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Supplemental Information

PSMA-Directed CAR T Cells Combined with Low-Dose Docetaxel Treatment Induce Tumor Regression in a Prostate Cancer Xenograft Model

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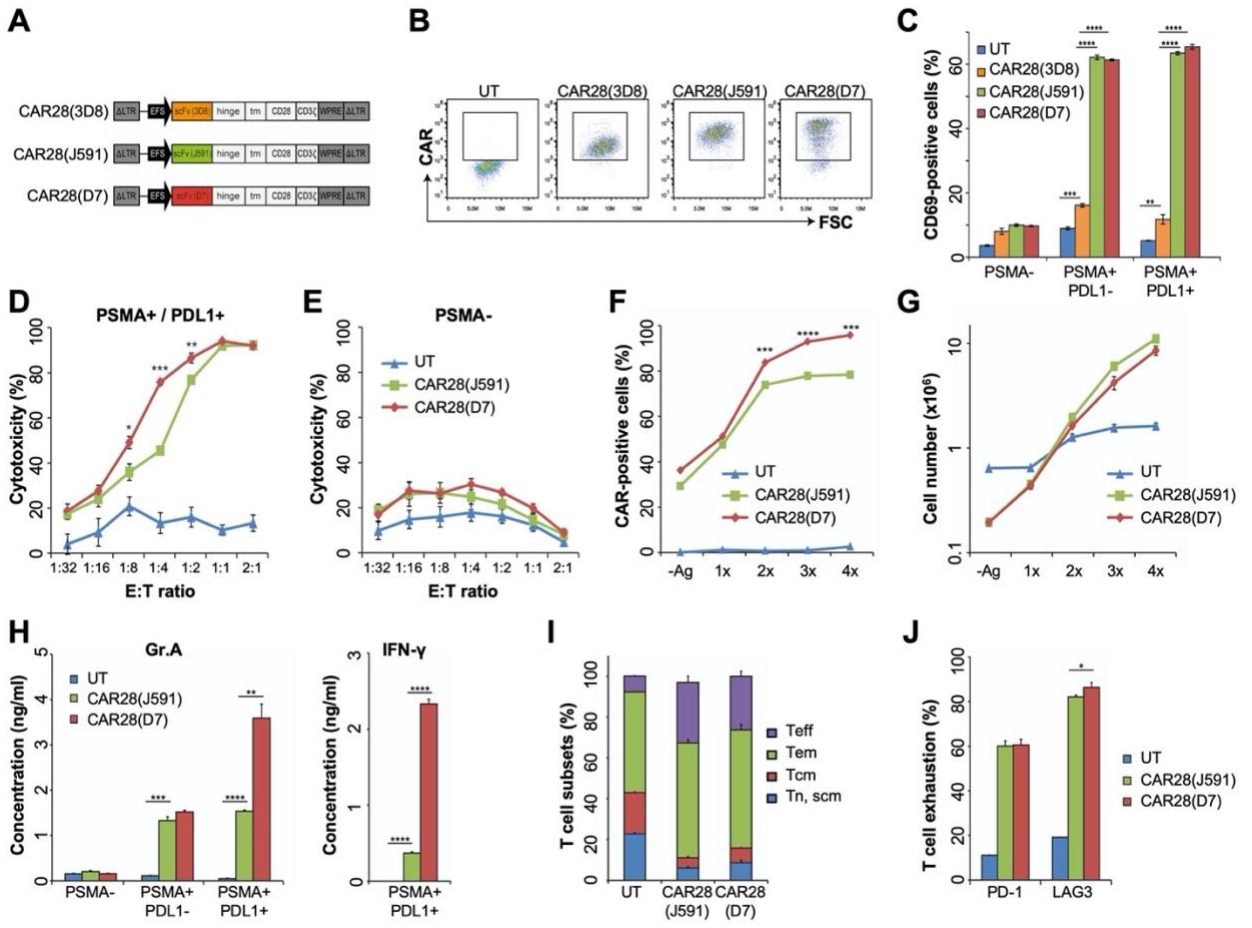

