PE anti-human CD269 (BCMA) antibody (clone 19F2, BioLegend). Measurement of sBCMA in plasma or cell culture supernatants was performed using the Human TNFRSF17/BCMA enzyme-linked immunosorbent assay kit (Boster Biological Technology, China).

Cell lines

The MM.1S, RPMI-8226, and HEK293T cell lines were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). MM.1S and RPMI-8226 cells were expanded in RPMI-1640 medium (Gibco, Waltham, MA) containing 10% fetal bovine serum (FBS; Gibco) and 100U/mL penicillin/streptomycin (Gibco) at 37°C with 5% CO₂. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS at 37°C with 5% CO₂. Mycoplasma was tested using the MycoAlert Detection Kit, according to the manufacturer's instructions (Lonza, Basel, Switzerland). Cell line authentication was performed based on short tandem repeat (STR) profiling according to the criteria established by the International Cell Line Authentication Committee.

CRISPR/Cas9 gene editing

CRISPR/Cas9 target sites were determined and sgRNAs were designed using Synthego's design tool (<http://www.synthego.com>). Oligonucleotides targeting the sequences in exon 3 of *PSENEN* were synthesized (GenScript Company, China). Gene knockout was performed in both MM.1S and RPMI-8226 cell lines using ribonucleoprotein (RNP)-based CRISPR/Cas9 gene editing methodology. Briefly, 10 µg Cas9 protein (Invitrogen, Carlsbad, CA, USA) and 5 µg sgRNA were mixed and incubated at room temperature for 15 min for hybridization. CRISPR-Cas9 ribonucleoprotein (RNP) complexes were then electroporated into cell lines at

450 volts. Cells were subcloned by limit dilution and cultured for 3 weeks. Finally, clones with homozygous frameshift mutations in exon 3 validated by Sanger sequencing were selected.

Lentiviral gene transfer

The open reading frames of WT and mutant *PSENE1*, which harbored the same missense mutation identified in the patient, were synthesized (GENWIZ company, China) and inserted into the pLVX-EF1 α -IRES-puro vector. HEK293T cells were co-transfected with the lentiviral vector and packaging system, psPAX2/pMD2. G, using Lipofectamine 3000 (Invitrogen). The lentiviral supernatant was purified via instantaneous centrifugation using centrifugal filters (Amicon, USA). Next, MM.1S-KO and RPMI8226-KO cells were transfected with wild-type or mutant *PSENE1* lentivirus, respectively. In addition, all cell lines were transfected with an in-house reserved firefly luciferase lentivirus.

Western blotting

Exponentially growing cells were harvested, washed with ice cold phosphate buffered saline (PBS), and then lysed in radioimmunoprecipitation (RIPA) lysis buffer (BioServe, China) supplemented with protease inhibitors (ThermoFisher Scientific, Waltham, MA, USA) for 20 min at 4°C with gentle shaking. After centrifugation for 15 min at 13000 \times g, the protein concentration of the collected lysate was determined using Bradford assays (Thermo Fisher Scientific). Equal amounts of lysates were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Freiburg, Germany) following incubation with primary antibodies and appropriate secondary antibodies. Protein bands were detected using the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, USA). Primary antibodies against presenilin enhancer-2 (PEN2), presenilin (PS1 and PS2), and nicastrin (NCT)

(Cell Signaling Technology, CST) were used.

Cell proliferation and apoptosis assays

For proliferation assays, cells were seeded in a 96-well plate at 30,000 cells/100 μ L per well and the cells were incubated at 37°C for 24 h, 48 h, and 72 h, as indicated. Cell viability was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Each experiment was performed in triplicate. For apoptosis analysis, cells were seeded at a density of 4×10^5 cells/mL in the presence of DMSO or bortezomib (Selleckchem) for 48 h. Cell apoptosis was assessed by staining the cells with Annexin V-FITC and propidium iodide (BD Biosciences, USA), and subsequently analyzed by flow cytometry on LSRFortessa (BD Biosciences).

CAR-T-cell manufacturing for in vitro functional studies

Briefly, after activation, human peripheral T cells were transduced with retroviral vectors encoding CAR and truncated epidermal growth factor receptor (tEGFR) for the specific recognition and cellular ablation of CAR-T cells. The expansion of CAR-T cells was determined by quantifying the average CAR transgene copies using droplet digital polymerase chain reaction (ddPCR, Bio-Rad QX200, Bio-Rad Laboratories).

