Declarations

Ethics approval

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Center for Safety Evaluation of Drugs (NCSED, IACUC approval No. IACUC-2019-009).

1. Specificity of the scFv of C-CAR088 CAR

The scFv of C-CAR088 was derived from the human IgG1 antibody BCMA-20. The binding affinity (KD) of BCMA-20 to recombinant human BCMA was determined using a surface plasmon resonance sensor (Biacore Inc., Uppsala, Sweden). The results were shown in Supplemental Table 1. Epitope mapping experiments suggested that scFv had a high binding affinity for the extracellular domain of BCMA (Supplemental Figure 1).

To assess the specificity of scFv, tissue cross-reactivity assays were performed using a BCMA-20 scFv/rabbit Fc chimeric antibody (B20-scFv-rFc). The polyclonal antibody B0807-50G was used as a positive control, and a rabbit IgG was used as an isotype control. Thirty-two human tissues from three donors were stained with this chimeric antibody. The results were presented in Supplemental Table 2. The antibody (20.0 μg/mL) was detected in lymphocytes in the thymus, spleen, lymph nodes, bone marrow, thyroid glands, adrenal glands. The antibody was also detected in plasma cells or lymph cells in the thymus, lymph nodes, and bone marrow. In addition, B20-scFv-rFc and B0807-50G-positive cells were scattered throughout the thyroid gland and adrenal

gland (Supplemental Figures 2 and 3), suggesting the presence of plasma cells but not parenchymal cells. To confirm this hypothesis, C-CAR088 T cells were co-cultured with H6040 primary human thyroid epithelial cells (Cell Biologics) or P10482 primary human adrenal cortical cells (Innoprot) overnight. IFN-γ was not detected in the supernatant of these primary cell lines by enzyme-linked immunosorbent assay (ELISA). In contrast, IFN-γ levels were high in the supernatant of C-CAR088 T cells co-cultured with H6040 cells or P10482 cells (Supplemental Figure 4).

Off-target screening was performed using a Membrane Proteome Array (Integral Molecular), an expression array of >6,000 full-length human membrane proteins. The library was screened in duplicate in a matrix format to facilitate testing and analysis[1]. TNFRSF17(BCMA), MAG, CR2, CXADR, and DDR2 had moderate binding affinity to B20-scFv-rFc at 20.0 μg/mL (Supplemental Figure 5). To exclude false-positive results, C-CAR088 T cells were co-cultured with 293T cells transfected with MAG, CR2, CXADR, or DDR2. 293T cells transfected with BCMA and an empty vector served as positive and negative controls, respectively. C-CAR088 cells were activated only by BCMA (Supplemental Figure 6).

2. Construction of C-CAR088 CAR

The coding sequence of C-CAR088 CAR was composed of scFv against BCMA, components from the CD8a signaling and CD8a transmembrane regions, the intracellular domain of 4-1BB, and a CD3-ζ chain-derived signaling domain. The BCMA-specific CAR described by Kochendofer et al.[2] was used as a positive control

in preclinical studies. The coding sequences of both CARs were cloned into a replication-defective lentiviral vector.

3. Efficiency of C-CAR088 CAR transduction

The lentiviral vector containing C-CAR088 CAR was transfected into 293T cells for viral packaging, and the viral supernatant was collected and purified. Healthy donor PBMCs were activated with OKT-3 antibody and transduced with the lentiviral vector. Transduction efficiency was assessed by BCMA/Fc fusion protein staining (Supplemental Figure 7). Briefly, the extracellular domain of the BCMA protein was fused with the Fc fragment, and C-CAR088 CAR expressed on T cells was detected by the binding of the BCMA/Fc fusion protein and a secondary anti-Fc antibody.

4. Activation and toxicity of C-CAR088 T cells in vitro and in vivo

a. CD137 was highly expressed on activated human T cells. C-CAR088 T cells were efficiently activated by CD137, as shown previously[3]. CAR T cells were co-cultured with K562-BCMA+E7 (a K562 cell line stably expressing BCMA), stained with CD137 antibody, and analyzed by flow cytometry. C-CAR088 showed antigen-specific upregulation of CD137 expression in response to K562-BCMA+E7, a stably transduced K562 cell line expressing BCMA molecules (Supplemental Figure 8).

b. IFN-γ levels increased in C-CAR088 cells upon antigen stimulation. IFN-γ levels in C-CAR088 cells and T cells co-cultured with K562-BCMA+E7 were analyzed by ELISA.

