

Anti-BCMA CAR-T cells therapy for a patient with extremely high membrane BCMA expression: a case report

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ABSTRACT

B cell maturation antigen (BCMA)-directed CAR-T cell therapy is a disruptive approach for treating relapsed/refractory multiple myeloma (R/R MM); however, optimization is necessary to maximize patient benefit. We report the case of a 61-year-old woman with primary refractory MM who presented with high expression of membrane BCMA and low expression of soluble BCMA (sBCMA), experienced grade 4 cytokine release syndrome, and died from severe pneumonia after receiving anti-BCMA CAR-T (CT103A) therapy. This case highlights the importance of assessing the expression range of BCMA for its efficacy and safety in patients receiving BCMA CAR-T therapy. For patients who present with extremely high membrane BCMA expression and extremely low sBCMA expression, the presence of γ -secretase-related gene mutations should be considered. Special attention should also be paid to the prevention and treatment of cytokine release syndrome in such patients.

INTRODUCTION

B cell maturation antigen (BCMA, also referred to as TNFRSF17 or CD269) is expressed almost exclusively in plasma and B cells, and its overexpression and activation are associated with multiple myeloma (MM) progression. BCMA-directed immunotherapies, especially BCMA CAR-T cell therapy, have shown remarkable activity in relapsed/refractory MM (R/R MM).^{1,2} Despite significant efficacy, the use of CAR-T cell therapy is limited by potentially severe toxicities. The most frequent life-threatening adverse events following CAR-T cell infusion are cytokine release syndrome (CRS) and CAR-related encephalopathy syndrome. The γ -secretase complex cleaves membrane-bound BCMA from the surface of plasma cells to a soluble form (sBCMA).³ Recent studies have shown that γ -secretase inhibitors improve the recognition of anti-BCMA CAR-T cells, increase surface BCMA levels, and enhance antitumor activity.⁴ Nevertheless, there have been no reports on the effect of BCMA expression

on anti-BCMA CAR-T cell therapy. In this report, we present an in-depth analysis of the high surface expression of BCMA in an R/R MM patient who had severe adverse events after receiving anti-BCMA CAR-T (CT103A) therapy.

CASE PRESENTATION

A 61-year-old Chinese woman was diagnosed with IgG lambda MM (DS IIIA, ISS II, and R-ISS II) in April 2018. RB-1 (93%+) was detected by fluorescence in situ hybridisation (FISH), but no other myeloma-associated chromosomal abnormalities were detected. Over the following 8 months, the patient received three chemotherapy regimens: VRD (bortezomib, lenalidomide, and dexamethasone), VCD (bortezomib, cyclophosphamide, and dexamethasone), and VDCEP (bortezomib, dexamethasone, etoposide, cisplatin, and cytarabine), but never achieved partial remission (figure 1A). The best response was the minimal response. In January 2019, after the failure of three prior lines of treatment, the patient was enrolled in the CT103A clinical trial (phase I). Before CAR-T cell infusion, serum protein electrophoresis revealed an immunoglobulin level of 46.34 g/dL. Bone marrow cytology showed 26% plasma cells, and 2.46% of cells were considered monoclonal plasma cells in flow cytometry. FISH analysis revealed 1q21 amplification, Rb1 deletion, MAF deletion, and a few tetraploid karyotype complex changes. Notably, high surface expression of BCMA (mean fluorescence intensity=51 025) was observed by flow cytometry, whereas significantly decreased serum sBCMA (sBCMA=7.12 ng/mL) was detected by ELISA (figure 1B). At enrolment, the patient was assigned to the high-dose group. After lymphodepletion with fludarabine and cyclophosphamide, a target dose

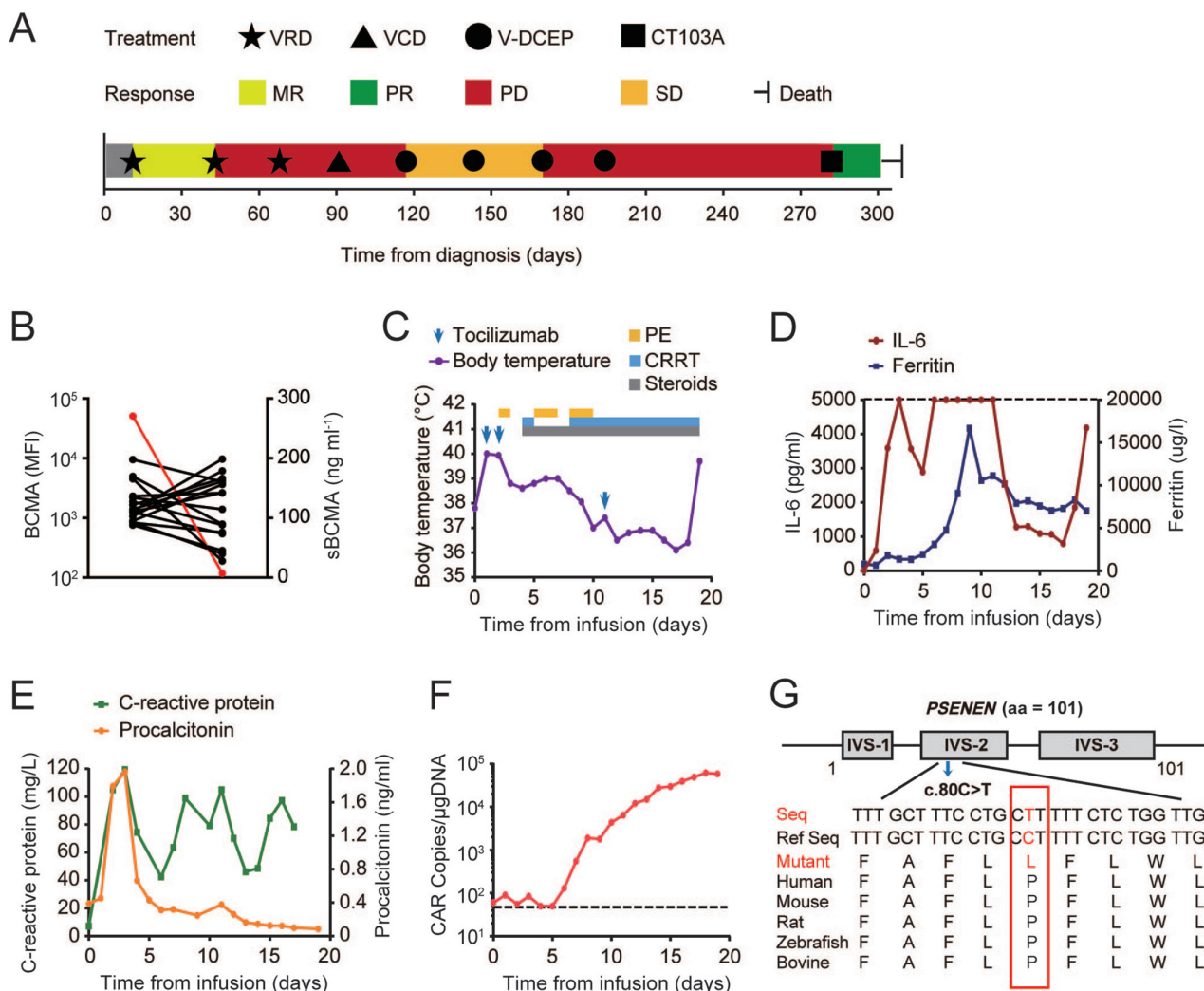


Figure 1 Clinical course of the patient. (A) Timeline of treatment and response. StAR, triangle, circle, and square markers indicate the start time of VRD, VCD, V-DCEP, and CT103A regimen chemotherapy, respectively. (B) Paired plots diagrams of MFI of BCMA and sBCMA concentrations from an MM patient cohort. The red plot indicates the patient with extremely high BCMA expression and with low sBCMA levels. (C) Body temperature and treatment for CRS of the patient after CAR-T cell infusion. (D) Levels of IL-6 and ferritin after CAR-T cell infusion. The horizontal line denotes the higher limit of quantitation (5000pg/mL). (E) Levels of procalcitonin and C reactive protein after CAR-T cell infusion. (F) Human CAR BCMA transgene copy numbers detected by ddPCR. The horizontal line denotes the lower limit of quantitation (50 copies/μg). (G) Location of the monoallelic *PSENEN* missense mutation c.80G>T, p.Pro27Leu. Each gray rectangle indicates an exon of the *PSENEN* gene. The red font illustrates the altered codon and protein. CAR, chimeric antigen receptor; CRRT, continuous renal replacement therapy; CRS, cytokine release syndrome; ddPCR, droplet digital PCR; GC, glucocorticoid; MFI, mean fluorescence intensity; MR, minimal response; PD, progression disease; PE, plasma exchange; PR, partial response; sBCMA, soluble B cell maturation antigen; SD, stable disease.

of 6×10^6 /kg of CAR-T cells was infused. The patient was febrile for 10 days, with a maximum temperature of 40.0°C on the first day postinfusion (figure 1C). Serum IL-6 (>5000pg/mL) and ferritin (16680μg/L) levels peaked on the 3rd and 10th day postinfusion, respectively (figure 1D). C reactive protein and procalcitonin (PCT) levels continued to increase at an early stage, while PCT levels returned to normal on the fifth day (figure 1E). Cellular kinetic analysis showed substantial expansion and persistence of CAR-T cells (figure 1F). The patient experienced grade 4 CRS, requiring steroids, tocilizumab, plasma exchange, and continuous renal replacement therapy during the entire treatment period. Eventually,

the patient achieved a partial response according to the current International Myeloma Working Group guidelines, but died 20 days after infusion caused by severe pneumonia.

