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Supplemental Information

The Vascular Disrupting Agent CA4P Improves the Antitumor Efficacy of CAR-T Cells in Preclinical Models of Solid Human Tumors

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Supplemental Figure Legends

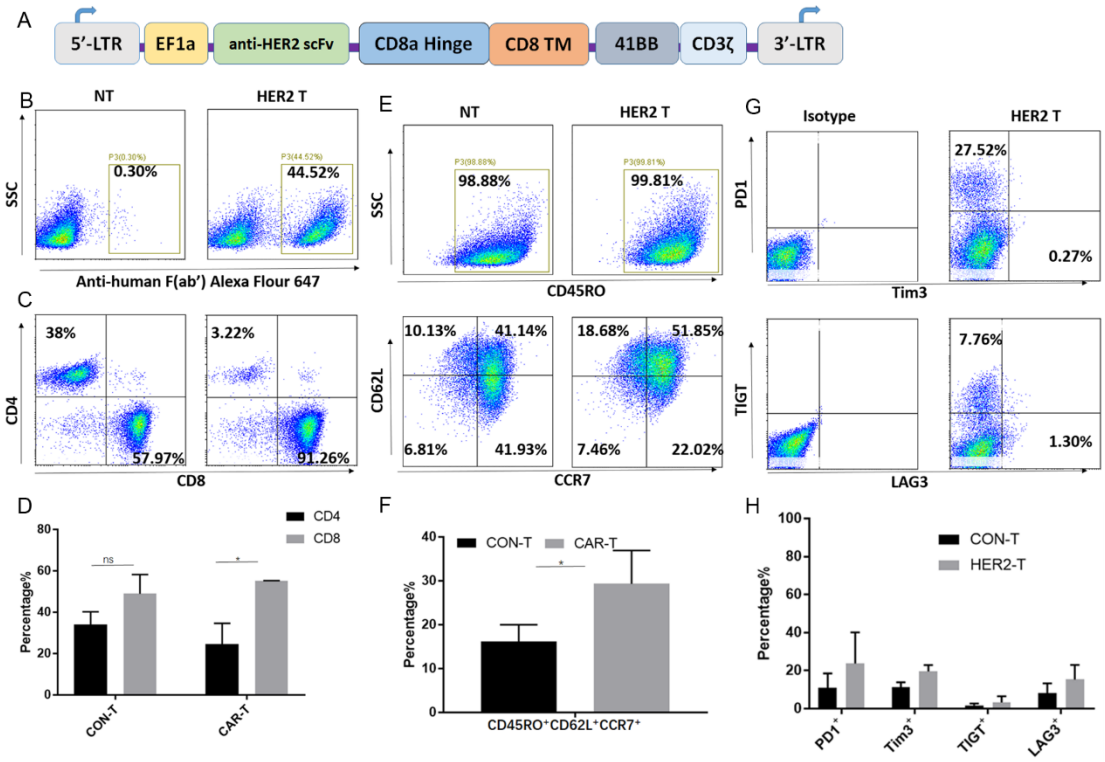

Figure S1. Construction and expression of a CAR to create HER2-CAR-T cells. (A) The structure of HER2-CAR comprised an Ig kappa signal peptide, an anti-HER2 (4D5) scFv, a CD8α hinge and transmembrane region, and a 4-1BB costimulatory signaling domain fused to the cytoplasmic region of the CD3ζ chain. This second-generation CAR was inserted into a self-inactivating (SIN) pRRL lentiviral vector with Xba I and Sal I sites. The expression of HER2-CAR was driven by the EF1-alpha promoter. (B) The expression of HER2-CAR in T cells was examined by flow cytometry with an Alexa Fluor 647-conjugated anti-human IgG, F(ab')2 antibody (BioLegend) 6 days after transduction. (C) The phenotypic subset composition of the CAR-T cell population was determined by flow cytometry 14 days after transduction. (D) The statistical analysis of the ratio of subset composition of the HER2-CAR-T cells in C generated from peripheral blood of three different donors (n=3).(E)The proportion of central memory T cells ($CD45RO^+CCR7^+CD62L^+$) was 51.85%, and the proportion in a control sample was 41.14%. (F) The statistical analysis of the ratio of central memory T cells of the HER2-CAR-T cells in E generated from peripheral blood of three different donors (n=3).(G) The proportions of HER2-CAR-T cells expressing exhaustion markers ($PD1^+$ 27.52%, $LAG3^+$ 1.3%, $Tim3^+$ 0.27%, and $TIGT^+$ 7.76%) were assayed by flow cytometry using appropriate antibodies (Biolegend) for flow cytometry analysis.(H) The statistical analysis of the proportions of HER2-CAR-T cells expressing exhaustion markers in G generated from peripheral blood of three different donors (n=3).