Figure S1. Characterization of PSMA-CARs. (A) Schematic of γ-retroviral vectors. CAR expression is driven by an EFS promoter. The CARs consist of a single chain variable (scFv) fragment derived from 3D8, J591 or D7 antibodies, respectively, a hinge region (modified Fc IgG1 domain), a transmembrane domain, a costimulatory domain derived from CD28 with a mutated LcK binding moiety, and an intracellular signaling domain derived from CD3ζ chain. **(B)** CAR expression. Jurkat cells were transduced with retroviral particles, followed by expansion and staining with anti-human IgG antibody. (**C**) PSMA-mediated activation of CAR expressing Jurkat cells. Cells were co-cultured at a 1:1 effector-to-target (E:T) ratio with either C4-2 (PSMA-positive/PDL1-negative) or LNCap (PSMA-positive/PDL1-positive) tumor cells. PSMA-negative DU145 cells served as control. Activation was assessed by evaluating the percentage of CD69 positive cells (n=3). (**D-E**) Cytolytic activity. CAR T cells were co-cultured at indicated E:T ratios with either LNCaP (D) or DU145 (E) tumor cells. Cytotoxicity was determined by cell viability assay (% cytotoxicity = 100 – % viability). (**F-G**) Antigen-specific CAR T cell proliferation. CAR T cells were propagated without antigen stimulation (-Ag) or exposed to irradiated PSMA-positive C4-2 cells up to 4 times (1–4x) at E:T ratios of 1:1. Cells were stained with CD3 and anti-human IgG (CAR) antibodies to determine the fraction of CAR-positive cells (F) or the total number of CAR-positive cells (G). (**H**) Granzyme and cytokine release. Untransduced or CAR T cells were co-cultured at a 1:1 effector-to-target (E:T) ratio with either C4-2 (PSMA-positive/PDL1-negative) or LNCap (PSMA-positive/PDL1-positive) tumor cells. PSMA-negative DU145 cells served as control. Granzyme A (Gr.A) or IFNγ concentration in supernatant was quantified by cytometric bead array (n=3). (**I-J**) Untransduced T cells (UT) or CAR T cells were co-cultured at a 1:1 E:T ratio with C4-2 cells. (**I**) CAR T cell phenotype. Shown are the percentages of T cell subsets (n=3) based on CD62L and CD45RA expression. (**J**) CAR T cell exhaustion. Shown are the percentages of PD-1 or LAG3 positive cells (n=3). Cells were pre-gated on CD3 for UT, or CD3/CAR for CAR T cells. Statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), or ****(P<0.0001). UT, untransduced T cells; PDL1, programmed cell death ligand 1; ΔLTR, long-terminal repeat with deletion in U3 region; EFS, elongation factor 1α short promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; Tn/Tscm, T cell naïve or T stem cell memory; Tcm, T cell central memory; Tem, T cell effector memory; Teff, T cell effector.