Antigen-specific IFN-γ levels were significantly higher in co-cultured cells than in T cells alone and K562 cells not expressing BCMA (Supplemental Figure 9).

c. Toxicity of C-CAR088 in vitro

Cell proliferation assays and real-time cell analysis showed that C-CAR088 T cells were more toxic to BCMA-positive A549-BCMA-2E9 cells than BCMA-negative A549 cells, even at low effector to target ratios (Supplemental Figure 10A, Supplemental Figure 10B). Furthermore, the effect of C-CAR088 cells on BCMA-positive cells was not affected by soluble BCMA (R&D Systems) at concentrations of 100 ng/ml and 500 ng/ml. IFN-γ levels in C-CAR088 cells co-cultured with A549-BCMA-2E9 cells were not significantly affected by soluble BCMA (Supplemental Figure 10C).

5. *In vivo* treatment experiments using a murine model

a. B-NDG (NOD-*Prkdc*^{scid} *Il2rg*^{tm1}/Bcgen) mice (Biocytogen Pharmaceuticals; Beijing, China) were subcutaneously injected with RPMI-8226 cells or K562-BCMA cells to establish a tumor model. Tumors were allowed to grow to a volume of approximately 100 mm^3, and mice received a single intravenous infusion of 2.5-10.0 × 10^6 human T cells transduced with a lentiviral vector expressing C-CAR088 CAR. CAR T cell treatment cured all mice, with dramatic regressions of all tumors occurring between day 6 and 15 after the T-cell infusion (Supplemental Figures 11 and 12). In contrast, tumor volume increased in mice treated with naive T cells. Furthermore, mice receiving C-

CAR088 T cells had no signs of toxicity during treatment.

b. Toxicity of C-CAR088 T cells to RPMI-8226 cells *in vivo.* B-NDG mice (aged 6-8 weeks) received a subcutaneous injection of 2.5×10^6 RPMI-8226 cells to establish a tumor model. Tumors were allowed to grow to a volume of approximately 100 mm^3, and mice received a single intravenous infusion of (A) vehicle control, (B) untransduced T cells, or C-CAR088 CAR T cells at (C) 2.5×10^6 cells/mouse, (D) 5.0×10^6 CAR cells/mouse, and (E) 10×10^6 cells/mouse. Tumor volume was measured twice a week. (F) Survival of RPMI-8226 tumor-bearing mice. (Supplemental Figure 13).

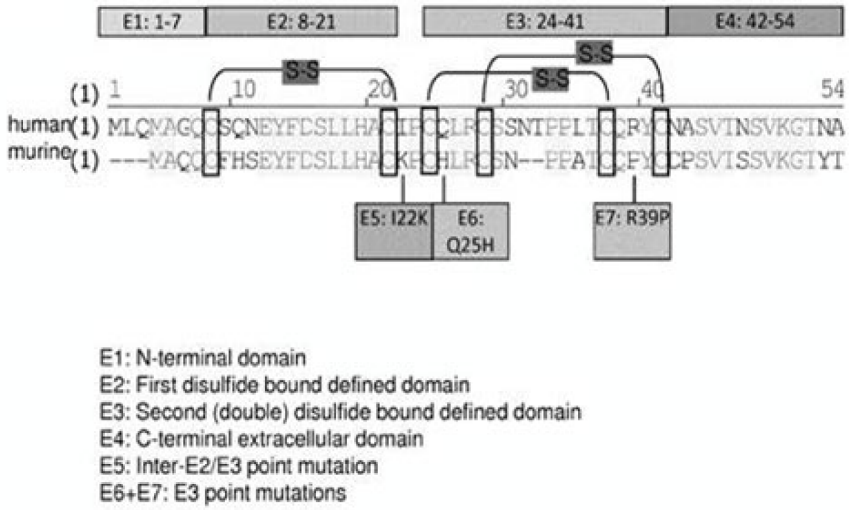
6. Clinical data of C-CAR088 in patients with MM from the first in human study

a. The rates of the common adverse events (rate ≥20%) as of the data cutoff date. (Supplemental Table 3)

b. The efficacy of C-CAR088 in patients with or without previous anti-CD38 antibody treatment in terms of PFS, DOR and OS. (Supplemental Figure 14)

Supplemental Table 1. Binding affinity (KD, in nM) of BCMA-20 to recombinant soluble B cell maturation antigen (BCMA). (Peter Kufer et al. Binding molecules for BCMA and CD3. US patent 2013/0156769 A1[P]. 2013)

