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Supplemental Information

Preclinical Evaluation of Allogeneic CAR T Cells Targeting BCMA for the Treatment of Multiple Myeloma

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SUPPEMENTAL INFORMATION

Supplemental Figures

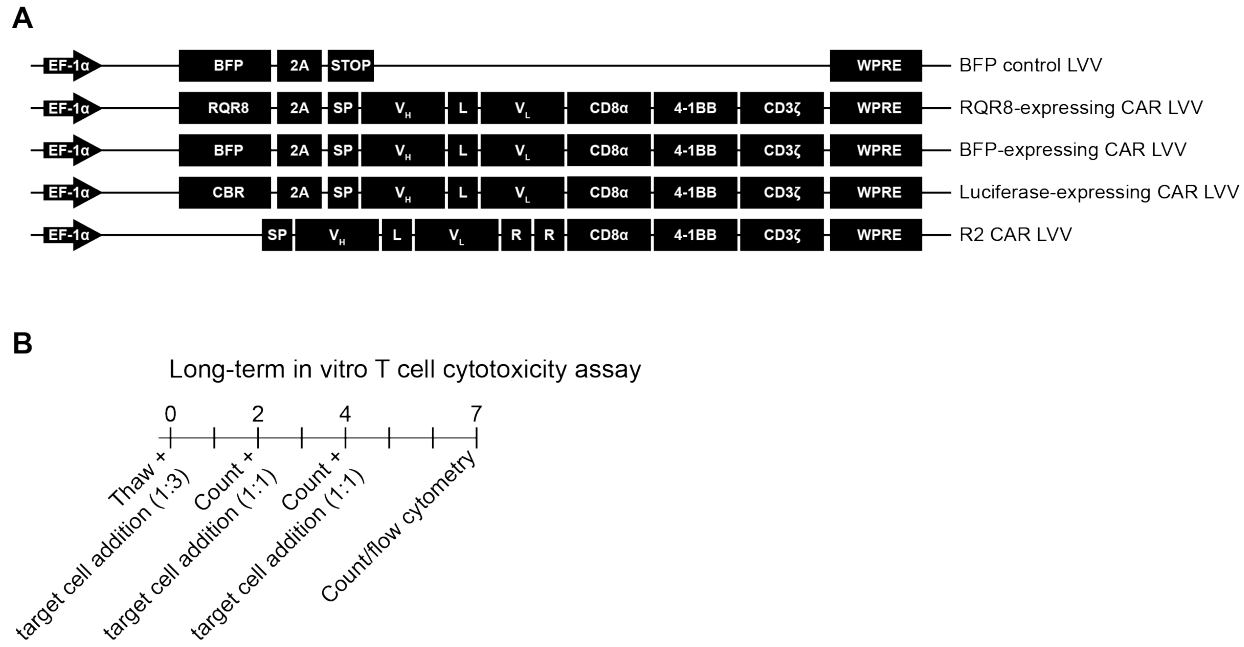

Figure S1. Constructs and experimental outlines. (A) Schematic representation of the lentiviral backbone coding for control or BCMA CAR under control of the human EF-1α promoter. SP: signal peptide, V_H: scFv heavy chain, V_L: scFv light chain: L: GS linker, R: rituximab mimotope flanked by GS linker, 2A: self-cleaving peptide, RQR8: rituximab/ QBEND10-binding fusion protein, CBR: click-beetle red luciferase, BFP: blue-fluorescent protein, WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element. (B) Schematic representation of the long-term cytotoxicity assay after recovery of CAR T from cryopreservation.