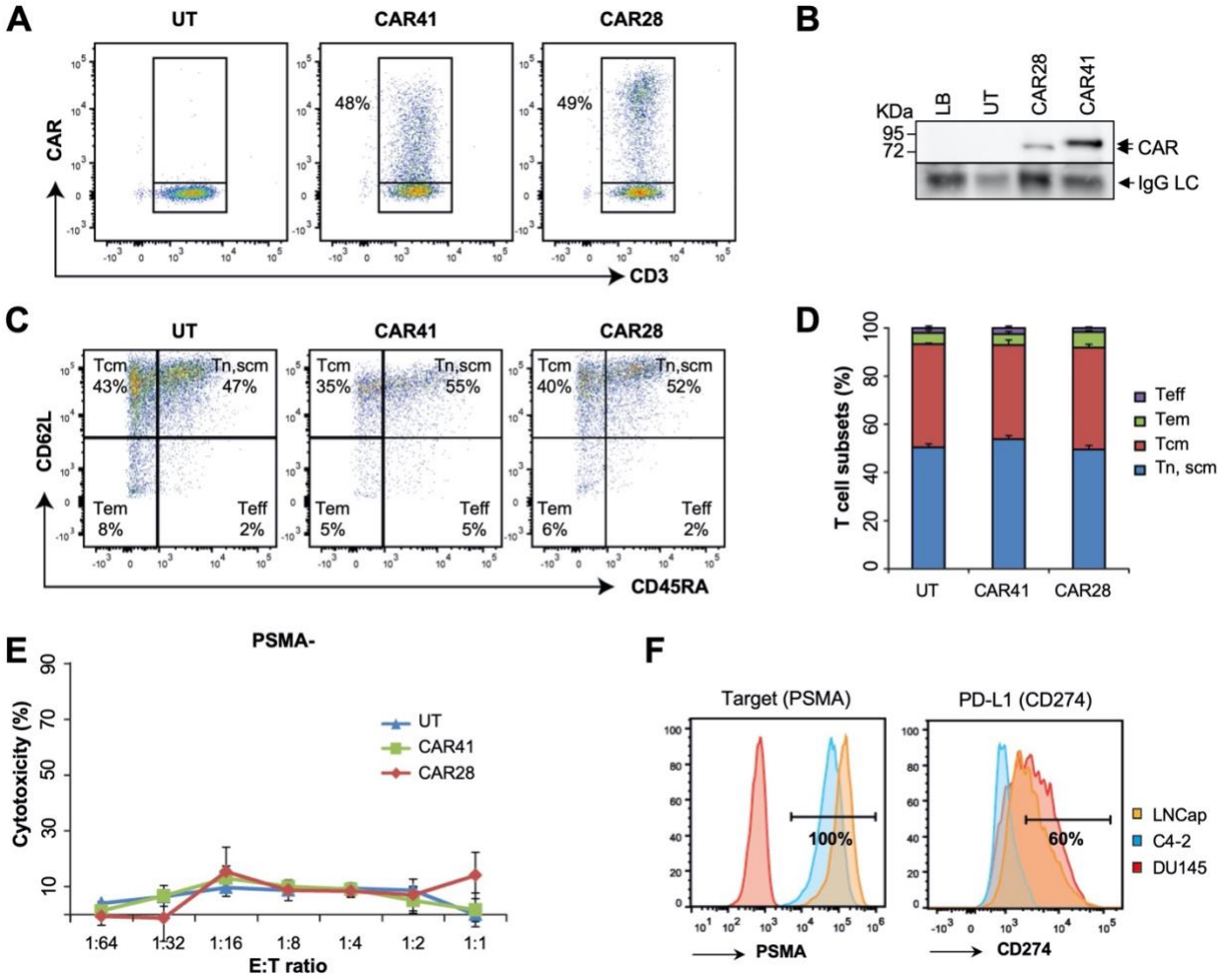

Figure S2. Quality assessment. (A) Evaluation of CAR surface expression. After transduction of activated T cells with γ-retroviral vectors, CAR T cell were expanded and stained with antibodies against anti-human IgG (CAR) and CD3. **(B)** CAR expression. CAR T cells were lysed, CARs immune-precipitated using anti-scFv antibody, and CAR expression visualized by Western blot analysis. Anti-IgG antibody staining served as a loading control. **(C)** CAR T cell phenotyping. The percentages of T cell subsets were determined at the end of the expansion phase by assessing the expression of CD62L and CD45RA by flow cytometry. Cells were pre-gated on CAR-/CD3+ for the UT or CAR+/CD3+ for of CAR T cells. **(D)** Quantitative assessment of CAR T cell phenotypes. Shown are the average percentages of T cell subsets (n=3 or 4). **(E)** Cytolytic activity. CAR T cells were co-cultured at indicated effector to target (E:T) ratios with PSMA-negative DU145 tumor cells. Cytotoxicity was determined by cell viability assay (% cytotoxicity = 100 – % viability). (**F**) Characterization of Prostate cancer cell lines. Cells were stained with anti-PSMA (3/F11) and CD274 (PD-L1) antibodies, respectively, and expression levels assessed by flow cytometry. PSMA, prostate-specific membrane antigen; PD-L1, programmed cell death ligand 1; LB, lysis buffer; UT, untransduced T cells; IgG LC, IgG light chain loading control; Tn/Tscm, T cell naïve or T stem cell memory; Tcm, T cell central memory; Tem, T cell effector memory; Teff, T cell effector.