Antibody	Human BCMA	Monkey BCMA
BCMA-20	0.008	0.016



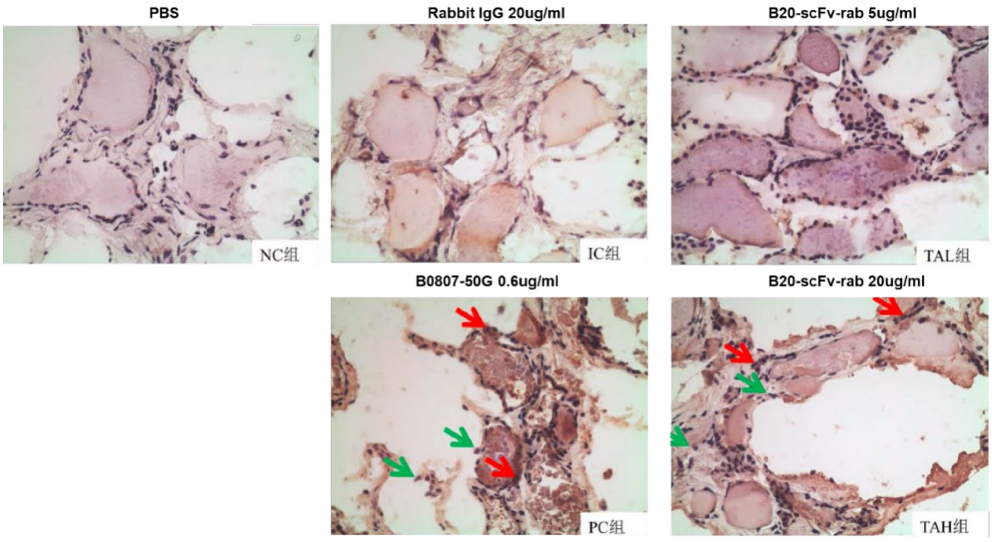
E1: N-terminal domain
E2: First disulfide bound defined domain
E3: Second (double) disulfide bound defined domain
E4: C-terminal extracellular domain
E5: Inter-E2/E3 point mutation
E6+E7: E3 point mutations

Supplemental Figure 1. Antigen-binding extracellular domain of B cell maturation antigen. (Peter Kufer et al. Binding molecules for BCMA and CD3. US patent 2013/0156769 A1[P]. 2013)

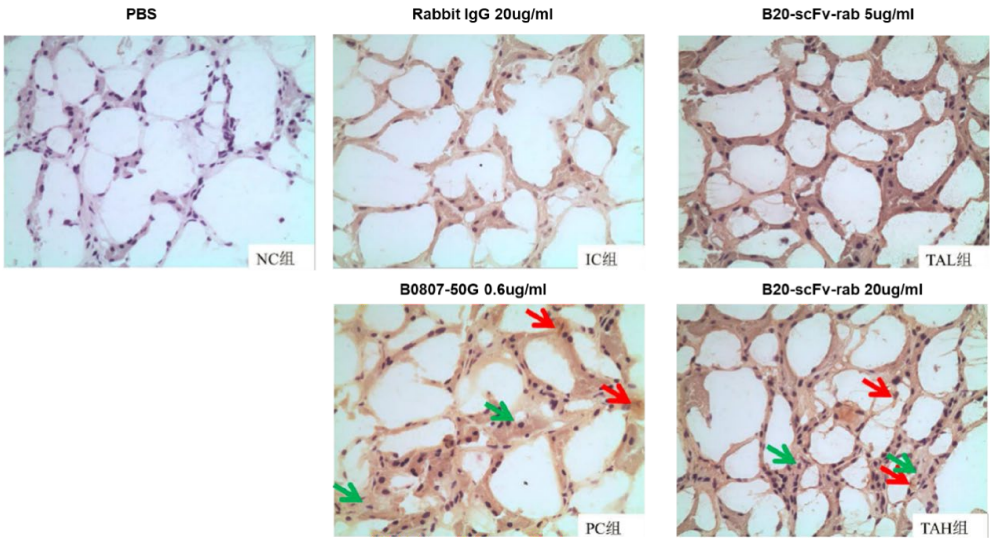
Supplemental Table 2. Tissue cross-reactivity assay results of B20-scFv-rabbit Fc staining in normal human tissues.

(a) Number of positive signals in three donors. (b) Staining intensity in positive cells and percentage of positive cells in each tissue. (–) no staining, (+) <10% of cells were stained, (++) 11–50% of cells were stained.