To elucidate the molecular mechanism underlying severe CRS, we performed whole-genome sequencing on the MM cells of the patient, which revealed a single-base missense mutation and deletion of *Psenen* alleles (figure 1G). To simulate the state of the patient based on the deletion of *Psenen* alleles, we constructed a *Psenen* knockout (KO) model in MM.1S and RPMI 8226 cell lines using CRISPR/Cas9 technology (online supplemental figure S1). Gene expression results were confirmed at

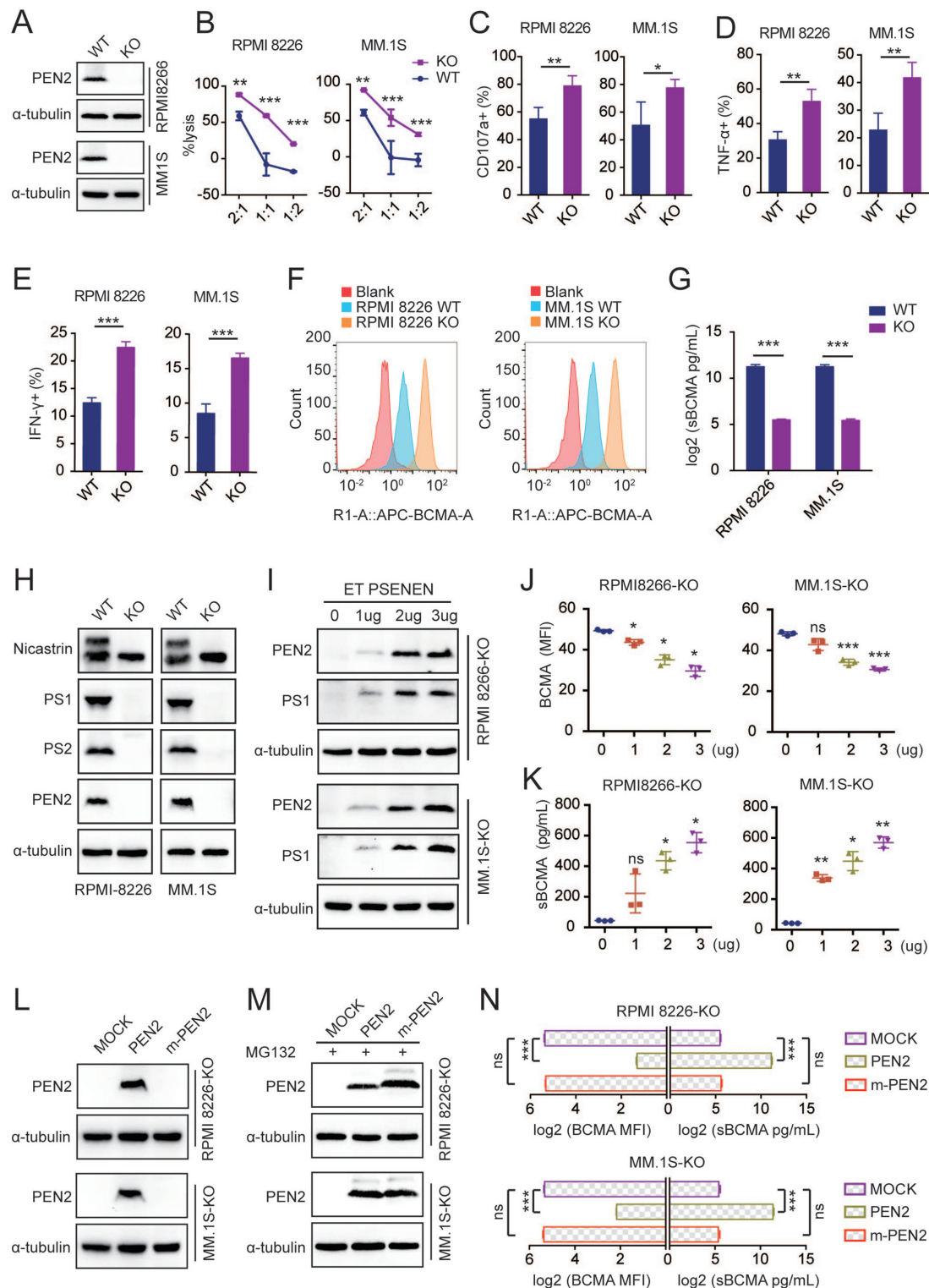


Figure 2 Phenotypic alterations associated with PSENEN deletion and missense mutation. (A) Pen2 protein levels detected by Western blotting. KO cell lines had no Pen2 protein expression. (B) Cytotoxicity of CAR-T cells at different effector-to-target ratios. (C–E) degranulation markers CD107a, TNF- α , and IFN- γ at the indicated effector-to-target ratio. (F) FACS analyses for membrane BCMA expression. (G) sBCMA concentrations on wild-type (WT) cells and Psenen KO cells. (H) Effects of PEN2 KO on the protein levels of different γ -secretase subunits. (I) Protein levels of PEN2 and PS1 in KO cell lines after plasmid electrotransfection. (J, K) MFI of BCMA and sBCMA concentrations in KO cell lines after plasmid electrotransfection. (L, M) expression of Pen2 protein in PSENEN and mPSENEN overexpressing cell lines. Proteasome inhibitor MG132 was added before protein extraction. Pen2 protein in mPSENEN-overexpressing cells could be detected only with MG132. (N) Pairing diagrams of MFI of BCMA and sBCMA concentrations in PSENEN and mPSENEN overexpression cell lines. BCMA, B cell maturation antigen; MFI, mean fluorescence intensity; ns, not significant; KO, sBCMA, soluble BCMA; WT, soluble BCMA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

the protein level using western blotting (figure 2A). Psenen KO induced high proliferation and a reduction in bortezomib-triggered apoptosis when compared with wild-type (WT) cells (online supplemental figures S1B and S1C). Rescue experiments were performed to re-express Psenen in the KO cells. Restoring Psenen expression reinhibited the proliferation and rescued the apoptosis induced by bortezomib owing to Psenen knockdown (online supplemental figures S1D and S1E). This suggests that the deletion of Psenen may reflect a higher malignant potential of MM cells, which concurs with the clinical characteristics of primary refractory disease. Notably, Psenen KO cells were more sensitive to CAR-T cell-mediated killing at different effector-to-target ratios (figure 2B). Higher lysis was positively correlated with the release of CD107a, TNF- α , and IFN- γ in BCMA CAR-T cell cocultures (figure 2C–E). γ -secretase acts as a multisubunit protease that directly cleaves BCMA from plasma cells, consisting of nicastrin, presenilin 1 or 2 (PS1 or PS2), Aph-1, and presenilin enhancer protein (PEN2, Psenen-encoded protein).⁵ Some studies have shown that PEN2 is required to stabilize the γ -secretase complex.⁶ Considering the essential role of PEN2, we assessed BCMA expression in the KO and WT cells. We found that KO cells expressed significantly higher levels of membrane BCMA and lower levels of sBCMA than WT cells (figure 2F,G). Moreover, knockdown of Psenen resulted in the downregulation of other γ -secretase subunits (figure 2H). To further verify the association between Psenen and BCMA, we conducted rescue experiments using plasmid electrotransfer in Psenen KO cells. Interestingly, we found that this markedly increased the expression of PEN2 and PS1 in a dose-dependent manner, concurrently and significantly decreased surface BCMA levels, and increased sBCMA concentrations with dose escalation (figure 2I–K). Given that surface BCMA is a determinant of tumor cell recognition by CAR-T cells, we speculate that the deletion of Psenen might affect the killing efficiency of CAR-T cells by regulating BCMA expression through γ -secretase.

Considering that the patient had a monoallelic Psenen deletion and a missense mutation (c.80C>T, p.Pro27Leu) on the remaining allele, we constructed an m-PEN2 cell line with single-base mutations using lentiviral overexpression technology in KO cells. However, while both DNA electrophoresis and real-time qPCR showed that the mutant Psenen (m-*Psenen*) was successfully transferred (online supplemental figures S2A and S2B), western blotting indicated that mutated PEN2 protein could not be expressed normally (figure 2L). Indeed, recent studies indicate that PEN2 is degraded via the proteasome pathway.⁷ We observed that mutated PEN2 was normally detected by western blotting only in the presence of the proteasome inhibitor MG132 (figure 2M). Furthermore, m-PEN2 cells expressed significantly higher levels of membrane BCMA and lower levels of sBCMA than the PEN2 cells (figure 2N). These results suggest that both the deletion and missense mutation of Psenen are

inactivating mutations, which increase BCMA expression in tumor cells and decrease sBCMA in peripheral blood.