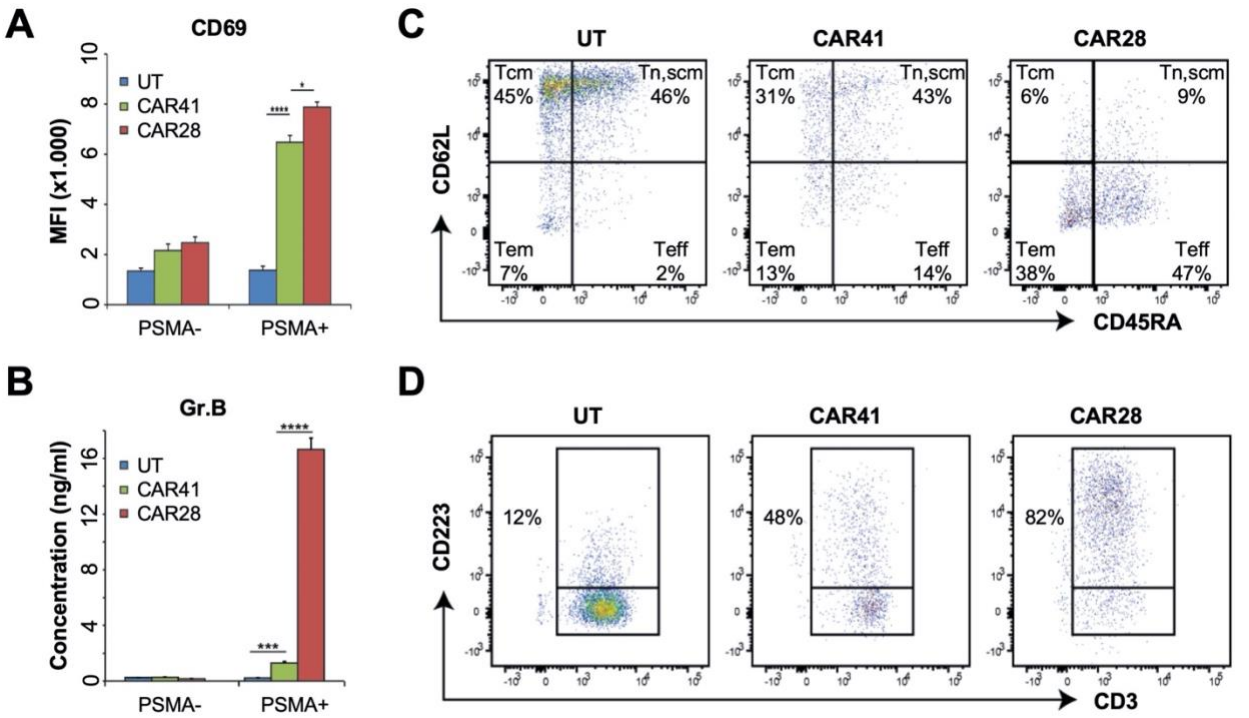

Figure S3. Extended phenotypic and functional analysis of PSMA-targeting CAR T cells. Untransduced T cells (UT), CAR41 or CAR28 T cells were co-cultured with PSMA-positive (C4-2) or PSMA-negative (Du145) tumor cells at a 1:1 effector-to-target (E:T) ratio. (**A**) PSMA-mediated activation. CAR T cell activation was assessed by evaluating CD69 expression. Indicated is mean fluorescent intensity (MFI, n=6). (**B**) Granzyme release. Granzyme B (Gr.B) concentration in supernatant was quantified by cytometric bead array (n=3). (**C**) Phenotype. CAR T cell phenotypes were assessed based on CD62L and CD45RA expression. Cells were pre-gated on either CD3 alone for UT control, or CAR and CD3 for CAR T cells. (**D**) CAR T cell exhaustion. Extent of exhaustion was assessed by measuring CD223 (LAG-3) expression. Cells were pre-gated on CD3 alone for UT control, or CAR and CD3 for CAR T cells. Statistically significant differences are indicated by *(P<0.05), ***(P<0.001), or ****(P<0.0001). Tn/Tscm, T cell naïve or T stem cell memory; Tcm, T cell central memory; Tem, T cell effector memory; Teff, T cell effector.