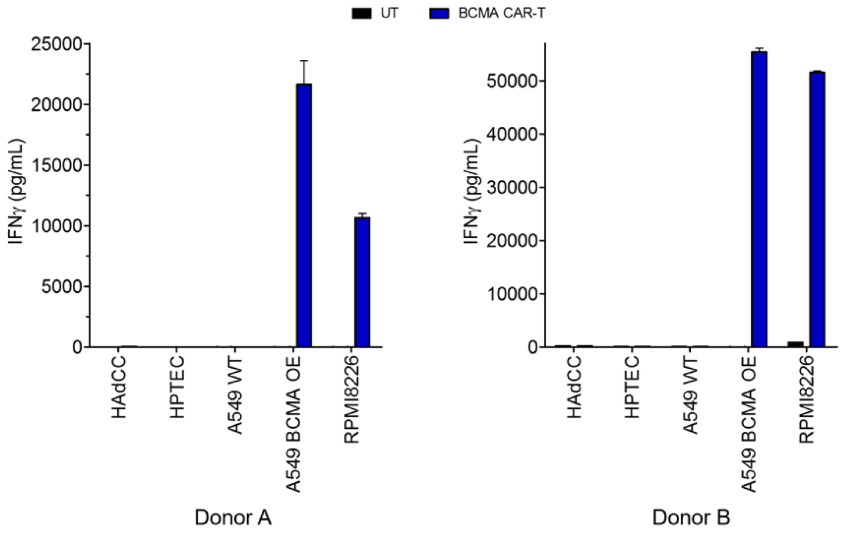
Staining site	Tissue	5.0 μg/mL	20.0 μg/mL
Nucleus	Esophagus, mucosal layer	1/3[a], +[b]	1/3, +
Cytoplasm	Stomach, mucosal layer	1/3, +	2/3, +
	Kidney, cortex	1/3, +	2/3, +
	Bladder, mucosal layer	-	1/3, +
Membrane	Thyroid	-	3/3, +
	Thymus, cortex, medulla	3/3, +	3/3, +
	Spleen, splenic cord	2/3, +	3/3, +~++
	Lymph node, medulla	3/3, +	3/3, +
	Adrenal gland, cortex	-	3/3, +
	Bone marrow	-	3/3, +



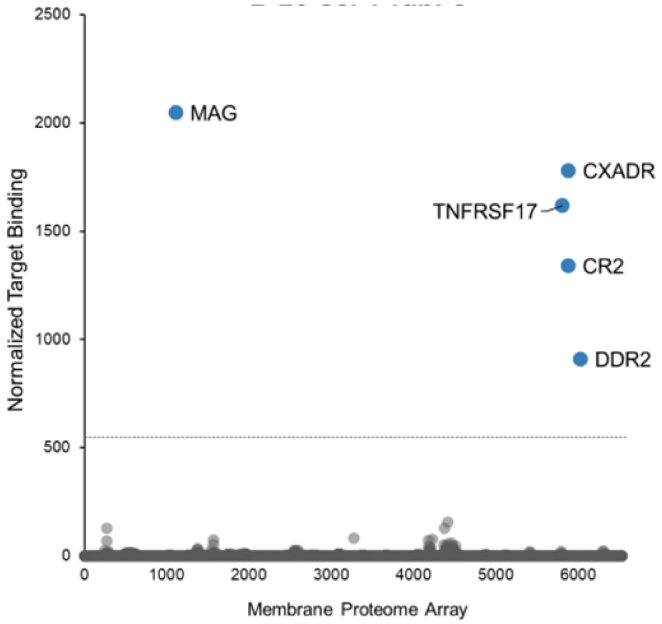
Supplemental Figure 2. Localization of B20-scFv-rFc and B0807-50G in thyroid gland sections by immunohistochemical staining.



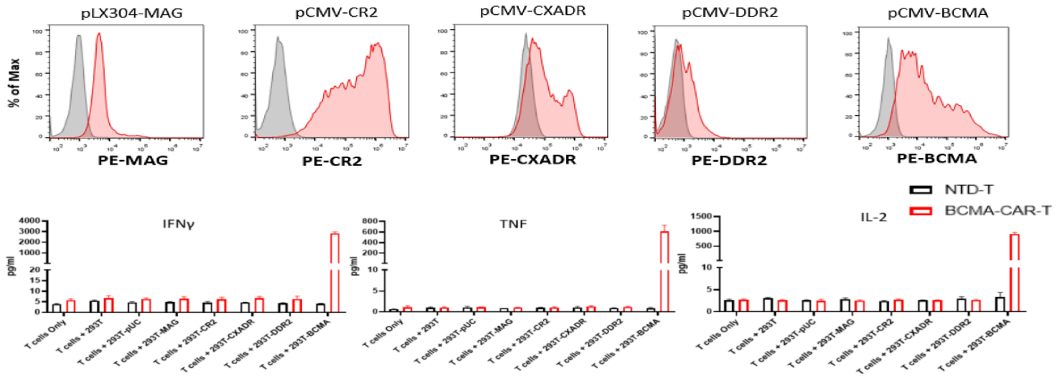
Supplemental Figure 3. Localization of B20-scFv-rabFc and B0807-50G in adrenal gland sections by immunohistochemical staining.