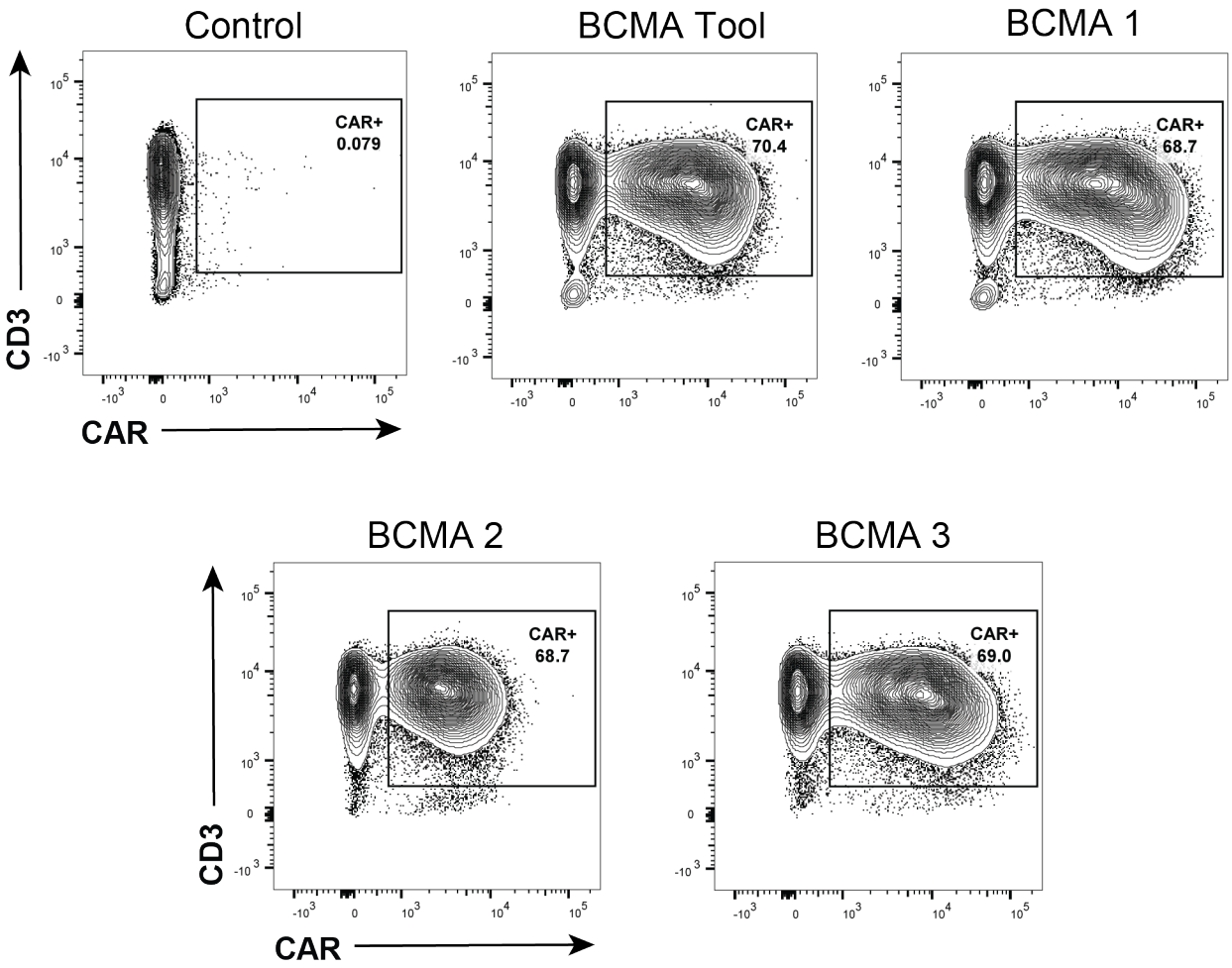

Figure S2. Flow cytometry analysis of CAR expression in BCMA CAR T. Activated T cells were transduced with lentiviral vectors encoding the BCMA CARs and CAR expression was detected by staining with biotinylated-soluble BCMA followed by PE-streptavidin. FACS plots from a donor with high transduction efficiencies are shown.

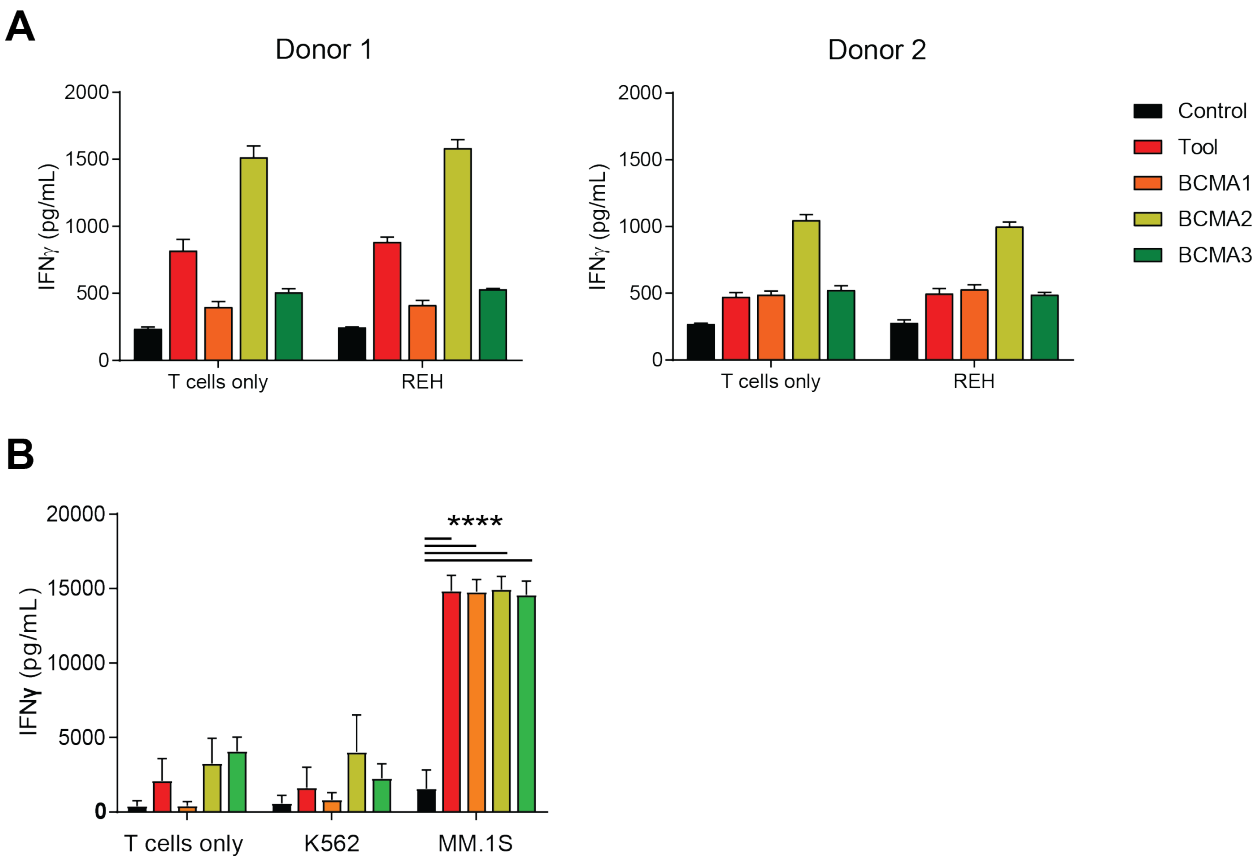

Figure S3. BCMA CAR T exhibit no changes in INFγ secretion when exposed to BCMA-negative cells. (A) IFNγ concentrations were measured on cryopreserved supernatants of BCMA CAR T cultured for 24 hours either alone or with an equal number of BCMA-negative REH cells using ELISA. (B) IFNγ concentrations were measured on cryopreserved supernatants of BCMA CAR T cultured for 24 hours either alone, with BCMA-negative K562 cells, or with BCMA-expressing MM.1S cells, n=4 donors, E:T = 1:1. Data were analyzed using Tukey's one-way ANOVA and all results are shown as mean ± SEM, ****p<0.0001.