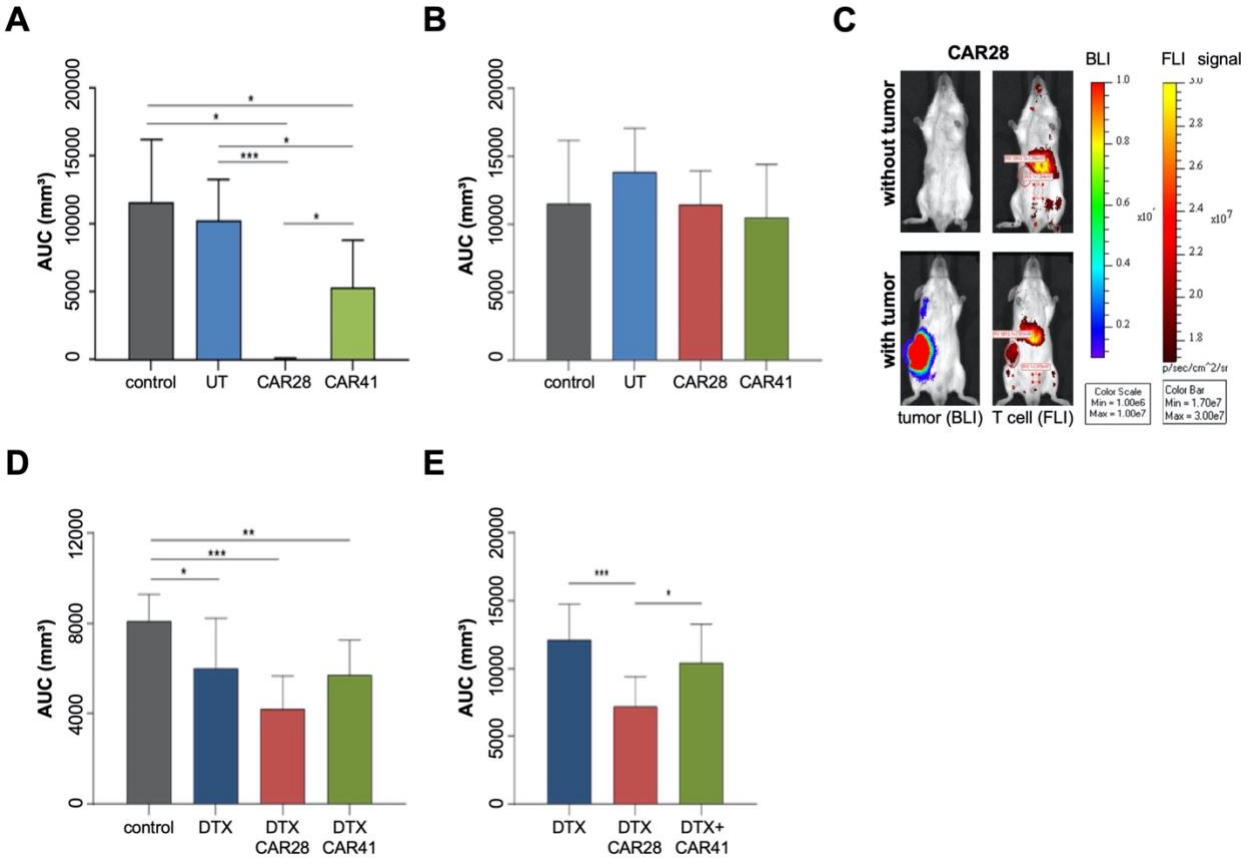

Figure S4. Therapy response of mice bearing human prostate tumors xenografts. Antitumor activity of anti-PSMA CAR T cells upon focal **(A)** or systemic application **(B)**. One dose of $5x10^6$ D7-based CAR28 or CAR41 T cells were injected either intratumorally (A) or intravenously (B). Tumor volumes from individual groups were monitored by calculating the area under the curve (AUC) over the entire course of the experiment, starting from day 1 of the therapy until the end at day 22. As controls, mice were left untreated (control) or treated with untransduced T cells (UT). **(C)** Biodistribution of anti-PSMA CAR28 T cells. CAR T cells were labelled with Xenolight DiR dye and injected intravenously into mice bearing PSMA-positive tumor xenografts or controls without tumor. 24 hours post-infusion, tumors were monitored by *in vivo* bioluminescence imaging (BLI) while labeled T cells were detected by fluorescence imaging (FLI). (**D**-**E**) Anti-tumor activity of combined therapy with DTX and anti-PSMA CAR T cells. Mice bearing tumors of ~150-200 mm^3 were treated with 2 cycles of docetaxel (DTX) followed by intravenous injection of $5x10^6$ CAR28 (n=9) or CAR41 T cells (n=8). As controls, mice were treated with DTX alone (n=9) of left untreated (control, n=8). Changes in tumor volumes from individual treated groups were monitored by calculating the AUC over the course of the entire experiment, starting from day 1 of the therapy until day 17 (D) or until the end of the therapy at day 22 (E). Statistically significant differences were determined by unpaired t-test: *p<0.05, **p<0.01 and ***p<0.001.