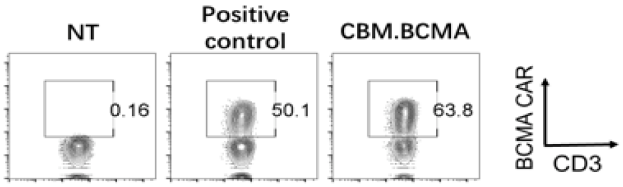
Supplemental Figure 4. IFN-γ levels in different cell types. UT: untransduced C-CAR088 CAR T cell. BCMA CAR-T: BCMA-targeting C-CAR088 CAR T cells co-cultured with primary human thyroid cells or adrenal cortical cells.



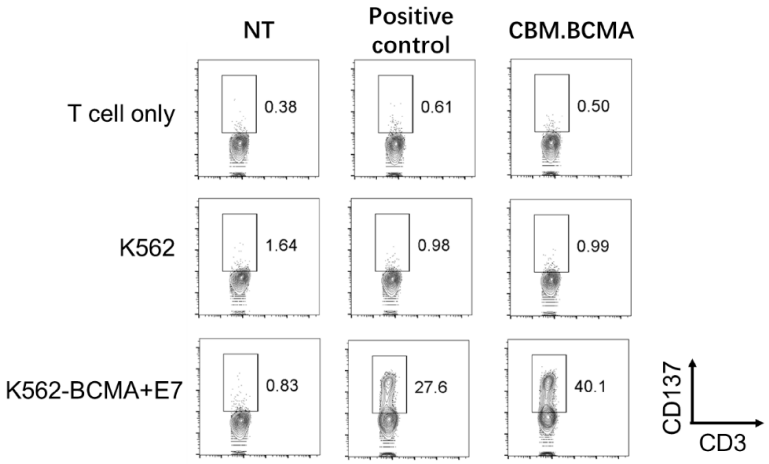
Supplemental Figure 5. Identification of targets of B20-scFv-rabFc chimeric antibody using a membrane proteome array.



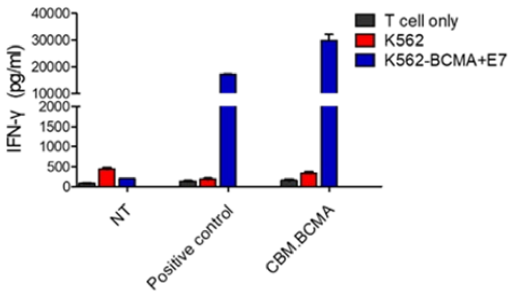
Supplemental Figure 6. C-CAR088 T cells co-cultured with 293T cells expressing MAG, CR2, CXADR, DDR2, or BCMA. Lower panel: Levels of IFN-γ, TNF, and IL-2 in T cells alone and co-cultures.



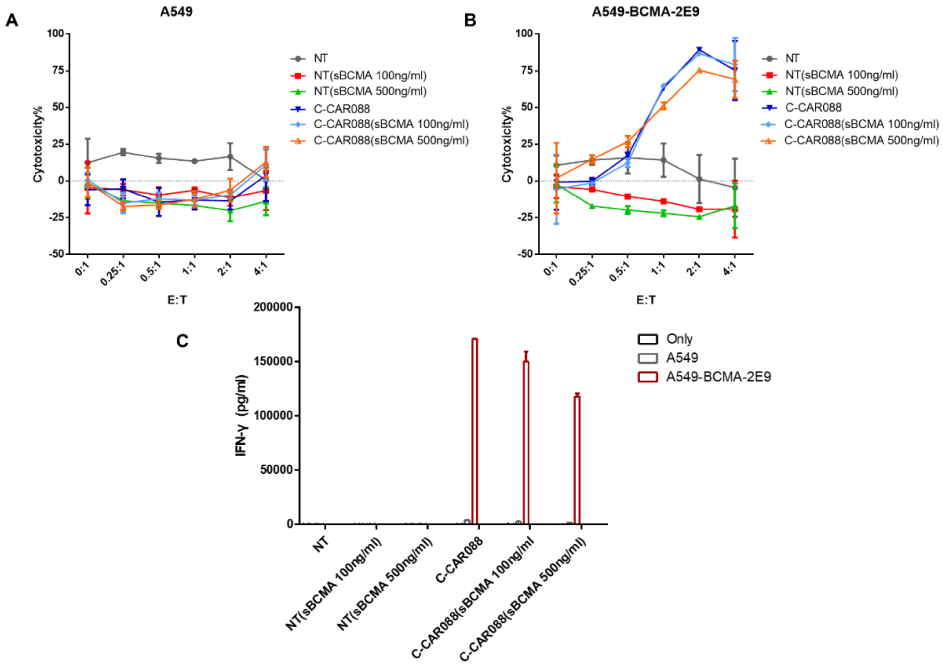
Supplemental Figure 7. Flow cytometry analysis of C-CAR088 CAR transduction efficiency. Healthy donor PBMCs were activated and transduced with a lentiviral vector expressing C-CAR088 CAR. Three days after transduction, CAR-transduced T cells (shown as CBM.BCMA) were stained with BCMA/Fc fusion protein. The anti-BCMA CAR T cells described by Kochendofer et al.[2] were used as a positive control.