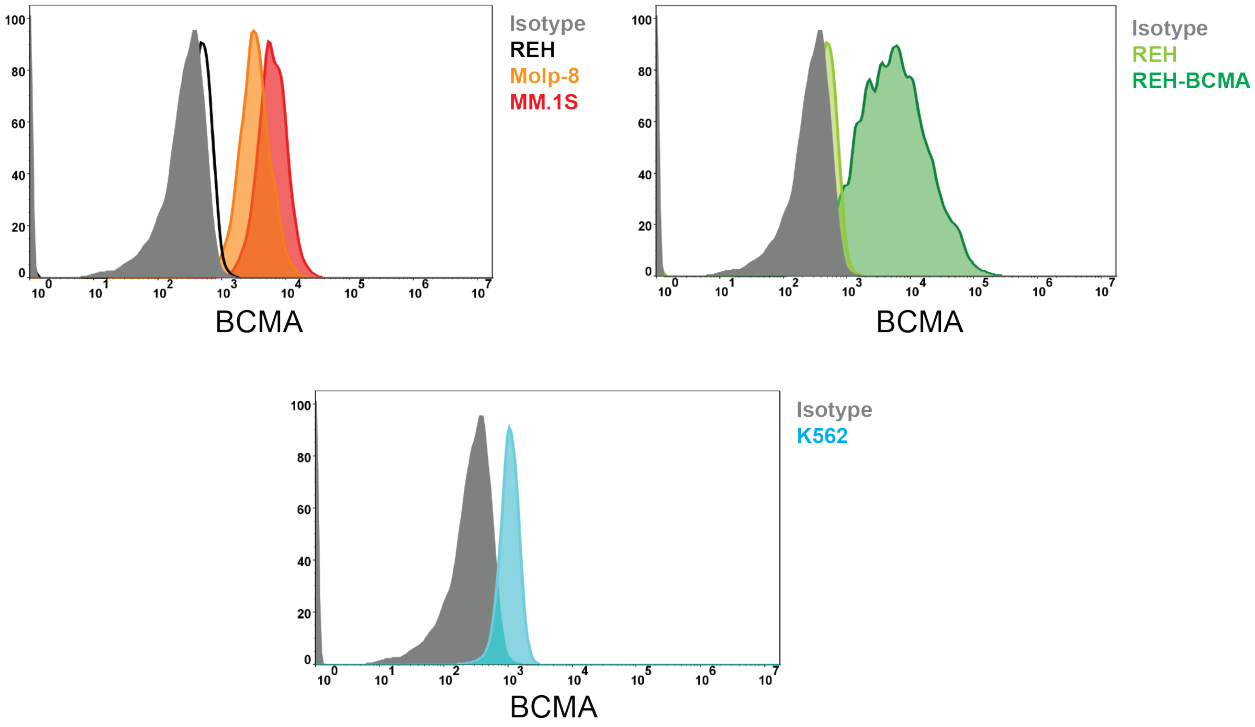

Figure S4. Surface expression of BCMA in target and control cell lines. The surface expression of human BCMA in multiple myeloma (MM.1S, Molp-8) and control cell lines (K562, REH), and in REH cells overexpressing BCMA was assessed by flow cytometry.