Cytotoxicity assay

Anti-BCMA CAR-T-cells were co-cultured with 1×10^4 reserved firefly luciferase-expressing MM cells at gradient effector-to-target ratios at 37°C for 24 h. Cell extracts were prepared using the Steady-Glo Luciferase Assay System (Promega Corp.). Luciferase activity was measured using a SpectraMax luminescence microplate reader (Molecular Devices) and calculated by normalizing the luciferase activity to cell viability.

Degranulation assay

Anti-BCMA CAR-T cells were co-cultured with target MM cells at different effector-to-target ratios at 37°C for 3 h. PE/Cyanine 7-conjugated anti-human CD107a antibody (clone H4A3, BioLegend) and monesin (BioLegend) were added 5 h before harvesting. Cells were harvested, washed, fixed/permeabilized, and then surface-stained with APC-conjugated anti-human CD8 antibody (clone RPA-T8, BD Biosciences) and Alexa Fluor 488 anti-human EGFR antibody (clone AY13, BioLegend). The surface expression of CD107a on CD8 and EGFR dual-positive cells was determined by flow cytometry using LSRFortessa (BD Biosciences, USA).

Intracellular cytokine analysis

Anti-BCMA CAR-T cells were stimulated for 6 h in the presence of CD107a monoclonal antibody and Golgi inhibitors brefeldin A and monensin (BD Biosciences, USA). The cells were harvested, washed, and surface-stained with APC-conjugated anti-human CD8 antibody (clone RPA-T8, BD Biosciences) and Alexa Fluor 488 anti-human EGFR antibody (clone AY13, BioLegend). Cells were then fixed, permeabilized, and intracellularly stained for PE/Cyanine 7-conjugated anti-interferon (IFN)- γ (clone B27, BioLegend) and Brilliant Violet 421 anti-tumor necrosis factor (TNF)- α (clone Mab11, BioLegend) antibodies. Intracellular IFN- γ and TNF- α levels were measured by flow cytometry using an LSRFortessa (BD Biosciences, USA).

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software) or R v3.6.2. Comparisons were performed using Fisher's exact test or Student's *t*-test, as appropriate. All tests were two sided. *P* values are reported as follows: ns, no significant; *, *p* < 0.05; **, *p* <

0.01; ***, $p < 0.001$; ****, $p < 0.0001$. Estimates of the variation within each group of data are presented as error bars.

Supplemental references

- [1] Chapuy B, Stewart C, Dunford AJ, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes[J]. *Nat Med*. 2018 May;24(5):679-690.
- [2] Lee Lichtenstein, Betty Woolf, Alyssa MacBeth, et al. ReCapSeg: Validation of somatic copy number alterations for CLIA whole exome sequencing. *Cancer Res*. 2016 July 15; 76 (14_Supplement): 3641.
- [3] Giannikou K, Malinowska IA, Pugh TJ, et al. Whole Exome Sequencing Identifies TSC1/TSC2 Biallelic Loss as the Primary and Sufficient Driver Event for Renal Angiomyolipoma Development. *PLoS Genet*. 2016 Aug 5;12(8):e1006242.