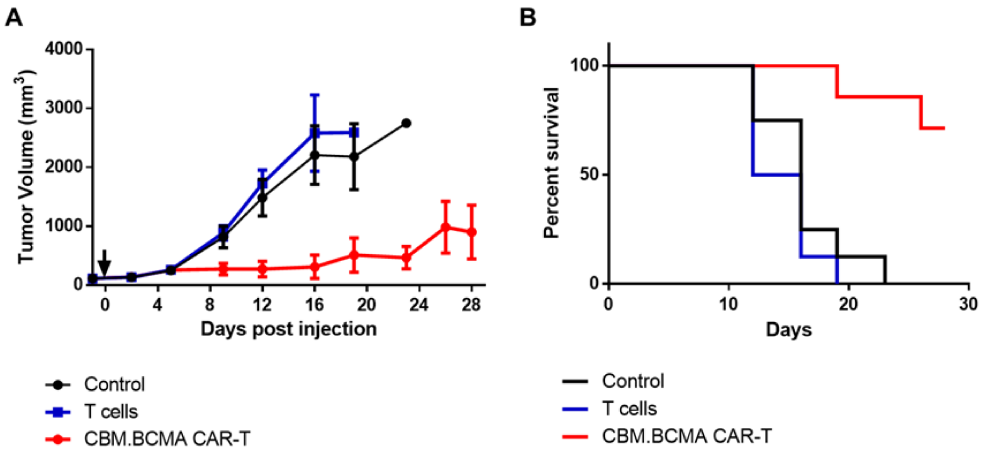
Supplemental Figure 8. Flow cytometry analysis of CD137 expression in activated C-CAR088 T cells, K562-BCMA+E7 (a K562 cell line stably expressing B cell maturation antigen [BCMA]) (positive control), and CAR T cells co-cultured with K562-BCMA+E7 (CBM.BCMA). Cells were stained with anti-CD137 and anti-CD3 antibodies.



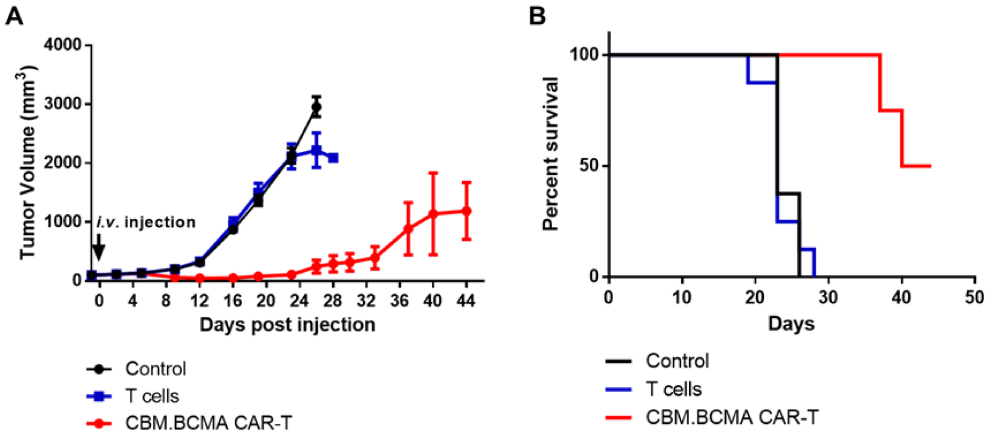
Supplemental Figure 9. IFN-γ levels in untransduced T cells, K562-BCMA+E7 (a K562 cell line stably expressing B cell maturation antigen [BCMA]) (positive control), and C-CAR088 cells co-cultured with K562-BCMA+E7 (CBM.BCMA). Cytokine levels in culture supernatants were measured by enzyme-linked immunosorbent assay.