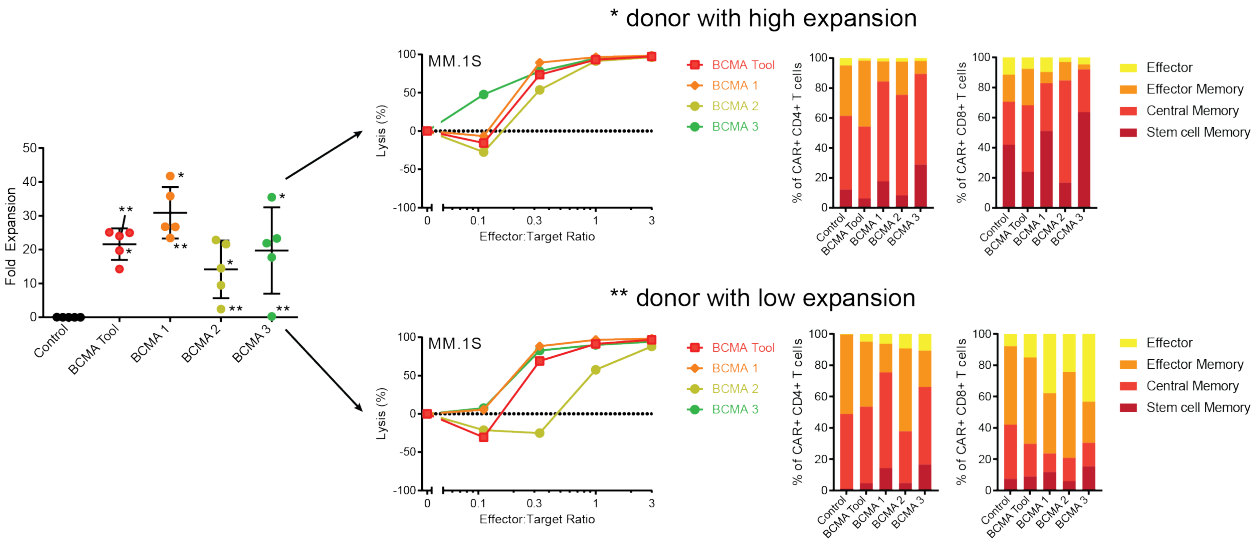

Figure S5. Differences in T cell phenotype affect target-dependent BCMA CAR T expansion and activity. CAR T were stained for CD45RO and CD62L 14 days after expansion and before cryopreservation and analyzed using flow cytometry. CAR T were thawed and expanded on BCMA-expressing target cells for 3 rounds in 7 days, and CAR T expansion was measured using flow cytometry. Expanded CAR T were then cultured with luciferase-expressing MM.1S target cells for 24 hours and cytotoxicity was assessed using bioluminescence readers. Two individual donor products with low and high expansion profiles and their correlating cytotoxicity and phenotype profiles are shown.

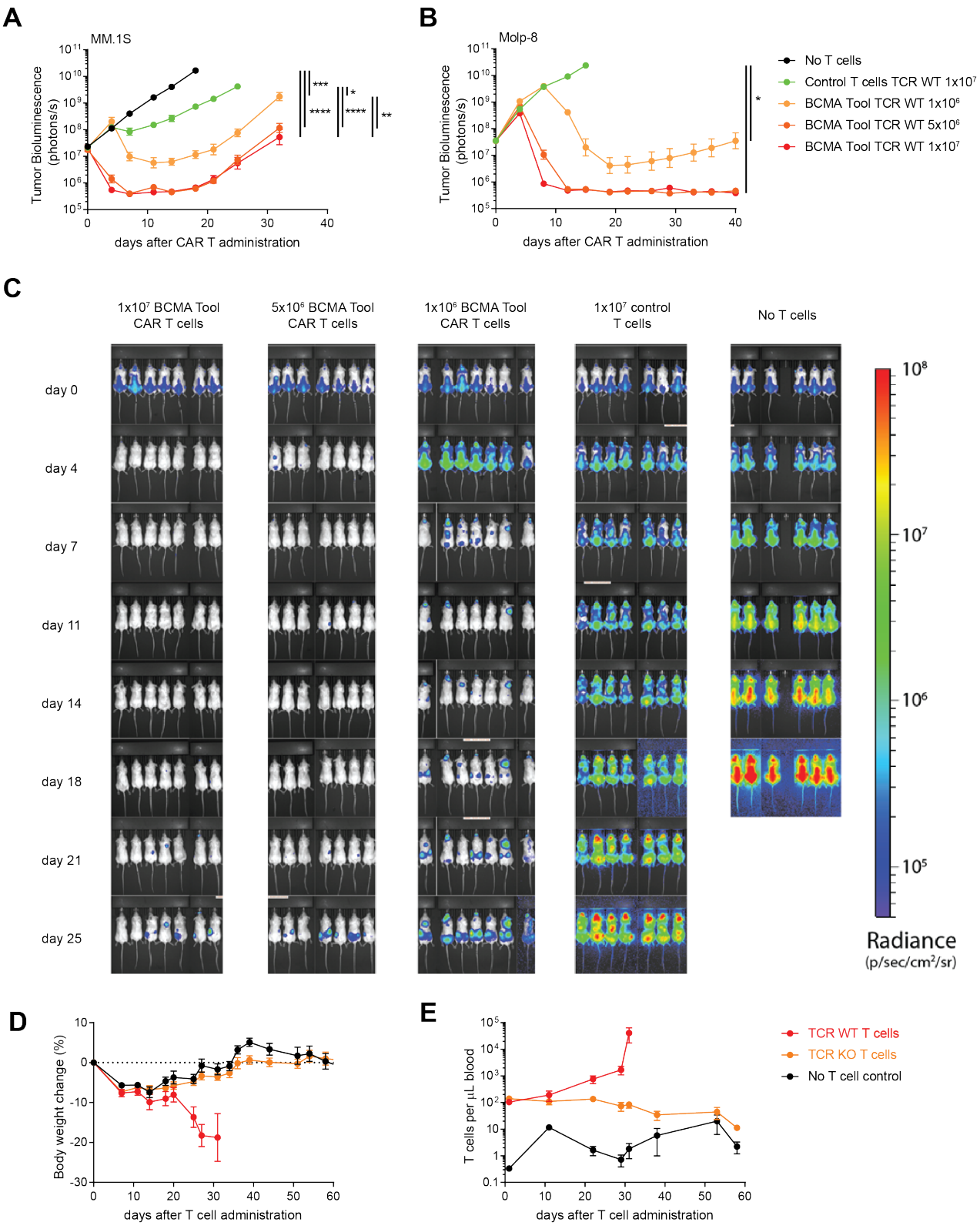

Figure S6. Development of animal models to test BCMA CAR T activity. (A-B) BCMA tool CAR T showed potent efficacy in two orthotopic tumor models. NSG mice were injected with luciferase-expressing MM.1S (A) or Molp-8 cells (B). Tumor-bearing mice received the indicated dose of CAR$^+$ cells together with control T cells to