Supplementary Figures

Figure S1

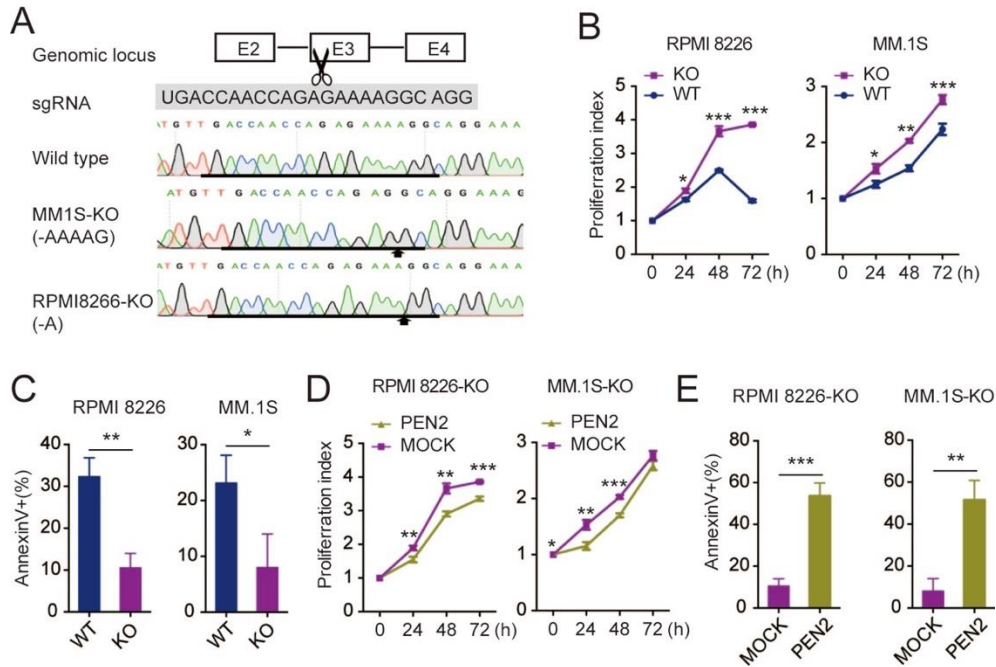


Figure S1. Construction of KO cells and alteration in phenotype. (A) PSENEN exon 3 was targeted by sgRNA. The monoclonal cells were confirmed by sequencing. (B-C) Effects of bortezomib on cell proliferation and apoptosis. (D-E) Effects of bortezomib on restoring Psenen cells.

Figure S2

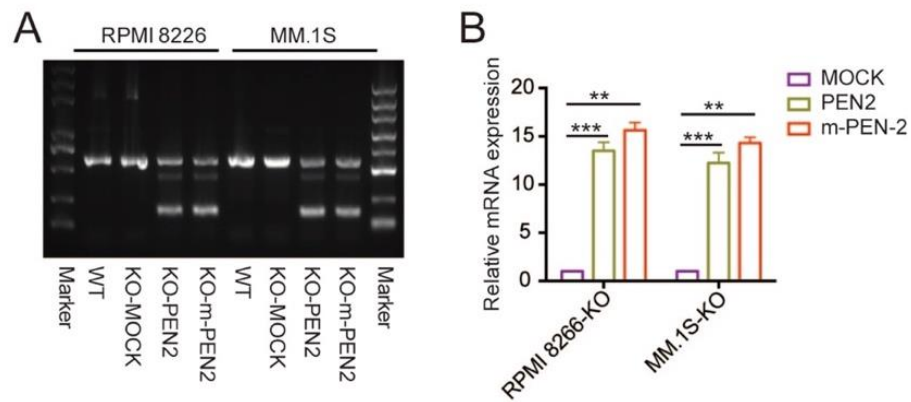


Figure S2. Electrophoresis and expression levels of PSENEN mRNA. (A) Electrophoresis of PCR products. PEN2 and mPEN2 were overexpressed in KO cells. The overexpressing cell lines produce shorter DNA fragments. (B) Expression levels of PSENEN mRNA detected by qPCR. KO-PEN2 and KO-mPEN2 had higher PSENEN mRNA levels.

Table S1. MFI value and sBCMA (ng/ml) levels of the patients receiving CT103A therapy

Patient No.	Age (years)	Gender	BCMA MFI	sBCMA, ng/mL
01-001	43	Male	1656	166.03
01-002	51	Male	1068	178.7
01-004	61	Female	1393	161.21
01-005	61	Female	4505	27.93
01-007	41	Female	1883	88.28
01-009	66	Female	1283	198.58
01-010	52	Male	1267	142.14
01-011	56	Male	2318	141.95
01-012	61	Female	51023	7.12
01-013	54	Male	9503	160.55
01-015	63	Male	2189	114.52
01-016	38	Female	4961	89.45
01-019	48	Male	888	156.02
01-020	53	Female	761	45.4
01-021	49	Male	849	39.65
01-022	60	Male	835	75.76
01-023	62	Female	NA‡	12.66
01-024	52	Male	1257	73.42

Abbreviations: BCMA, B-cell maturation antigen; MFI, Mean Fluorescence Intensity; NA, not applicable;

‡ MFI was not available for patient #23, an EMM patient who had no involvement in the bone marrow.