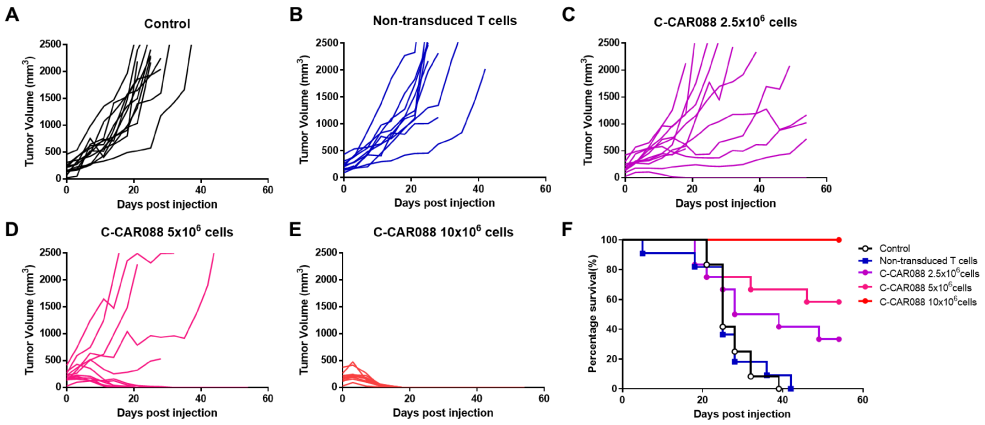
Supplemental Figure 10. Toxicity of C-CAR088 T cells in vitro. UT, untransduced; sBCMA, soluble BCMA.



Supplemental Figure 11. Cytotoxicity of anti-BCMA C-CAR088 T cells to K562 cells stably expressing B cell maturation antigen *in vivo*.



Supplemental Figure 12. Toxicity of C-CAR088 T cells to RPMI-8226 cells *in vivo.*



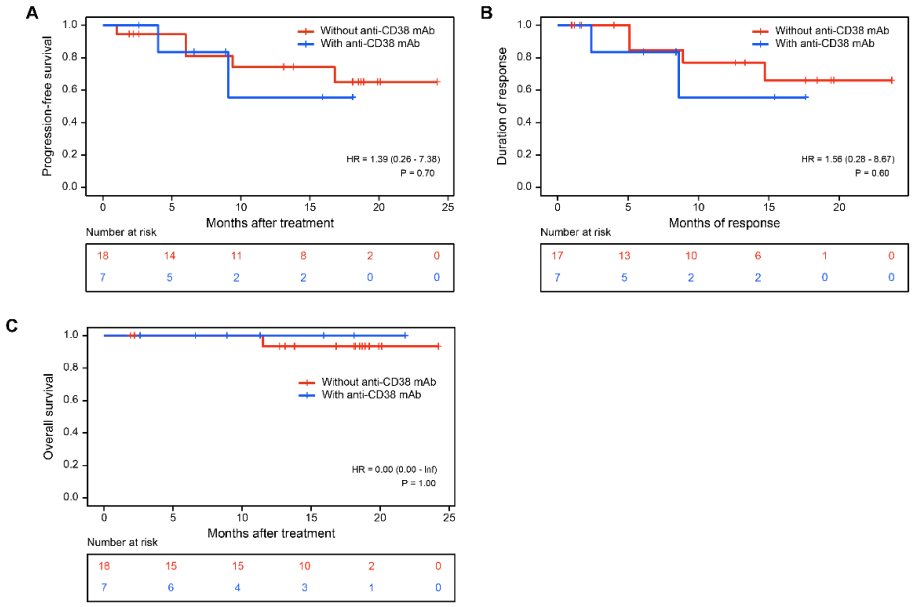
Supplemental Figure 13. Toxicity of C-CAR088 T cells to RPMI-8226 cells *in vivo.* B-NDG mice (aged 6-8 weeks) received a subcutaneous injection of 2.5×10^6 RPMI-8226 cells to establish a tumor model. Tumors were allowed to grow to a volume of approximately 100 mm^3, and mice received a single intravenous infusion of (A) vehicle control, (B) untransduced T cells, or C-CAR088 CAR T cells at (C) 2.5×10^6 cells/mouse, (D) 5.0×10^6 CAR cells/mouse, and (E) 10×10^6 cells/mouse. Tumor volume was measured twice a week. (F) Survival of RPMI-8226 tumor-bearing mice.