Supplemental Methods

Western blot

To verify the size of our CAR28 or CAR41, we have performed Immuno-purification and western blotting of chimeric antigen receptors from cellular lysates as previously described (REF: PMID 31064990). Briefly, 3×10^7 cells were lysed in 1 ml lysis buffer containing 20 mM Tris-HCl pH8, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1x protease inhibitor cocktail, 1 mM PMSF, 5 mM iodoacetamide, 0.5 mM sodium orthovanadate, 1 mM NaF, and 0.5% Brij96 for 30 min at 4 °C followed by 15 min centrifugation to pellet the nuclei and insoluble material. For immuno-purification of the chimeric antigen receptors, 400 µl cleared cell lysate was incubated with 7.5 µl streptavidin-coupled sepharose (#17-5113-01, GE Healtcare) and 20 µg biotin-coupled anti-mouse IgG (Fab´)2 biotin (#31803, ThermoFisher) for 2 h at 4 °C. After four washes, the immunoprecipitated material was separated by 12% reducing SDS-PAGE. The separated proteins were transferred to PVDF membranes by semi-dry transfer. After blocking with 5% milk in PBS containing 0.1% Tween-20 the membranes were incubated with HRPO-conjugated secondary antibodies (1:10000) (anti-rabbit IgG HRPO, #31460, ThermoFisher). Western blot signals were recorded using an Image Quant LAS 4000 Mini from GE Healthcare Life Sciences, Boston, MA.

Phenotyping of prostate cancer cells

To evaluate PSMA expression, cells were incubated with 2.8 µg/ml anti-PSMA mAb 3/F11 for 30 minutes at 4°C followed by staining with a 1:100 dilution of goat anti-mouse Ig-PE (Southern Biotech) for 30 minutes in dark at 4°C and evaluation by flow cytometery. To determine PD-L1 expression, prostate cancer cells were stained with anti-CD274-Bv421 (Biolegend) and evaluated by flow cytometery.

In vivo biodistribution of CAR T cells

In order to track CAR T cells in vivo, cells were labelled with Xenolight DiR dye (Caliper LifeSciences) according to the manufacturer's recommendations. Shortly, $2\text{-}3x10^6$ cells/ml of RPMI medium supplemented with 0.1% FCS were labelled with a 1:500 dilution of DiR (8.3 mg/ml) for 10 min. at 37°C. Then, cells were washed 3 times before i.v. injection into tumor bearing mice. After 24 h, mice were imaged under anesthesia using the In Vivo Imaging System IVIS 200 (Xenogen VivoVision) in the FLI channel.

Antigen-specific expansion of PSMA-targeting CAR T cells

CAR T cells were generated from PBMCs following retroviral transduction as described in Materials and Methods. At day 12 post-transduction, CAR T cells were co-cultured with irradiated PSMA-positive C4-2 tumor cells for 12 days at a 1:1 effector-to-target ratio in RPMI complete medium supplemented with 100 U/ml of IL-2, 25 U/ml of IL-7, and 50 U/ml of IL-15 (all Miltenyi Biotech). Every 3 days, cells were harvested, counted and plated over fresh irradiated C4-2 tumor cells. Cells were stained with anti-human IgG-PE (Southern Biotech) and anti-CD3-APC (Miltenyi) antibodies to determine CAR expression levels and the fraction of CAR-positive cells by flow cytometry (Accuri, BD Biosciences). The absolute number of CAR T cells was determined at indicated time points using a NucleoCounter (NC-250, ChemoMetec).