match the total number of T cells across all groups (total of $1.3x10^7$ cells). Tumor growth was assessed using whole-body luminescence imaging. n=7 in (A), n=10 in (B). (C) Tumor-bearing mice treated with BCMA CAR T relapse with discrete tumors outside of the bone marrow. Bioluminescence images of mice in (A). (D-E) Gene-edited BCMA CAR T did not cause GvHD in a xenogeneic model. (D) NSG mice received 2 Gy total body irradiation and $3x10^7$ T cells ($1.8\text{-}2.0x10^7$ CAR^+ cells) and body weight was measured as readout for GvH responses. n=10 animals. (E) Peripheral blood cells of mice in (D) were stained using CD45 antibodies and quantified using flow cytometry. A-B were analyzed using Tukey's repeated measures one-way ANOVA. All results are shown as mean ± SEM. Asterisks show statistical significance against the indicated condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

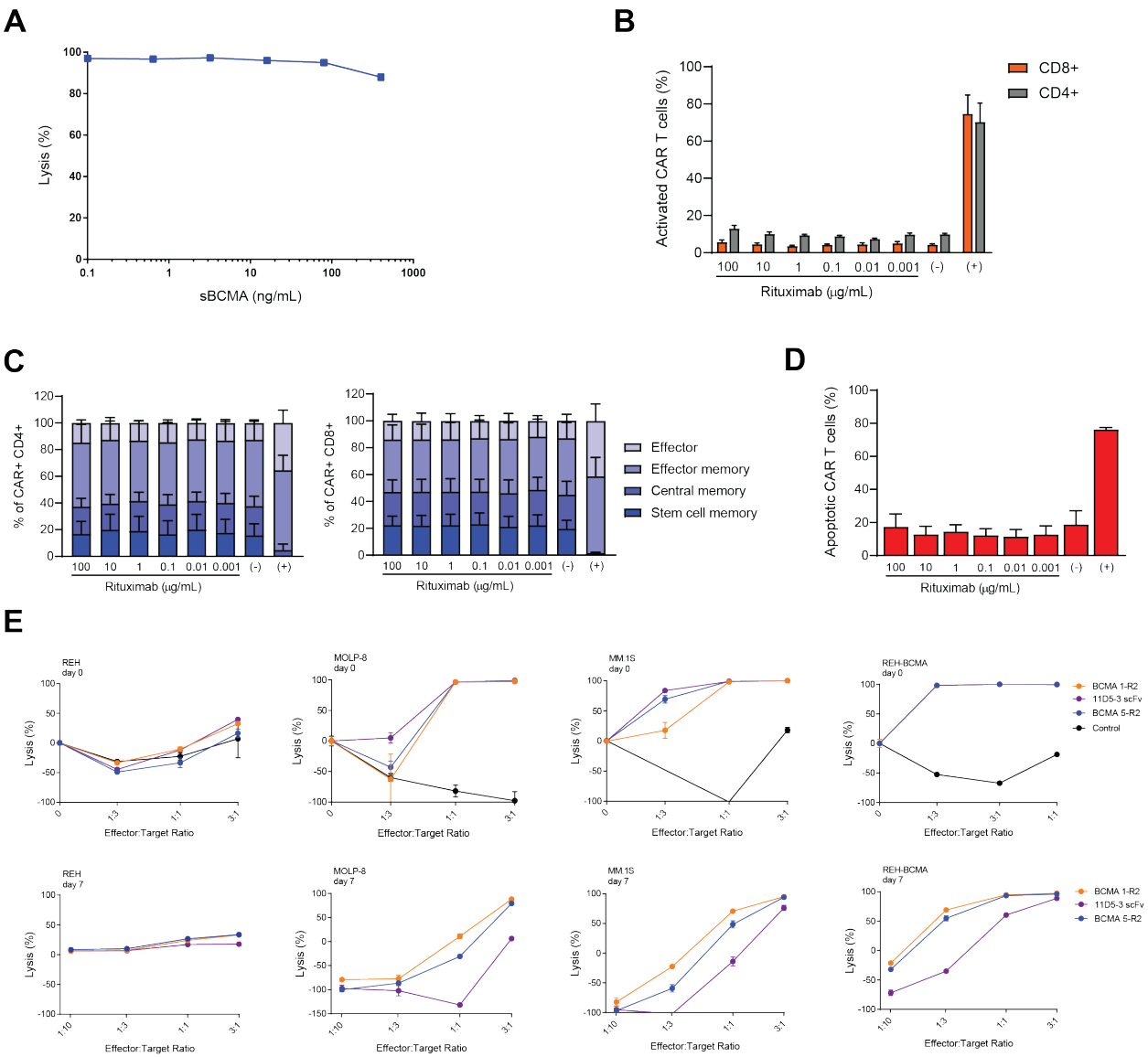

Figure S7. Effects of soluble BCMA (sBCMA) and rituximab on BCMA CAR T and comparison with 11D5-3 anti-BCMA CAR. (A) BCMA 1-R2 CAR T were cultured with luciferase-expressing MM.1S cells in the presence of increasing concentrations of sBCMA (up to 400 ng/mL) and residual target cell viability was determined at 24 hours by luminescence analysis. (B-C) Rituximab does not influence BCMA 1-R2 CAR T activation or differentiation, as evaluated by flow cytometry analysis of the expression of 4-1BB (B) and the differentiation markers CD62L and CD45RO (C) on CAR T cultured with increasing concentrations of the antibody for 24 hours. CAR T stimulated with PMA/ionomycin served as positive control (n=3 donors). (D) Rituximab does not influence the apoptotic rate of BCMA 1-R2 CAR T, as determined by flow cytometry analysis of CAR T cultured with increasing concentrations of the antibody for 24 hours using Annexin V protein conjugated with FITC. CAR T stimulated with PMA/ionomycin served as positive control (n=3 donors). (E) Comparable activity of BCMA 1-R2, BCMA 5-R2 and 11D5-3 CAR T. The 11D5-3 CAR consists of the 11D5-3 scFv followed by a CD8 hinge/TM region and 4-1BB and CD3ζ signaling domains. CAR T were cultured with luciferase-expressing target cell lines or a BCMA-negative control cell line and residual luciferase activity was measured at 24 hours (*Top panel*). The activities of the three CARs were also evaluated after seven days of continuous exposure to target (*Bottom panel*).