DISCUSSION AND CONCLUSIONS

CAR-T immunotherapies are a disruptive approach for treating hematological malignancies, but optimization is necessary to maximize patient benefit. Although the causes and consequences of BCMA decrease have been described in CAR-T cell therapy studies,^{8,9} the mechanisms and clinical outcomes of high BCMA expression have rarely been reported. Here, we report a case of a patient with very high membrane BCMA expression and very low sBCMA expression who experienced grade 4 CRS after high-dose BCMA CAR-T cell infusion. We conclude that BCMA expression should be concerned not only with the lower boundary, but also the upper line, to ensure the efficacy and safety of patients receiving BCMA CAR-T therapy. For patients with γ -secretase-related gene mutations who may present with extremely high expression of membrane BCMA and extremely low expression of sBCMA, special attention should be paid to the prevention and treatment of CRS.

Clinical manifestations of CRS after CAR-T-cell therapy include fever, hypotension, and coagulopathy, which range from non-specific to severe life-threatening symptoms.¹⁰ Currently, tocilizumab, steroids, and plasma exchange have been used to treat CRS once the symptoms become severe.¹⁰ However, despite the use of these regimens, the levels of cytokines, such as IL-6 and ferritin, increased after infusion and remained elevated in the patient experiencing CRS, while inflammatory markers, such as PCT, returned to normal on the fifth day. This indicates that the patient developed uncontrolled CRS rather than an infection.

BCMA is actively cleaved from the tumor cell surface by the multisubunit γ -secretase complex, which reduces the ligand density on tumor cells for CAR-T cell recognition and releases an sBCMA fragment capable of inhibiting CAR-T cell function.⁴ We further verified that both the deletion and the single-base missense mutation of Psenen resulted in γ -secretase inactivation, which in turn increased membrane BCMA expression and decreased sBCMA. Previous studies have shown that increasing BCMA surface expression on myeloma cells with small-molecule γ -secretase inhibitors correlates with increased CAR-T cell effector function in vitro, including cytokine release and proliferation, and improved in vivo anti-tumor activity in preclinical models.⁴ This is consistent with the CRS severity described above. For patients with γ -secretase-related gene mutations such as deletions or missense mutations in Psenen, special attention should be paid to the prevention and treatment of CRS when receiving anti-BCMA CAR-T therapy.

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Contributors JZ, LH, and MX designed and supervised the clinical study, enrolled the patient and took care of the patient. Moreover, they also supervised the CAR-T cell production for preclinical quality control. DL, WZ, and SD collected clinical data, performed the experiments, and performed statistical analyses. YQ wrote and revised the manuscript and figure. YQ and DL edited and formatted the document.

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Competing interests No, there are no competing interests.

Patient consent for publication Not applicable.

Ethics approval The study protocol (Online supplemental file 1) was approved by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and registered with the Chinese Clinical Trial Registry (<http://www.chictr.org.cn> Number, ChiCTR1800018137). Participants gave informed consent to participate in the study before taking part.

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Protocol

This trial protocol has been provided by the authors to give readers additional information about their work.

A Phase I Study of a Novel Fully Human BCMA-Targeting CAR (CT103A) in Patients with Relapsed/Refractory Multiple Myeloma

This supplement contains the following items:

1. Selection of patients, including both eligibility and ineligibility criteria
2. Schema and treatment plan, including administration schedule
3. Rules for dose modification
4. Measurement of treatment effect including response criteria, definitions of response and survival, and methods of measurement
5. Reasons for early cessation of trial therapy
6. Objectives and entire statistical section (including endpoints)

Clinical study protocol

An open-label, single-center, and single-arm phase I trial of anti-BCMA CAR-T cell (CT103A) therapy for R/R plasma cell neoplasms

Protocol Number: XL-LCYJ-0003

Protocol Version: Version 3.0

Version Date: 2019-10-31

Principle Investigator: Dr. Jianfeng Zhou

Study Site: Tongji Hospital of Tongji medical school, Huazhong University of Science and Technology

CAR-T Producer: Nanjing IASO Biotherapeutics Ltd.

Study Sponsor: Nanjing IASO Biotherapeutics Ltd.

Study Objectives:**1.Primary Objectives**

Evaluate the tolerability and safety of different doses of CT103A in patients with relapsed / refractory plasma cell tumors, observe the characteristics of dose-limiting toxicity (DLT), determine the maximum tolerated dose (MTD), and confirm the recommended phase II dose (RP2D). To provide a basis for the dosage and dosing regimen for subsequent clinical trials

2.Secondary Objectives

- 1) Preliminary evaluation of the clinical efficacy of CT103A infusion
- 2) Evaluate the pharmacokinetic characteristics after CT103A administration
- 3) Evaluate the pharmacokinetic characteristics after CT103A administration

3.Exploratory Objectives

- 1) Evaluate the in vivo expansion characteristics and persistence of CT103A infusion
- 2) Characteristics of lymphocyte depletion in the subject
- 3) Evaluation of immunogenicity after administration of CT103A

Study Population and Number of Subjects

This trial enrolls patients with relapsed/refractory plasma cell tumors without formal sample size estimation.

In the dose-escalation phase, each dose group level will include 3-6 subjects, and the total number of subjects depends on increased dose levels. The estimated number of enrollment at this stage will be 12-15 cases in total.

In the dose-expansion stage, the first dose group will expand 6-20 cases at 1×10^6 / kg. An interim analysis is set and reviewed by an independent data review committee in the dose-expansion phase. The results will decide whether to increase the number of subjects or to adjust the dose.

Study Design

Study XL-LCYJ-0003 is a single-center, open design study, divided into two stages of dose escalation and dose expansion.

Study Design of Dose escalation

The initial dose was set to 1.0×10^6 CAR⁺ cells/kg with the maximum dose of 8.0×10^6 CAR⁺ cells/kg based on the data from multiple international clinical trials of the same target. Subjects will be enrolled into the first dose (initial dose) group 1.0×10^6 / kg, the second dose group 3.0×10^6 / kg, the third dose group 6.0×10^6 / kg, and the fourth dose (maximum dose) 8.0×10^6 / kg group.

A traditional “3 + 3” dose-escalation protocol (see Figure 1) is used to observe the tolerance and safety of different doses of CT103A in patients with relapsed/refractory plasma cell tumors (membrane expressing BCMA antigen), and observe the dose limitation Toxicity (DLT) characteristics, to determine the maximum tolerated dose (MTD).

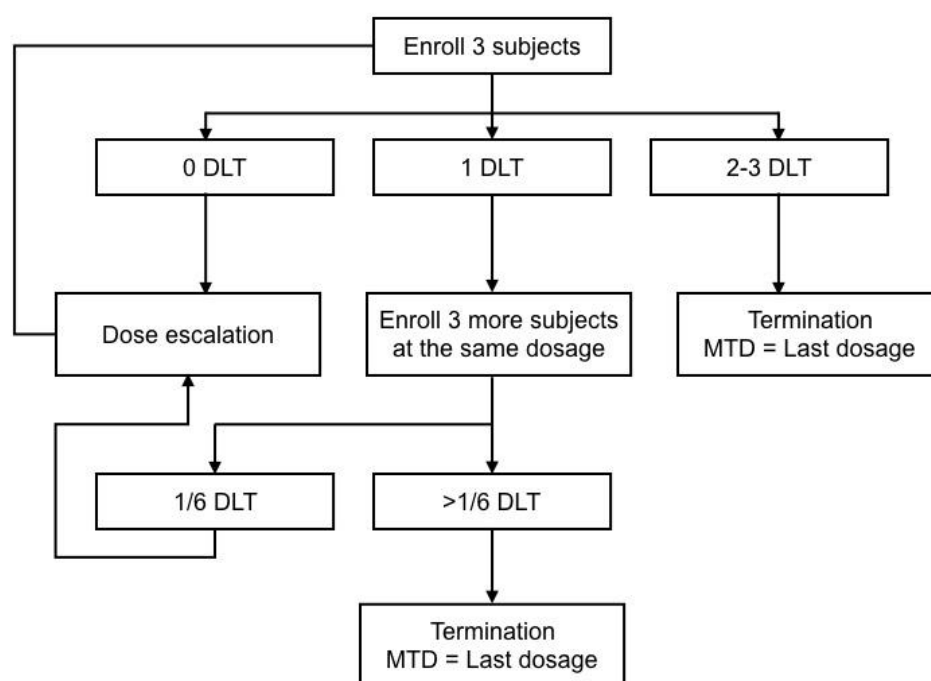


Figure 1. “3 + 3” dose-escalation protocol

During the actual dose escalation of CT103A, one DLT occurred in one of three patients in the third dose group (6.0×10^6 CAR⁺ T cells/kg), and it was decided to stop the dose escalation. Three more DLT evaluable patients were further enrolled in the second dose group (3.0×10^6 CAR⁺ T cells/kg), and no DLT was observed, suggesting a dose of $\leq 3.0 \times 10^6$ CAR⁺ T cells/kg was well tolerated in subjects.