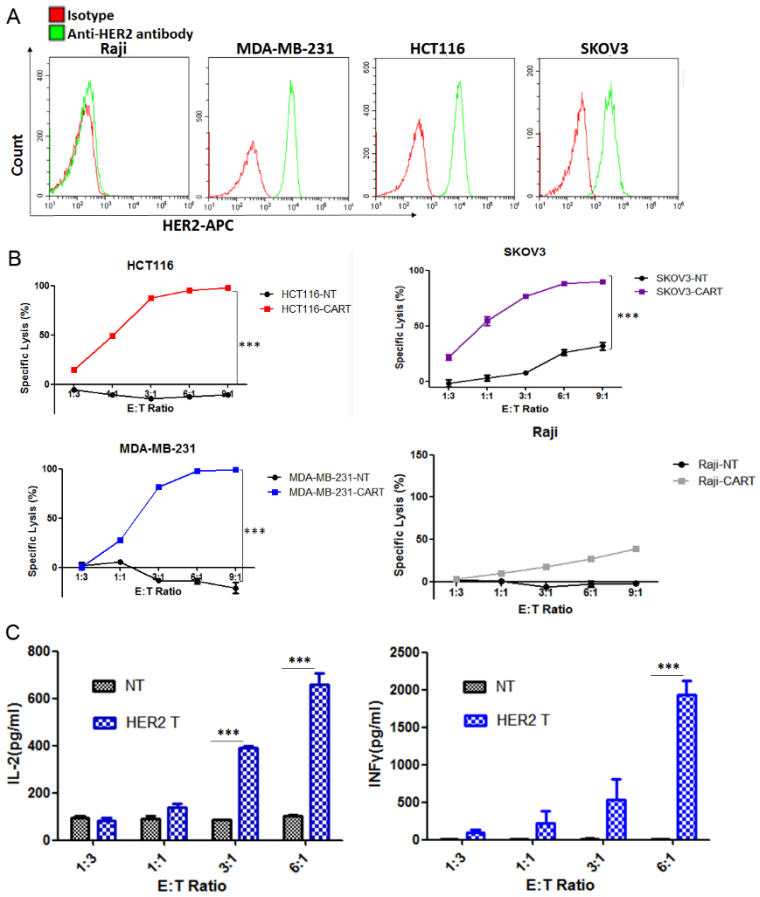

Figure S2. HER2-CAR-T cells kill target cells and secrete cytokines in a coculture system. HER2 antigen expression on the surface of tumor cells was determined by flow cytometry. (A) HER2 expression in the tumor cell lines HCT116 (colorectal cancer), SKOV3 (ovarian cancer), MDA-MB-231 (breast tumor) and Raji (lymphoma) was evaluated. (B) Normal T (NT) or CAR-T cells and $HER2^+$ or $HER2^-$ tumor cells were cocultured in a 96-well plate for 20 hours at the indicated effector-to-target (E:T) ratio, and then cytotoxicity was evaluated by a standard bioluminescence assay based on luciferase ($n = 3$, $p < 0.001$). (C) Normal T (NT) or CAR-T cells and $HER2^+$ SKOV3 tumor cells were cocultured in a 96-well plate for 24 hours at the indicated E:T ratio. The supernatants were collected, and an ELISA was used to determine the levels of secreted IL-2 and IFN-γ. The mean ± S.D. of triplicate cultures is shown ($n = 3$, $p < 0.001$).