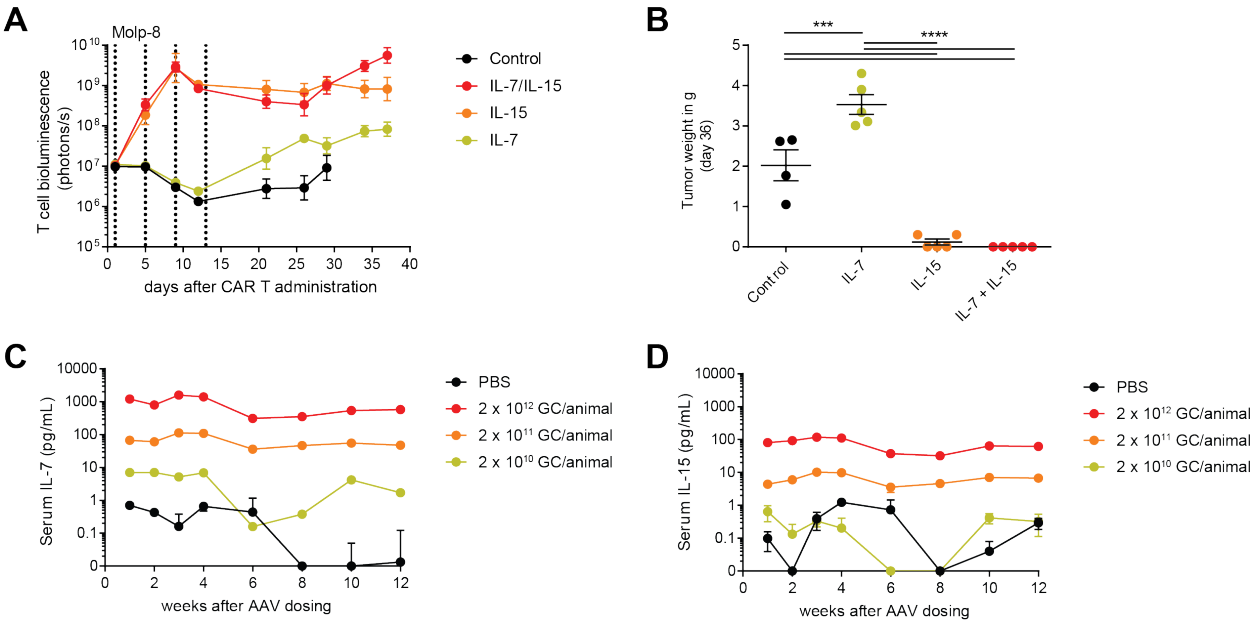

Figure S8. Exogenous homeostatic cytokines support expansion of BCMA CAR T in NSG mice. (A-B) Exogenous IL-15 enhanced proliferation and activity of BCMA Tool CAR T in an orthotopic Molp-8 model. (A) NSG mice were injected with Molp-8 cells and $1x10^6$ click-beetle red luciferase-expressing BCMA Tool CAR T (total of $8.3x10^6$ T cells) 8 days later. 500 ng of human recombinant IL-7 and IL-15 were complexed in vitro with equimolar amounts of either IL-7 or IL-15Rα-Fc for 30 minutes before i.p. administration to mice as indicated by dotted lines. CAR T expansion and persistence were assessed using whole-body luminescence imaging. n=5 mice per group. (B) Total tumor mass of animals in (A) was determined during necropsy on day 36 after CAR T administration. Visible tumors were collected from all body parts except the brain. n=4-5 animals, Tukey's one-way ANOVA. (C-D) Mice injected with AAV9 pseudotyped viruses coding for human IL-7 or IL-15/IL-15Rα fusion proteins stably express human cytokines at near physiological levels. Viruses were injected at the indicated titers (genomic contents per animal) into the tail vein of NSG mice and the cytokine concentration in peripheral blood was determined using ELISA. n=3 animals.