Study Design of Dose Expansion

Because the preliminary efficacy data shows that the ORR of the 1×10^6 CAR⁺ cells/kg dose group is 100%, in order to explore further the possible efficacy and safety differences between different dose groups and to determine the optimal dose, it is decided to establish a lower dose group during the dose-expansion phase (0.5×10^6 CAR⁺ cells/kg). Two dose groups of 0.5×10^6 CAR⁺ cells/kg and 1×10^6 are chosen to carry out the dose-expansion phase to explore the safety, tolerability, pharmacokinetics, pharmacodynamics, and immunogenicity of the drugs in different dose groups, and

to preliminary observe the initial efficacy of the chosen dose group of study drugs in small samples of patients with relapsed/refractory multiple myeloma.

Subjects who signed the informed consent form will be screened for inclusion/exclusion criteria. It is expected to enroll 3-6 subjects in the 0.5×10^6 CAR⁺ cells / kg and 6 subjects in the 1×10^6 CAR⁺ cells/ kg dose group. Subjects will receive the drug once.

The treatment plan is the same as that of the dose-escalation study.

If any safety risk occurs in the dose-expansion phase, the number of subjects and the dose can be adjusted after the evaluation of the data committee.

Eligibility, Exclusion and Withdrawal Criteria

Inclusion Criteria

Subjects must meet all of the following criteria to be selected:

- 1) 18~70 years of age, regardless of gender, at the time of voluntarily signing an informed agreement consent form(s)
- 2) Diagnosed as plasma cell neoplasms (including multiple myeloma, plasma cell leukemia, POEMS syndrome, monoclonal immunoglobulinemia of unknown significance, primary Macroglobulinemia or primary amyloidosis) according to the IMWG classifications, having accepted at least three lines of prior therapies (must include proteasome inhibitor and immunoregulator-based chemotherapy) and relapsed or relapsed to the most recent therapy
- 3) The expression of BCMA must be positive for the patient's clonal plasma cells
- 4) Ineligible for autologous hematopoietic stem cell transplantation or relapsed after autologous hematopoietic stem cell transplantation, but further treatment is needed by the investigator's judgement
- 5) ECOG score is 0 or 1
- 6) Expected survival time ≥ 12 weeks
- 7) Essentially adequate organ function is necessary for eligibility
 - a) Blood routine: neutrophil $\geq 1.0 \times 10^9$ /L; hemoglobin ≥ 70 g/L; platelet $\geq 50 \times 10^9$ /L
 - b) Aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) $\leq 2.5 \times$ upper limit of normal (ULN); serum total bilirubin $\leq 1.5 \times$ ULN
 - c) Serum creatinine $\leq 2.5 \times$ ULN; or creatinine clearance rate (CrCl) (calculated according to Cockcroft-Gault formula) ≥ 60 ml / min
 - d) Electrolytes: blood potassium ≥ 3.0 mmol/L; blood calcium ≥ 2.0 mmol/L; blood magnesium ≥ 0.5 mmol/L
 - e) Coagulation: fibrinogen ≥ 1.0 g/L; activated partial thromboplastin time (APTT) \leq ULN + 10s; prothrombin time (PT) \leq ULN + 3s

- 8) The subject must be willing to provide valid initial diagnostic evidence and accept bone marrow examination before and after treatment
- 9) Female subject of childbearing age or male subject with fertility should agree to take one of the following contraception measures from the date of signing informed consent until one year after CT103A cell reinfusion: abstinence, double-barrier contraception, intrauterine device, or hormonal contraception drug
- 10) Male subject is forbidden to donate sperm from the date of signing informed consent until one year after CT103A cell reinfusion
- 11) Written informed consent
 - a) The subject (or his/her legal representative) should voluntarily sign the informed consent
 - b) Ability and willingness to adhere to the study visit schedule and all protocol requirements

Exclusion Criteria

Subjects who meet any of the following criteria will be excluded:

- 1) Treatment with the following therapies within the specified period:
 - a) Any hematopoietic stem cell transplant within two months prior to the start of infusion of CT103A, or any immunosuppressive therapy due to graft-versus-host disease after hematopoietic stem cell transplantation within the screening period
 - b) Any chemotherapy, immunotherapy, radiotherapy, or major surgery within four weeks prior to screening
 - c) Any live vaccination within four weeks prior to the start of infusion of CT103A and/or plan to receive live vaccines after participation in the trial
 - d) Any clinical trial therapy within four weeks prior to the start of infusion of CT103A, or ongoing participation in other clinical trials
- 2) Following disease or surgical history:
 - a) Subjects with a central nervous system invasion of plasma cell tumors
 - b) Hypertensive patients with hypertension history whose disease is uncontrollable by drug therapy (blood pressure $\geq 140/90$ mmHg)
 - c) Left ventricular ejection fraction (LVEF) $< 50\%$ evaluated by Doppler ultrasound examination
 - d) \geq grade 2 arrhythmia according to NCI CTCAE 5.0 grade or QTc > 450 ms (male), QTc > 470 ms (female) (QTc is calculated using Fridericia correction formula $QTc = QT / RR^{0.33}$)
subjects with a history of Torsades de pointes ventricular tachycardia or congenital prolonged QT syndrome
 - e) Patients with any of the following diseases within 12 months before the screening: including but not limited to unstable angina pectoris, myocardial infarction, congestive heart failure (New York Heart Disease Association [NYHA] classification \geq Grade III) and severe

- arrhythmia, coronary artery bypass grafting or peripheral artery bypass grafting surgery, cerebrovascular events (including transient ischemic attacks), etc.;
- f) Uncontrollable and active infections during the screening period regarded by the investigators
 - g) Subjects infected with human immunodeficiency virus (HIV)
 - h) The hepatitis C virus (HCV) antibody is positive, and the peripheral blood HCV RNA is positive
 - i) Subjects with severe electrolyte disturbance regarded by the investigators
 - j) Subjects with a clear gastrointestinal bleeding tendency, including the following: active local ulcer lesions, and fecal occult blood ($\geq ++$); subjects with a history of melena and hematemesis within two months prior to screening; Subjects who may have a major gastrointestinal bleeding history
 - k) Subjects with a history of solid organ transplantation
 - l) Subjects with other acute, severe, or chronic medical or psychological conditions regarded by investigators or sponsors as not suitable for enrollment
 - m) Pregnant or lactating women.
- 3) Prohibited treatment and/or medication
- a) Ongoing therapy with other anti-tumor drugs, including traditional Chinese medicine
 - b) On-going therapy with drugs that extend the QT interval (including Class Ia and III antiarrhythmic drugs)
 - c) Subjects who need to receive oxygen daily
 - d) Long-term use of corticosteroids (except for local inhalation)
- 4) Others
- a) Subject with a history of psychotropic substance abuse who are unable to quit or have mental disorders
 - b) Subject with a habit of drinking grapefruit juice or excessive tea, coffee, and/or caffeinated beverages which are unable to quit during the period of clinical trial
 - c) Subjects with concomitant diseases or comorbidities that could seriously endanger the safety of the patient or affect the completion of the trial as judged by the investigators.

Withdrawal Criteria

- 1) Subjects are unwilling to continue to participate in clinical trials. According to the "Helsinki Declaration" and informed consent, subjects have the right to withdraw at any stage of the trial, and their subsequent treatment and follow-up will not be affected by the withdrawal of this trial
- 2) CT103A cells are not successfully prepared, which may include blood collection failure, preparation process failure, product cryopreservation failure, etc.

- 3) Medical examinations indicate that the disease progression requires an alternative treatment plan, or the subject cannot benefit from the trial treatment as judged by the investigator
- 4) The occurrence of adverse events or serious adverse events that have not improved after treatment or intolerable, or the circumstances that the trial treatment need to be interrupted or affected (including pregnancy or planned pregnancy) as judged by the investigator
- 5) The investigator and/or the subject's authorized personnel believe that discontinuation of CT103A treatment is in the best interests of the subject (e.g., poor tolerance, poor protocol compliance)

Treatment Plan

Investigational drug

- Drug name: Fully Human BCMA Chimeric Antigen Receptor Autologous T Cell Injection
- Administration dosage: According to different study groups, the dosage is 1×10^6 CAR⁺ cells/kg, or 3×10^6 CAR⁺ cells/kg
- Major ingredients: Fully human BCMA chimeric antigen receptor autologous T Cells
- Names of excipients: Compound Electrolyte Injection, Human Serum Albumin, Glucose Injection, Vitamin C Injection, Compound Amino Acid Injection (18AA-II), Dimethyl sulfoxide (DMSO)
- Packing specifications: 1×10^6 CAR⁺ cells/kg, 12~50 mL; 3×10^6 CAR⁺ cells/kg, 12~80mL, depending on the bodyweight of the subject and the effective content of the cell preparation
- Appearance: White or yellowish frozen solid, yellowish suspension liquid after thawing
- Storage and transportation conditions: Stored in gas-phase liquid nitrogen container (< -130°C); Transported in liquid nitrogen container (< -130°C)
- Manufacturer: Nanjing IASO Biotherapeutics Ltd.