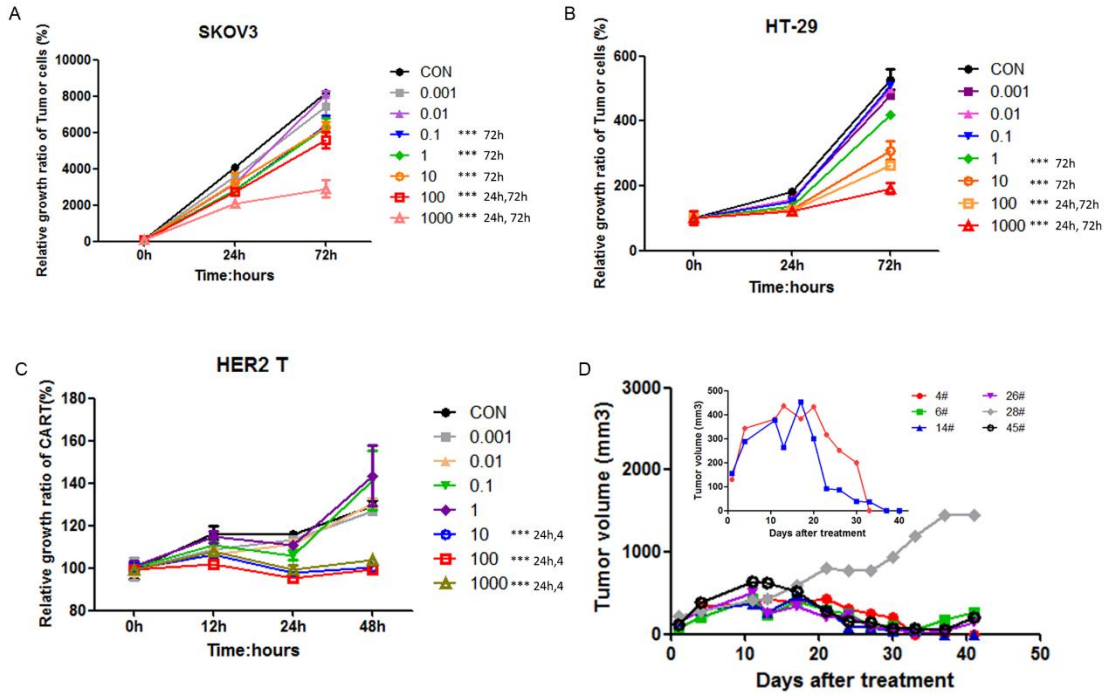

Figure S3. CA4P inhibits tumor cell, HER2-CAR-T cell proliferation. SKOV3 cells (A), HT29 cells (B), HER2-CAR-T cells (C) were seeded at a density of 2 x10^4 cells in each well of 96-well plates and cultured overnight, with CA4P added at different concentrations(ng/ml). The proliferation of the cells was measured using a cell counting kit-8 (CCK-8) assay at the indicated time points. The absorbance of each well was measured with a microplate reader at dual wavelengths of 450 nm and 690 nm. All experiments were performed three times, and the results are presented as the mean ± standard deviation (ns, no significance; *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$; n ≥3).(D) SKOV3 cell-derived xenograft (CDX) model treated with HER2-CAR-T cells in combination with multiple CA4P treatments. Tumor growth curves of different groups. Compared with the other groups, the HER2-CAR-T cells combined with CA4P group showed significantly reduced tumor volumes (n=6, $p < 0.01$).Two (#4 and #14) of the 6 mice in the group showed complete tumor elimination.