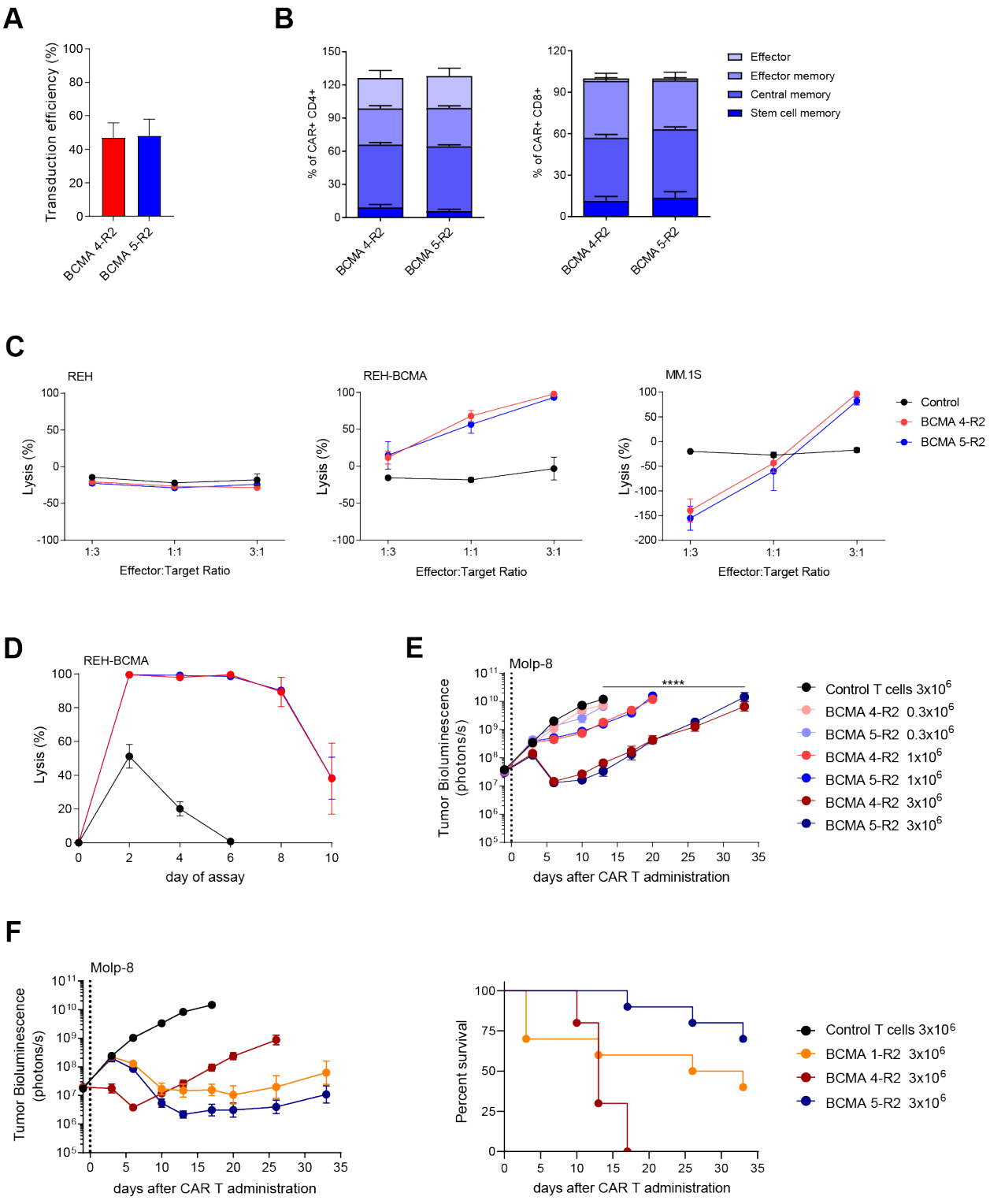

Figure S9. Two additional BCMA CAR T candidates have equivalent activity in vitro and in vivo. (A) Both BCMA CAR T candidates demonstrated similar transduction efficiencies as detected by soluble BCMA staining or BFP expression via flow cytometry at day 16 of expansion. n=5 donors. (B) BCMA CAR T candidates showed similar differentiation phenotypes. CAR T were analyzed using flow cytometry 16 days after activation and phenotypes were assigned according to CD62L and CD45RO expression within the CAR^+ population. n=5 donors.

(C) BCMA CAR T candidates showed similar cytotoxicity against BCMA-expressing target cells. CAR T were cultured with luciferase-expressing BCMA-negative REH cells, REH cells overexpressing BCMA, or the MM cell line MM.1S. Target cell luminescence was assessed after 24 hours. n=5 donors. (D) Candidates maintained cytotoxicity after repeated exposure to target cells. $1x10^6$ BCMA CAR T were mixed with $1x10^6$ GFP-expressing REH-BCMA cells and incubated for 48 hours. 5% of the culture was removed and GFP^+ tumor cells were quantified using flow cytometry. $1x10^6$ REH-BCMA cells were added to the culture and the assay was repeated for a total of 5 times. n=4 donors. (E) BCMA CAR T candidates performed similarly in an orthotopic Molp-8 tumor model. Tumor-bearing animals received TCR-wild type CAR^+ cells at the indicated doses in a total of $6.9x10^6$ cells. Tumor growth was assessed using whole-body luminescence imaging (n=10). (F) BCMA 4-R2 and BCMA 5-R2 were tested against BCMA 1-R2 CAR T in an orthotopic Molp-8 tumor model as in (E). n=8-10. A-C were analyzed using Tukey's one-way ANOVA. E and F were analyzed using Tukey's repeated-measures one-way ANOVA. All results are shown as mean ± SEM. Asterisks show statistical significance against the indicated condition, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Table S1. Binding affinities of anti-BCMA antibodies

Antibody	K_D (nM)
BCMA 1	0.19 nM
BCMA 2	0.54 nM
BCMA 3	0.04 nM
BCMA 4	0.038 nM
BCMA 5	5.57 nM

The affinities of the anti-BCMA antibodies for human BCMA were determined at 37°C by surface plasmon resonance. K_D = equilibrium dissociation constant.