PBMC collection

The healthy mononuclear cells of the subjects were extracted from the peripheral blood, and then sent to Nanjing IASO Biotherapeutics Ltd. for CT103A cell preparation (at least 18 days from the arrival of PBMC to the company to the Certificate Of Analysis issued, slightly delayed if due to holidays)

Bridging chemotherapy

After the PBMC collection and before lymphocyte clearance pretreatment, bridging chemotherapy is allowed to control the disease if the subject's tumor burden is high.

Lymphocyte clearance pretreatment

The recommended scheme for lymphocyte clearance pretreatment is as follows:

Cyclophosphamide 20 mg/kg/d × 3d, fludarabine 25 mg/m²/d × 3d,

If a patient has a moderate renal impairment (creatinine clearance rate: 30 to 70 mL/min/1.73 m²) before lymphocyte clearance, the recommended medications for lymphocyte clearance pretreatment are:

Cyclophosphamide 20 mg/kg/d × 3d, fludarabine 20 mg/m²/d × 3d,

CT103A reinfusion

After the lymphocyte clearance pretreatment, CT103A cell reinfusion is given to the subjects according to the assigned dose group once. CT103A injection, intravenous administration, one package each time (according to individual differences, reinfusion volume = patient body weight × administration dose ÷ CAR⁺ T cell concentration)

Cytokine Release Syndrome (CRS) Management

This protocol will follow the recommendations and management for CRS, as defined by Lee et al. (2014).

For subjects highly suspected of CRS, early and timely intervention and monitoring evaluation should be carried out. CRS scoring is performed twice a day, and the frequency of evaluation should be increased if the disease condition changes.

Mild CRS is only manifested as transient fever, fatigue, muscular pain, nausea, etc., and can often be relieved automatically. Symptomatic supportive treatment should be given. Moderate to severe CRS can be manifested as respiratory difficulty, progressive hypotension, leakage syndrome, acute renal failure, and other multiple organ failure and neuropsychiatric abnormalities (including convulsions and epilepsy). The critical points of treatment for moderate and severe CRS are early identification and timely treatment. The early manifestations of moderate and severe CRS are sensitive to Tocilizumab and/or glucocorticoids, while the late manifestations are tolerant to Tocilizumab and/or glucocorticoids. Therefore, the essence of CRS is immunosuppressive therapy, and it is essential to control the progression of CRS in time and effectively. The accommodations in Table 2 will help to control CRS. If the above treatments fail to relieve the symptoms, or IL-6 is > 500-1000 pg/ml within three days, or IL-6 rises sharply within one day, high fever is not reduced. Symptoms continue to worsen, Tocilizumab and glucocorticoids should be applied together regardless of the CRS classification, and glucocorticoids can be used until the symptoms are relieved. If ferritin is > 20,000 ug/L, high fever persists, and symptoms and signs continue to worsen for a patient, such patient should be treated with both Tocilizumab and glucocorticoids, regardless of CRS classification, and glucocorticoids can be used until the symptoms are relieved. For severe leakage syndrome and cardiac insufficiency, continuous hemofiltration (CRRT) and/or plasma exchange are used as effective treatment measures. If the patient's IL-6 decreases and rises sharply again during treatment, severe infection such as septicemia should be suspected. Boosting pressure, mechanical ventilation, and plasma exchange are effective measures to treat severe CRS.

1) Fever: Non-steroidal drugs (such as acetaminophen and ibuprofen) and physical cooling measures are generally used for control. Infection is determined through laboratory tests and imaging

examinations. If a patient develops a high fever after CT103A infusion, normally broad-spectrum and powerful antibiotics will be used for empirical anti-infection treatment. Meanwhile, active efforts will be made to search for pathogenic bacteria. When the pathogenic bacteria are not clear, the possibility of atypical pathogenic bacteria infection or reactivation should be considered.

2) Hypotension: Before infusion of CT103A, blood pressure, electrocardiogram, and echocardiogram tests should be conducted to determine the baseline level of blood pressure and assess the cardiac function. After infusion, the investigators may make an additional assessment based on the clinical conditions. CRS-related hypotension after CT103A infusion should be treated at an early stage. The possibility of vascular exudation and pulmonary edema should be considered during volume resuscitation, and corresponding contingency plans should be made. Patients whose blood pressure cannot be maintained by active fluid infusion need vasoactive drugs for boosting therapy. If a patient's condition requires transfer to the ICU ward, it is suggested that the study doctor in charge and ICU doctor make joint diagnosis and treatment.

3) Hypocytosis: One or more series of hypocytosis caused by CT103A infusion require component transfusion or supplement of corresponding blood cell growth factors. However, it is not suggested that cell growth factors (especially GM-CSF) be infused in the first three weeks after the product infusion or be infused after CRS is resolved. If neutropenia or deficiency occurs after CT103A infusion, granulocyte stimulating factors can be used, and patients are advised to be transferred to laminar flow wards to avoid infection. If hemoglobin is lower than 80 g/L or platelets are lower than $20 \times 10^9/L$, component transfusion will be required. The number of platelets should be closely monitored and maintained at $20 \times 10^9/L$, or a higher level if there is active bleeding.

4) Coagulation dysfunction: It is mainly manifested as interspersed petechiae, thrombosis, and abnormal laboratory indexes, such as thrombocytopenia, an increase of D-dimer, decrease of fibrinogen, increase of fibrin degradation products, the extension of activated partial thromboplastin time, etc. Regular monitoring of blood routine and coagulation indexes is helpful for the timely detection of coagulation abnormalities. Early and correct treatment can prevent the occurrence of disseminated intravascular coagulation (DIC). The main treatment measures include supplementation of platelets and coagulation factors, appropriate application of anticoagulant or antifibrinolytic drugs, etc. If the APTT is prolonged for more than 1.5 times the normal value or the patient has a bleeding tendency, the fresh frozen plasma may be infused. When fibrinogen is $< 1.0g/L$, blood products such as cryoprecipitate and/or fibrinogen should be infused to make fibrinogen $> 1.0g/L$. If there is active hemorrhage, it needs to be infused to a higher level.

Symptoms and signs of CRS in patients should be closely monitored. The diagnosis of CRS requires the exclusion of other systemic inflammatory reactions, including concurrent infection.

In general, the principles of handling CRS are as follows:

1. Severity grading for CRS
2. Different treatment measures are adopted for CRS of different grades

3. For high-risk patients with severe CRS, Tocilizumab, and glucocorticoids should be applied simultaneously regardless of classification, and glucocorticoids can be used until the symptoms are relieved
4. For severe leakage syndrome and cardiac insufficiency, continuous hemofiltration (CRRT) and/or plasma exchange are used as effective treatment measures
5. For CRS, attention should be paid to the possibility of concurrent severe infection

CAR-T cell-related encephalopathy syndrome (CRES) Management

- 1) Carry out nervous system evaluation twice a day, and increase the evaluation times when the condition changes. Use the CARTOX-10 scoring system for the subjects
- 2) If necessary, the cell number and cytokine level of CT103A CAR-T in cerebrospinal fluid can be monitored, and other factors that may cause neurological abnormalities can be excluded by cranial MRI/CT to identify whether neurotoxicity is caused by intracranial CRS, to give appropriate treatment
- 3) For CRES of Grade 1, mainly supportive treatment is given. The head of the bed should be raised by at least 30 degrees to reduce the risk of aspiration and increase the cerebral venous blood supply. Anti-IL-6 therapy is recommended for patients with CRES of Grade 1 or above complicated by CRS. For patients with CRES of Grade 2 and above not complicated by CRS, corticosteroid therapy is preferred, and the dose can be gradually reduced when CRES improves to Grade 1. During the dose reduction of corticosteroids, the recurrence of neurotoxicity symptoms in patients should be closely monitored. Patients with CRES of Grade 3 accompanied by increased intracranial pressure should be treated with corticosteroids in time, and the neurology department should be requested to assist in the treatment in time. Patients with CRES of Grade 4 complicated by encephaledema should be treated with high doses of corticosteroids, oxygen inhalation, and dehydration

For the severity grading and clinical intervention measures for neurotoxicity that occurs in this study, refer to the consensus grading for immune effector cell-related cytokine release syndrome and neurotoxicity issued by 2019 ASTCT, and the guidelines for evaluation and management of cytotoxic therapy issued by the University of Texas MD Anderson Cancer Center in 2017

Study period

Including screening period, PBMC collection, lymphocyte clearance pretreatment, CT103A cell reinfusion, and in-hospital observation period, disease progression follow-up, and survival follow-up.