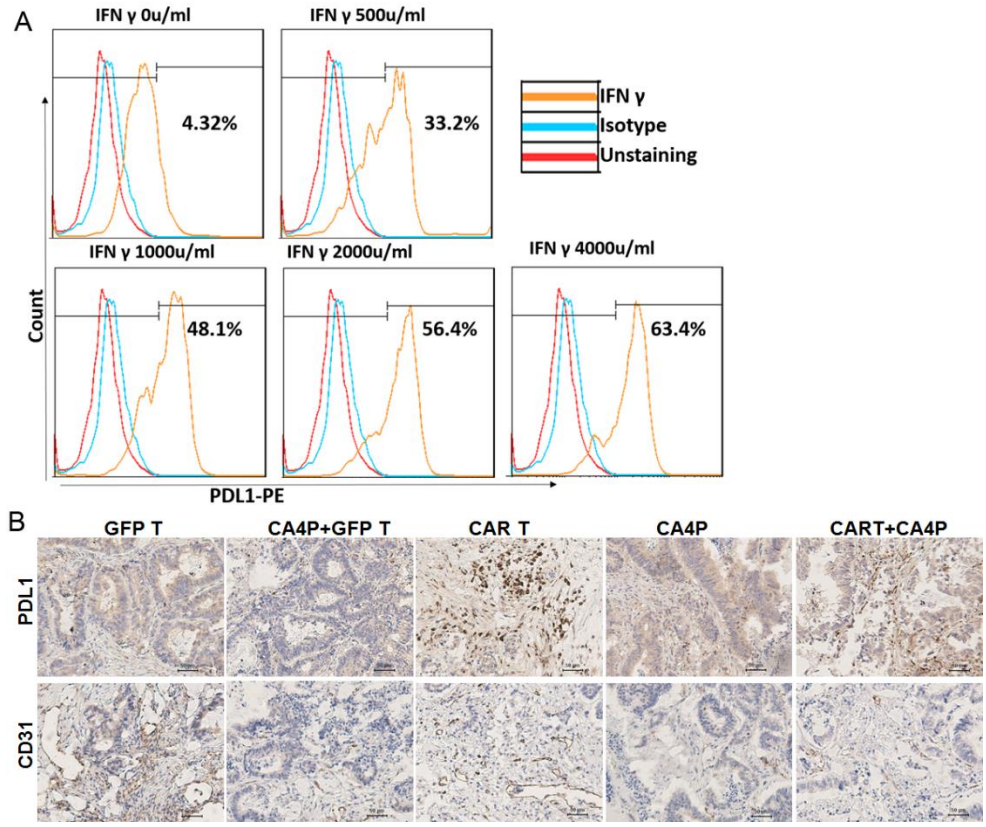

Figure S4. Effect of IFN-γ on the expression of PDL1 in tumors *in vivo* and PDL1 and CD31 expression in tumor tissue samples from patient-derived xenotransplantation (PDX) mouse models treated with HER2-CAR-T cells alone or in combination with CA4P. (A) Expression of PDL1 in SKOV3 cells induced by different concentrations of IFN-γ, as measured by flow cytometry. In total, 2 x 10^5 SKOV3 ovarian cancer cells were cultured in advance in a 6-well plate, and different concentrations of IFN-γ (5 concentrations between 0 and 4,000 U/ml) were selected. After 24 hours, the expression level of PDL1 was analyzed by flow cytometry, and the corresponding conditions were repeated three times. (B) PDL1 and CD31 expression in tumor tissue samples from PDX mouse models treated with HER2-CAR-T cells alone or in combination with CA4P. HER2-CAR-T cell (or GFP-T cell) infusions with or without CA4P treatments were carried out in PDX mouse models of HER2-positive colorectal cancer. Immunohistochemical analysis of the tumor tissue samples showed that PDL1 and CD31 expression differed among the GFP-T cells alone, CA4P alone, HER2-CAR-T cells alone, GFP-T cells combined with CA4P and CAR-T cells combined with CA4P groups (Scale bar, 50 μm).