Supplemental Table 3. Common adverse events (rate ≥20%).

	Low-dose group N=4	Medium-dose group N=13	High-dose group N=14	Total (n=31)
Hematologic				
Leukopenia	4 (100)	13 (100)	14 (100)	31 (100)
Lymphopenia	4 (100)	13 (100)	14 (100)	31 (100)
Neutropenia	4 (100)	13 (100.0)	14 (100.0)	31 (100.0)
Thrombocytopenia	4 (100)	11 (84.6)	13 (92.9)	28 (90.3)
Anemia	4 (100)	11 (84.6)	11 (78.6)	26 (83.9)
Coagulative				
Prolonged APTT	1 (25.0)	4 (30.8)	3 (21.4)	8 (25.8)
Metabolic				
Elevated ALT	1 (25.0)	4 (30.8)	4 (28.6)	9 (29.0)
Elevated AST	2 (50.0)	8 (61.5)	6 (42.9)	16 (51.6)
Elevated LDH	3 (75.0)	6 (46.2)	9 (64.3)	18 (58.1)
Elevated ALP	2 (50.0)	4 (30.8)	6 (42.9)	12 (38.7)
Elevated GGT	1 (25.0)	5 (38.5)	2 (14.3)	8 (25.8)
Hypoalbuminemia	3 (75.0)	3 (23.1)	4 (28.6)	10 (32.3)
Hypocalcemia	4 (100.0)	8 (61.5)	2 (14.3)	14 (45.2)
Hypokalemia	4 (100.0)	7 (53.8)	6 (42.9)	17 (54.8)
Hyponatremia	2 (50.0)	7 (53.8)	3 (21.4)	12 (38.7)
Hypophosphatemia	2 (50.0)	7 (53.8)	5 (35.7)	14 (45.2)
Hypomagnesemia	2 (50.0)	5 (38.5)	3 (21.4)	10 (32.3)
Gastrointestinal disorders				
Diarrhea	0 (0.0)	5 (38.5)	6 (42.9)	11 (35.5)
Constipation	2 (50.0)	3 (23.1)	4 (28.6)	9 (29.0)
Nausea	2 (50.0)	3 (23.1)	4 (28.6)	9 (29.0)
Vomiting	2 (50.0)	3 (23.1)	3 (21.4)	8 (25.8)
Abdominal discomfort	1 (25.0)	3 (23.1)	3 (21.4)	7 (22.6)
Others				

Pyrexia	3 (75.0)	13 (100.0)	14 (100.0)	30 (96.8)
Fatigue	1 (25.0)	3 (23.1)	3 (21.4)	7 (22.6)
Pneumonia	0 (0.0)	4 (30.8)	7 (50.0)	11 (35.5)
Cough	2 (50.0)	3 (23.1)	8 (57.1)	13 (41.9)
Productive cough	0 (0.0)	4 (30.8)	4 (28.6)	8 (25.8)
Hypoxia	0 (0.0)	4 (30.8)	3 (21.4)	7 (22.6)
Hypotension	1 (25.0)	4 (30.8)	2 (14.3)	7 (22.6)
CRS	3 (75.0)	13 (100.0)	13 (92.9)	29 (93.5)

Abbreviations: APTT, activated partial thrombin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; CRS, cytokine release syndrome.



Supplemental Figure 14. (A) The progression-free survival (PFS) estimation of patients with or without previous anti-CD38 monoclonal antibody(mAb); (B) The duration of response (DOR) estimation of patients with or without previous anti-CD38 mAb; (C) The overall survival (OS) estimation of patients with or without previous anti-CD38 mAb.

References:

1.Tucker DF, Sullivan JT, Mattia KA, et al. Isolation of state-dependent monoclonal antibodies against the 12-transmembrane domain glucose transporter 4 using virus-like particles. *Proc Natl Acad Sci USA* 2018;115:E4990-E99.

2.Carpenter RO, Evbuomwan MO, Pittaluga S, et al. B-cell maturation antigen is a promising targ et for adoptive T-cell therapy of multiple myeloma. *Clin Cancer Res* 2013;19:2048-60.

3.Wolfl M, Kuball J, Ho WY, et al. Activation-induced expression of CD137 permits detection, is olation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiri ng knowledge of epitope specificities. *Blood* 2007;110:201-10.