- screening period: About -42 days to -14 days before CT103A reinfusion, the length varies, depending on the preparation time of CT103A cells after the PBMC collection
- PBMC collection: 1 day
- lymphocyte clearance pretreatment: -4 days to -2 days before CT103A reinfusion
- CT103A cell reinfusion and in-hospital observation period: The day of CT103A cell reinfusion is recorded as day 0. The patient needs to be admitted to the hospital for safety monitoring for 17-30 days after reinfusion according to the specific physical condition of the patient.
- disease progression follow-up: after CT103A cell reinfusion, follow up once a month for the first three months, and once every three months thereafter until disease progression, death, or withdrawal from the trial
- survival follow-up: once every three months until the patient's death, prolapse, withdrawal of informed consent due to various reasons, or the end of the study

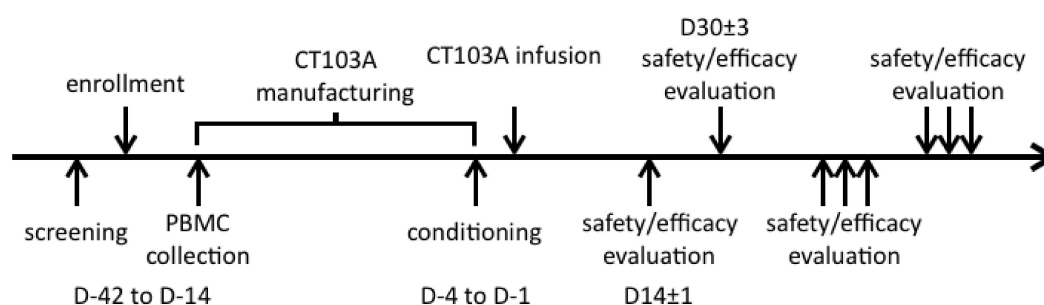


Figure 2. Study flow of the trial

Table 1. Study Flow Chart

Content of visit		Screening ¹	PBMC collection (enrollment) ¹	Lymphocyte clearance ²	Rest assessment	Cell reinfusion	In-hospital observation ³												Follow-up period ⁴			Expanded follow-up period ⁵	Withdrawal ⁶
		-8w~-8d		-7~-2 d	-1d	0d	1d	3d	5d	7d	10d	14d	21d	28d	2m	3m	Once every quarter to years ²	3y-15y					
Informed consent		×																					
Inclusion/Exclusion criteria ⁷		×		×	×																		
Demographics/past medical history/current medical history/medication history		×																					
Height/weight/body surface area ⁸		×		×																			
Physical examination/vital signs/ECOG score ⁹		×		×	×	×	×	×	×	×	×	×	×	×	×	×	×			×			
Echocardiography		×		×	×	If applicable																	
12-lead ECG		×				If applicable ×																	
Laboratory tests	Blood oxygen saturation test	×		×	×	If applicable																	
	Blood pregnancy test ¹⁰	×			×	If applicable																	
	Virology/syphilis testing ¹¹	×																					
	Blood routine ¹²	×	×	×	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×			
	Blood biochemistry ¹³	×		×	×		×	×			×		×	×	×	×		×	×				
	Urine routine ¹⁴	×		×	×		×	×			×		×	×	×	×		×	×				
Blood coagulation		×		×	×									×	×	×	×	×	×				

Content of visit	Screening ¹	PBMC collection (enrollment) ¹	Lymphocyte clearance ²	Rest assessment	Cell reinfusion	In-hospital observation ³										Follow-up period ⁴			Expanded follow-up period ⁵	Withdrawal ⁶
	-8w~-8d		-7~-2 d	-1d	0d	1d	3d	5d	7d	10d	14d	21d	28d	2m	3m	Once every quarter to years ²			3y-15y ²	
function ¹⁵																				
Peripheral blood lymphocyte subgroup	x			x									x		x					
Inflammatory factor ¹⁶				x		x	x	x	x	x	x	x	x		x					
PBMC collection		x																		
CT103A infusion					x															
Medication to prevent infusion reaction ¹⁷					x															
Efficacy evaluation	Peripheral blood smear	x		x							x		x	x	x					x
	β2-microglobulin	x		x							x		x	x	x					x
	Quantitation of serum immunoglobulin	x		x							x		x	x	x					x
	Serum protein electrophoresis	x		x							x		x	x	x					x
	Serum immunofixation electrophoresis	x		x							x		x	x	x					x
	Serum total light chain, free light chain	x		x							x		x	x	x					x
	Quantitation of 24 h urine protein ¹⁸	x		x							x		x	x	x					x
	Urine protein electrophoresis	x		x							x		x	x	x					x
	Urine protein	x		x							x		x	x	x					x

Content of visit	Screening ¹	PBMC collection (enrollment) ¹	Lymphocyte clearance ²	Rest assessment	Cell reinfusion	In-hospital observation ³												Follow-up period ⁴			Expanded follow-up period ⁵	Withdrawal
	-8w~-8d		-7~-2 d	-1d	0d	1d	3d	5d	7d	10d	14d	21d	28d	2m	3m	Once every quarter to years ²	3y-15y					
immunofixation electrophoresis																						
Urine light chain	×		×								×		×	×	×	×		×				
Radiological evaluation ¹⁹	×	If applicable																				
Bone marrow examination ²⁰	×												×		×							
BCMA CAR-T cell detection in peripheral blood ²¹						×	×	×	×	×	×	×	×	×	×	×						
Free BCMA detection in peripheral blood ²¹						×	×	×	×	×	×	×	×	×	×	×						
Immunogenicity test ²²				×									×		×	×						
RCL detection ²³				×											×	×	×	×				
Concomitant medication/adverse events/serious adverse events ²⁴		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×		×				

The window period for 21 days and 28 days of in-hospital observation is ± 1 day, the window period for all follow-up visits is ± 7 days, and the window period for extended follow-up visits is ± 14 days;

- After the subjects complete corresponding examinations in the visits according to the visit schedule during screening, they will enter the PBMC collection procedure according to the standard operating procedure (SOP) for PBMC collection after evaluation by the investigators. For example, in the screening period and the cell preparation period (-8w~-8d), if a subject delays the lymphocyte clearance pretreatment and/or cell reinfusion and causes the time from the subject's signing of the informed consent to the reinfusion time to exceed eight weeks specified in the protocol, it will not be regarded

as a protocol violation.

2. Lymphocyte clearance pretreatment: cyclophosphamide (20mg/kg/d×3d) and fludarabine (25 mg/m²/d ×3d) are preferred for the pretreatment scheme. If a patient has moderate renal function impairment (creatinine clearance rate: 30 to 70 mL/min/1.73 m²), the recommended medications for lymphocyte clearance pretreatment are: cyclophosphamide 20 mg/kg/d×3d and fludarabine 20 mg/m²/d×3d. The test time for evaluating test indexes before pretreatment is 1 day before the start of lymphocyte clearance.
3. At least 14 days of in-hospital observation should be completed, and 14-28 days of in-hospital observation should be conducted according to local diagnosis and treatment standards;
4. Please refer to the following time points of each visit for the visit arrangement during the follow-up period for details, or the follow-up period should last till withdrawal of informed consent, withdrawal from the study, disease progression, start of new anti-tumor treatment, or death, whichever occurs first;
5. The extended follow-up visit should be conducted once a year, or last till the withdrawal of informed consent, withdrawal from the study and refusal to receive the extended follow-up visit, or death, whichever occurs first;
6. Withdrawal visits should be conducted within seven days of patients' withdrawal from the study due to disease progression, death, or other reasons other than the loss to follow-up. If patients have been evaluated for efficacy within four weeks from the last examination, there is no need to repeat it; if the RCL detection is conducted within one year from the last examination after reinfusion, there is no need to repeat it; if other examinations are more than one week from the time of withdrawal from the study, there is a need to conduct them;
7. During the screening period, subjects must meet all the inclusion criteria and must not meet any exclusion criteria before joining the study. Before lymphocyte clearance, the investigators must re-evaluate the subjects, and only those subjects that meet the criteria for lymphocyte clearance can receive the lymphocyte clearance pretreatment. Before cell reinfusion, the investigators must re-evaluate the subjects, and only the subjects meeting the cell reinfusion criteria can receive cell infusion.
8. The height and weight should be re-measured one day before administration for lymphocyte clearance, and the body surface area should be calculated on this basis. Calculation formula of body surface area: BSA (m²) = 0.00616 x Height (cm)+0.01286 x Weight (kg)-0.1529;
9. On the day of reinfusion, vital signs (body temperature, respiration rate, heart rate, and blood pressure) will be measured 15 ±2 minutes before the start of the infusion, at the beginning of infusion, and within 1 hour after the start of infusion (every 15 ±2 minutes) until the indicators are stable and safe. First