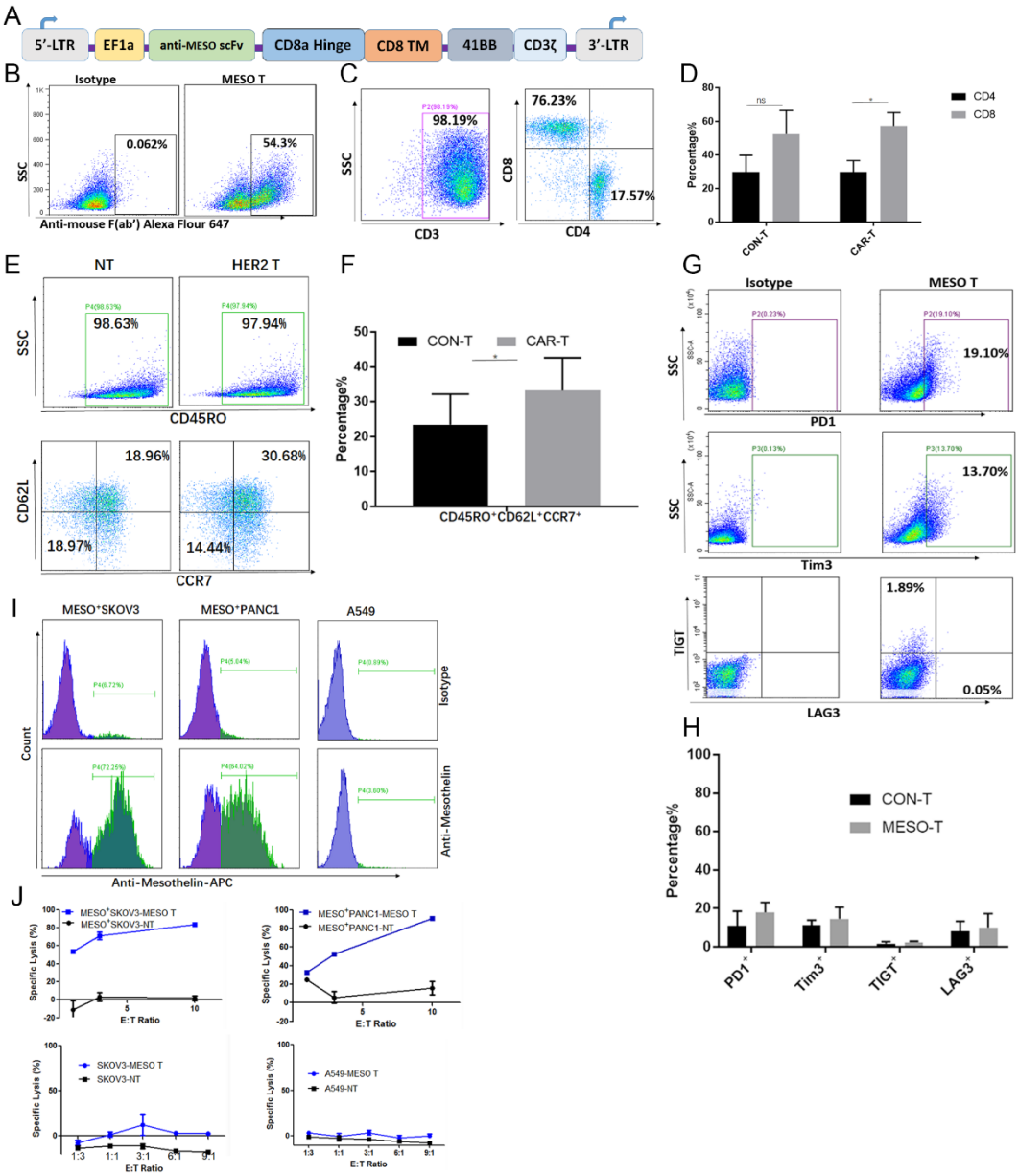

Figure S5. Construction, phenotype detection and cytotoxicity test of Meso-CAR-T cells. (A) The structure of Meso-CAR comprised an Ig kappa signal peptide, an anti-mesothelin (SS1) scFv, a CD8α hinge and transmembrane region, and a 4-1BB costimulatory signaling domain fused to the cytoplasmic region of the CD3ζ chain. (B) The expression of Meso-CAR in T cells was examined by flow cytometry with an Alexa Fluor 647-conjugated anti-mouse IgG, F(ab′)2 antibody (BioLegend) 6 days after transduction. Approximately 54.3% of the T cells were CAR positive. The phenotypic subset composition of the CAR-T cell population was assayed by flow cytometry 14 days after transduction. (C) $CD8^+$ T cells (76.23%) were dominant in the CAR-T cell population. (D)The statistical analysis of the ratio of subset composition of the Meso-CAR-T cells in C generated from peripheral blood of three different donors (n=3).(E)The proportion of central memory T cells ($CD45RO^+CCR7^+CD62L^+$) was 30.68%, and the proportion in a control sample was 18.96%.(F) The statistical analysis of the ratio of central memory T cells in Meso-CAR-T cells generated from peripheral blood of three different donors performed using GraphPad Prism5 software compaired with the control T cells(n=3).

(G) The proportions of Meso-CAR-T cells expressing exhaustion markers ($PD1^+$ 19.10%, $LAG3^+$ 0.05%, $Tim3^+$ 13.7%, and $TIGT^+$ 1.89%) were assayed by flow cytometry using appropriate antibodies (Biolegend) for flow cytometry analysis.(H) The statistical analysis of the proportions of Meso-CAR-T cells expressing exhaustion markers in G generated from peripheral blood of three different donors (n=3).(I)To identify the functionality of the CAR-T cells *in vitro*, a lentiviral expression vector for high expression of the Mesothelin antigen was constructed, and then target cells(PANC1 and SKOV3) were transfected. The expression of the corresponding antigen was confirmed by flow cytometry. Mesothelin was overexpressed in the tumor cell lines $MESO^+$PANC1 and $MESO^+$SKOV3. (J)Normal T or CAR-T cells and $Mesothelin^+$ or $mesothelin^-$ tumor cells were cocultured in a 96-well plate for 20 hours at the indicated effector-to-target (E:T) ratio, and then cytotoxicity was evaluated by a standard bioluminescence assay based on luciferase ($n = 3$, $p < 0.001$).