Supplemental Methods

Cells and culture conditions

Cell lines and primary cells were cultured in a humidified incubator at 37ºC and 5% CO_2. The cell lines HEK293T (CRL-3216), K562 (CCL-243), MM.1S (CRL-2974), and REH (CRL-8286) were acquired from ATCC (Manassas, VA). Molp-8 cells (ACC-569) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Tumor cell lines were engineered to express Luciferase and GFP using Luc2AGFP lentivirus (AMSbio, Cambridge, MA). Peripheral blood mononuclear cells (PBMC) were sourced from Stanford Blood Center (Palo Alto, CA) or Stemcell Technologies (Vancouver, BC, Canada) and T cells were isolated using the human Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA).

Protein Reagents

The extracellular domain of the human BCMA protein (NM_001192.2) fused to a C-terminal 10x His Tag and Avi Tag was expressed in Expi293 cells and purified from culture supernatants. The recombinant protein was biotinylated using the Bulk BirA ligase reaction kit (Avidity, Aurora, CO). Rituximab and alemtuzumab sequences were obtained from public sources. An anti-idiotype antibody against the BCMA 1 scFv was raised in Balb/c mice and was purified from hybridoma cell culture supernatants. Antibodies were labeled with R-PE (Prozyme, Hayward, CA) using sulfhydryl-maleimide reaction chemistry.

Lentiviral vector production

HEK293T cells were transfected with lentiviral transfer vectors, psPAX2, and pMD.2G (École Polytechnique Fédérale de Lausanne, France) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). Twenty-four hours after transfection, the medium was replaced with X-Vivo 15 medium (Lonza, Basel, Switzerland) supplemented with 10% FCS (GE Healthcare, Pittsburgh, PA). Virus-containing supernatants were harvested 24 hours later.

Production of allogeneic CAR T

T cells were isolated from healthy donor PBMC using T cell negative selection kits (Miltenyi Biotec, Auburn, CA Biotec) and cryopreserved in FCS/10% DMSO. Immediately after thawing, T cells were cultured in X-Vivo 15 medium (Lonza) supplemented with 10% FCS (GE Healthcare, Pittsburgh, PA) and 20 ng/mL IL-2 (Miltenyi Biotec, Auburn, CA) and activated using T cell TransAct™ (Miltenyi Biotec, Auburn, CA). 2 days after activation, T cells were transduced for 24 hours, then resuspended in X-Vivo 15 medium supplemented with 5% human AB serum (Gemini Bio-Products, West Sacramento, CA). IL-2 was supplemented every 2 days. Plasmids encoding TALEN® monomers for TRAC and CD52 were obtained from Cellectis, Paris, France (Poirot et al, 2015) and used for TALEN® mRNA synthesis (TriLink Biotechnologies, San Diego, CA). 7 days after activation, T cells were electroporated with 25 μg/mL per TALEN® mRNA using AgilePulse MAX electroporators (BTX, Holliston, MA). T cells were cultured in X-Vivo 15 medium with 5% human serum and 20 ng/mL IL-2 at 30ºC for 24 hours before continuing expansion at 37ºC. 17 days after activation, depletion of $TCR\alpha^+$ cells was performed using CliniMACS TCRαβ kits (Miltenyi Biotec, Auburn, CA), followed by recovery in X-Vivo 15 medium supplemented with 5% human serum and 20 ng/mL IL-2. 18 days after activation, T cells were cryopreserved in 90% FCS/10% DMSO using rate-controlled freezing chambers and stored in liquid nitrogen vapor phase. For large-scale production, the same protocol was followed with adaptations: Healthy-donor PBMC (Hemacare, Los Angeles, CA) were thawed and activated 24 hours later with T cell Transact™ (Miltenyi Biotec, Auburn, CA) and IL-2. 3 days after activation, T cells were transduced with concentrated lentiviral vector (Miltenyi Biotec, Auburn, CA) and electroporated with TALEN® mRNA 48 hours later. 7 days after activation, cells were expanded in 2L WAVE bag bioreactors (GE Healthcare, Pittsburgh, PA). Depletion of $TCR\alpha^+$ cells was performed 17 days after activation and T cells were frozen 18 days after activation in CS5 cryoprotectant (Stemcell Technologies, Vancouver, BC, Canada). A schematic of T cell culture timelines is given in Figure 6A. All functional assays were performed with cells after recovery from cryopreservation.

Determination of the binding affinities of anti-BCMA antibodies to human BCMA

Surface Plasmon Resonance (SPR) experiments were performed as follows. An anti-human IgG Fc capture chip was prepared by amine-coupling of goat anti-human IgG Fc (Southern Biotech, Birmingham, AL) to a Biacore Series S sensor chip CM4 (GE Lifesciences, Marlborough, MA) surface at 25°C. Kinetic assays were conducted at 37°C in running buffer HBST+ supplemented with 1 mg/mL bovine serum albumin. IgG-containing cell culture supernatants were injected for 2 minutes at 10 µL/min onto a downstream flowcell (resulting in different IgGs being immobilized in flowcell 2, 3 or 4). In all experiments, flowcell 1 was used as a reference surface. Human BCMA was diluted into running buffer at concentrations of 5 and 25 nM, injected as analyte for two minutes at 30 µL/min and dissociation was monitored for 20 minutes. The anti-human IgG Fc surfaces were regenerated using three 60-second injections of 75 mM phosphoric acid between each analyte binding cycle. All sensorgrams were double-referenced and fit to a 1:1 Langmuir binding with mass transport model using Biacore T200 Evaluation Software (Version 2.0).

References

Poirot L, Philip B, Schiffer-Mannioui C, et al. Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies. Cancer research. 2015;75(18):3853-3864.