- aid facilities should be provided during the infusion for timely treatment in case of severe allergic reactions, severe hypotension, or other reactions;
10. Only for women of childbearing age. If pregnancy is suspected during the study, the investigators may repeat the blood pregnancy test;
 11. Including HCV antibody, HBV five items, HIV antibody, EBV DNA, CMV DNA, and syphilis. Subjects with a positive HCV antibody should be tested for HCV-RNA, and the subjects can be enrolled only when RNA is negative. Both HBV surface antigen and HBV core antibody should be negative. If any of the above is positive, peripheral HBV-DNA testing is required, and the subjects can be enrolled only when DNA is within the normal range. If subjects have completed some or all of the above virological/syphilis tests within four weeks before the screening, the investigators will decide whether to repeat some or all of the tests.
 12. Blood routine includes: red blood cells count, hemoglobin, hematocrit, leukocyte count, platelets count, and leukocyte differential count (including the percentages and absolute counts of neutrophils, eosinophils, basophils, lymphocytes, and monocytes);
 13. Blood biochemistry includes: total bilirubin, ALT, AST, γ -glutamyltranspeptidase (γ -GT), total bilirubin (TBIL), alkaline phosphatase (AKP or ALP), albumin, total protein, lactate dehydrogenase (LDH), BUN, Cr, blood uric acid, Na, K, Cl, Ca and P;
 14. Urine routine includes pH, urine white blood cells, urine protein, urine red blood cells, and urine sugar;
 15. Coagulation function tests include: plasma prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (FIB), and international normalized ratio (INR);
 16. The examination of inflammatory factors in the study sites should include at least IL-6, CRP, and ferritin. In addition to the inflammatory factor test in the study sites, another 3 mL of venous blood should be collected and sent to the central laboratory for other inflammatory factor tests (IFN- γ , TNF- α , IL-2, GM-CSF, and IL-10);
 17. Promethazine and/or diphenhydramine hydrochloride will be administered before CAR-T cell infusion;
 18. Testing is conducted only when urine protein is +;
 19. The radiological evaluation includes: whole-body X-ray plain film (including skull, pelvis, femur, humerus, thoracic spine, lumbar spine, and cervical spine), CT (local or systemic low dose) or MRI (whole or local including the cervical spine, thoracic spine, lumbosacral vertebrae, and head), and PET-CT would be performed for imaging evaluation only when necessary.

20. Bone marrow examinations include: bone marrow smear, bone marrow flow cytometry, immunophenotyping examination, and bone marrow BCMA CAR-T detection (flow cytometry and PCR); the immunophenotyping during the first screening should include BCMA. In terms of bone marrow immunohistochemistry, it is suggested that antibodies against the following molecules be included: BCMA, CD19, CD20, CD38, CD56, CD138, κ light chain, and γ light chain. It is suggested that the bone marrow flow antibody labeling include: BCMA, CD19, CD38, CD45, CD56, CD20, CD138, cytoplasmic κ light chain, and cytoplasmic γ light chain. If conditions allow, the following may be added: CD27, CD28, CD81, CD117, and D200. The FISH examination is optional. It is suggested that the testing sites include IgM ectopia, 17p-(p53 deletion), 13q14 deletion, and 1q21 amplification. If IgH translocation is positive in the FISH test, it is suggested that further testing be conducted (4; 14), t (11;14), t (14;16), t (14;20). The results of bone marrow examinations four weeks before screening are acceptable.
21. 3mL of venous blood should be collected and sent to the central laboratory for peripheral blood BCMA CAR-T test and free BCMA test.
22. 3mL of venous blood should be collected and sent to the central laboratory for the immunogenicity test. During the follow-up period, the immunogenicity test time points are 3m, 6m, 12m, and 24m;
23. 4mL of venous blood should be collected and sent to the central laboratory for RCL testing. During the follow-up period, the time points for RCL testing are 3m, 6m, 12m, and 24m. If there are clinical indications to suspect the existence of RCL, RCL testing can be carried out as required. The RCL test is performed once a year during the long-term follow-up. If a subject withdraws from the study, has disease progression, or starts new anti-tumor therapy, it is still suggested that the investigators carry out RCL follow-up according to the protocol unless the subject refuses to accept it;

The medication before PBMC collection and the abnormal symptoms and signs of the subjects should be recorded as a history of past medication and present illness. The medication after PBMC collection and the abnormal symptoms and signs of the subjects should be recorded as concomitant medication/adverse events/serious adverse events.

Rules for dose modification

It is not allowed to adjust the dose of CT103A.

Measurement of treatment effect, including response criteria, definitions of response and survival, and methods of measurement

Response assessments will be made according to the IMWG Uniform Response Criteria for Multiple Myeloma. Response assessments include the following:

- Serum and urine immunofixation, Serum Free Light Chain (FLC, kappa and lambda), Quantification of Ig (IgG, IgM, IgA), Serum (SPEP) and urine (24-hour collection) (UPEP) electrophoresis for M-protein measurement
- Skeletal Survey: At baseline and at any time post cell infusion if the treating investigator believes there are signs or symptoms of increased or new skeletal lesions
- Radiographic Disease Assessment: Should be performed in any subjects with the documented extramedullary disease, according to the schedule of assessments. The same imaging modality used for screening (MRI, PET, CAT, or PET/CAT) should be used throughout the study
- Percent of plasma cells and BCMA expression will be assessed on bone marrow biopsy and aspirate samples collected per Schedule of Events and as clinically indicated to assess response according to the IMWG Uniform accurately
- Bone marrow assessments should include flow cytometry, fluorescence in situ hybridization (FISH), Cytogenetics, and morphology. Bone marrow aspirate will also be used for the evaluation of MRD at appropriate time points. If a bone marrow biopsy or aspirate is performed at any time during the study, biopsy and aspirate samples should be collected in the clinical response assessments and potential research if available

Objectives and entire statistical section (including endpoints)**Study endpoints****Primary endpoints**

- Safety endpoints: adverse events, ECOG performance status score, laboratory tests, vital signs, and physical examination, etc.
- Efficacy endpoints:
- Overall response rate (ORR): The percentage of patients who achieved PR or better response.
- Overall survival (OS): The time from the start of CT103A treatment to death (for any reason) for subjects
- Duration of response (DOR) after administration: It refers to the time from the first assessment of sCR or CR or VGPR or PR to the initial evaluation of disease recurrence or progression or death for any reason
- Progression-free survival (PFS): The time from the start of CT103A treatment for the subjects to

the first disease progression or death for any reason

- Time to response (TTR): The time interval between the first treatment of CT103A of a subject and the time of first recording of sCR or CR or VGPR or PR

Secondary endpoints

- PK endpoints: The highest concentration (C_{\max}) of BCMA CAR-T cells amplified in peripheral blood after administration, the time to reach the highest concentration (T_{\max}) and the area under the curve AUC_{0-28d} in 28 days, and the area under the curve AUC_{0-90d} in 90 days
- PD endpoints: the content of free BCMA in peripheral blood at each time point; the concentration levels of CAR-T-related serum cytokines such as CRP and IL-6
- Immunogenicity endpoints: positive rate and antibody level of human anti-CAR antibody

Statistical Methods

Sample Size Estimation

Dose escalation phase: 12-15 patients with relapsed / refractory plasma cell tumors. In the dose-expansion phase, each dose group level included 3-6 subjects, for a total of approximately 9-12 subjects. After the mid-term analysis of the dose extension trial, an independent data review committee reviews and decides whether to increase the number of subjects or adjust the dose based on the results.

General Methods

All statistical analysis is done using SAS 9.4 (or higher version)

Statistical analyses will be primarily descriptive.

For continuous variables, descriptive statistics will include the number of non-missing observations, mean, median, standard deviation, coefficient of variation (if applicable), maximum and minimum values, and so on. For categorical variables, descriptive statistics will include the number and percentage of subjects. The time-to-event variables will include the number, median, minimum, and maximum of non-missing observations. For specific time-to-event variables, Kaplan-Meier median time, 25th and 75th percentiles, and related 95% confidence intervals (CI) will also be provided.

Handling of missing values

Efficacy indicators: All the missing values of the primary efficacy indicators caused by early withdrawal are regarded as “un-evaluable” in the analysis. In the calculation of the involved time-event variables (e.g., PFS and OS) and the blind data verification, the subjects with no post-treatment efficacy evaluation will be checked on a case-by-case basis to determine the censoring time.

Unless otherwise specified, the baseline, safety data, and pharmacokinetic data are not filled in for missing values

The extreme value of laboratory data caused by improper handling of specimens, blood samples, etc., will use the corresponding data of unplanned visits during the analysis or will not participate in the analysis for the misconduct of processing

Populations for Analysis

The analysis population of this study includes the screening subject data set (All Subjects Enrolled Set), safety analysis set (Safety Set), effectiveness analysis set (Efficacy Set), pharmacokinetic analysis set (PK set), and drug efficacy Kinetic analysis set (PDS, PD set)

The principal statistical analysis set is as follows:

All Subjects Enrolled Set: All subjects with informed consent, regardless of whether the screening is successful or whether CT103A treatment has been received

Safety Analysis Set (SS, Safety Set): In the screening subject data set, all subjects enrolled who received the trial medication and had at least one subsequent safety visit. The safety analysis set will be used for the analysis of safety data and other baseline characteristics

Efficacy Set (ES, Efficacy Set): All subjects enrolled who received the trial medication, had a baseline tumor evaluation, and had at least one post-medication efficacy evaluation. Efficacy analysis set is an analysis data set that summarizes efficacy data

Pharmacokinetic analysis set (PKS, PK set): All enrolled subjects who receive the trial drug with at least one pharmacokinetic data of whom is available, and without major protocol deviation/violation considered to affect the pharmacokinetics data significantly

Pharmacodynamic analysis set (PDS, PD set): All enrolled subjects who received the trial drug with at least one pharmacodynamic data, and without major protocol deviation/violation considered to significantly affect the pharmacodynamic data

Analysis of content

Case distribution

The number of cases (percentage) is used to describe the subjects' enrollment and completion of the study. The distribution of cases in each data set lists of medication for dropout and excluded patients and reasons for early withdrawal.

Demographic data and baseline analysis

Descriptive statistical, demographic data, and other baseline characteristic values. Continuous variables are used to calculate the number of cases, mean, standard deviation, median, minimum, and maximum of non-missing observation results; counting and ranked data are used to calculate frequency and composition ratio.

Safety analysis

Any patient who has received a study drug needs to be assessed for safety. The evaluation time should be from the beginning of the administration to the end of the study or 28 days after the end of the administration. The drug safety/tolerance evaluation is conducted through analysis of adverse events related to treatment, the incidence of serious adverse events, and laboratory abnormalities of clinical significance;

According to the NCI CTCAE v5.0 and NCCN immunotherapy guidelines, the incidence and severity distribution of AEs, ADRs, SAEs, etc. are summarized, including but not limited to the following;

The subjects with medication termination due to adverse events, subjects with SAEs, and dead subjects will be tabulated.

- Calculation of incidence of adverse reaction;
- Listing of the occurrence frequency and frequency number of AEs by the system, and calculation of the percentage;
- A detailed list of adverse events;
- A detailed list of adverse reactions;
- Listing of the abnormal laboratory indicators and physical examination cases, and clinical explanations;
- Positive rates of CRS and neurotoxicity.

Efficacy Analysis

Overall response rate (ORR): The percentage of patients who achieved PR or better response. For ORR, the two-sided 95% confidence interval analysis of Clopper-Pearson distribution is used.

Duration of response (DOR) after administration: It refers to the time from the first evaluation of sCR or CR or VGPR or PR to the first evaluation of relapse or death from any cause. If a subject dies from other causes before the relapse of the disease is observed, the number of days from the date of cell reinfusion to the date of death will be calculated. The actual time of tumor evaluation will be used for calculation. For the subjects who do not have disease recurrence or death at the time of analysis, the duration of remission will take the time of the last tumor evaluation as the deadline and censoring will be performed for the subjects. For subjects who have not been evaluated for tumors since the baseline period, one day will be used as the deadline, and censoring will be performed for the subjects. The investigators' judgment will be used to determine the recurrence time. Missing or invalid tumor evaluation will not be used to calculate the duration of remission.

Disease progression-free survival (PFS): The time from the time a subject receives CT103A cell therapy to the time of the first disease progression or death from any cause. For surviving patients without disease progression, the date of the last tumor evaluation is set as the analysis deadline, and the patients will be treated as censored data. For patients receiving non-research anti-cancer therapy before the disease progresses, the date of the last evaluable tumor evaluation before the non-research anti-cancer therapy is set as the analysis deadline, and the patients will be treated as censored data. The Kaplan-Meier method is used to fit the survival curve, and the median PFS and its 95% confidence interval are statistically estimated.

Time to response (TTR): The time interval between the treatment of CT103A of a subject and the time of first recording of sCR or CR or VGPR or PR.

Overall survival (OS): The time from cell reinfusion to death caused by any reason. For the subjects lost to follow-up, the last known survival date is set as the analysis deadline, and the subjects will be treated as censored data. For patients who are still alive at the time of analysis, the survival time at the time of analysis will be taken as the deadline, and the patients will be treated as censored data. The Kaplan-Meier method is used to fit the survival curve, and the median OS and its 95% confidence interval are statistically estimated.

Minimal Residual Disease (MRD):

Use descriptive statistics to calculate the number and percentage of cases. Also, calculate the 95% confidence interval for the percentage.

PK/PD analysis

The PK/PD analysis will use the PK analysis set, and the PK blood sample collection schedule and derived sampling time deviation will be provided.

Descriptive statistics will be used to summarize the drug concentration data at each time point, and individual blood drug concentration-time curves and arithmetic mean concentration-time data (and standard deviation) charts will be given on the linear and semi-logarithmic scales of the treatment

groups. According to the actual blood collection time, WinNonlin software is used to calculate PK parameters through the non-compartment model.

Main PK parameters: The highest concentration (C_{max}) of BCMA CAR-T cells amplified in peripheral blood after administration, the time to reach the highest concentration (T_{max}) and the area under the curve AUC_{0-28d} in 28 days, and the area under the curve AUC_{0-90d} in 90 days;

PD analysis: Free BCMA is detected at different blood collection time points before and after cell infusion, and the concentration levels of CAR-T related serum cytokines such as CRP and IL-6 are detected at each time point. The content, percentage, copy number of CAR⁺ T cells, and their 95% confidence interval (CI), free BCMA count, and its 95% CI at different time points are calculated.

Immunogenicity analysis

The immunogenicity analyses will be conducted on the safety set.

In this study, the human anti-CAR antibody will be detected. The immunogenicity evaluation includes a positive rate and antibody level. Immunogenicity positive rates at different time points are summarized according to the treatment groups.

Interim Analyses

The interim analysis will be conducted in both the dose-escalation phase and the dose-expansion phase.

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 *PSENEN*, 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 *PSENEN* 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

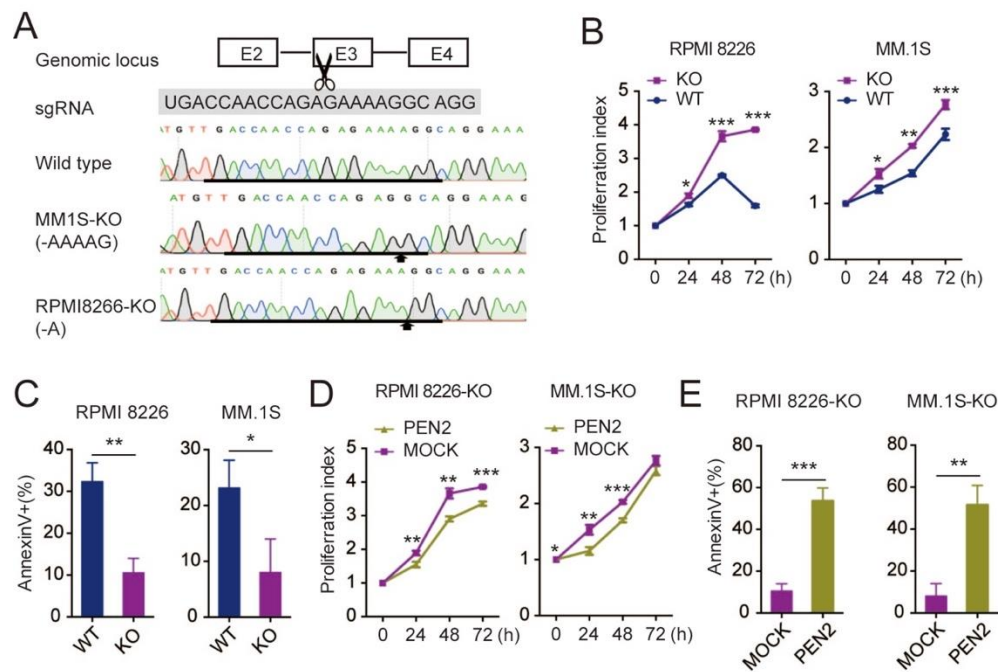


Figure S1. Construction of KO cells and alteration in phenotype. (A) PSENEN exon 3 was targeted by sgRNA. The monoclonal cells were confirmed by sequencing. (B-C) Effects of bortezomib on cell proliferation and apoptosis. (D-E) Effects of bortezomib on restoring Psenen cells.

Figure S2

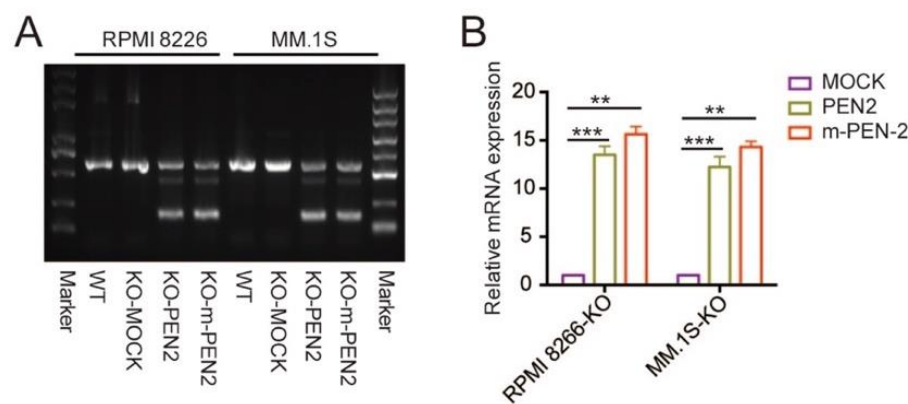


Figure S2. Electrophoresis and expression levels of PSENEN mRNA. (A) Electrophoresis of PCR products. PEN2 and mPEN2 were overexpressed in KO cells. The overexpressing cell lines produce shorter DNA fragments. (B) Expression levels of PSENEN mRNA detected by qPCR. KO-PEN2 and KO-mPEN2 had higher PSENEN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