Supplementary Data

Shed antigen-induced blocking effect on CAR-T cells targeting Glypican-3 in hepatocellular carcinoma

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Role of Shed Tumor Antigens in Immunotherapies

1. Materials and Methods

Immunohistochemistry.

Immunohistochemical staining of formalin-fixed and paraffin-embedded tissue sections was carried out using a two-step protocol. Briefly, 4 μm sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was blocked by 10 minutes of incubation in 3% hydrogen peroxide in double distilled water. Antigen retrieval was performed by microwave treatment in citrate buffer (pH 6.0). Primary antibodies (anti-GPC3, clone SP86, Abcam, Cambridge, MA; anit-Ki-67, rabbit polyclone, Abcam, Cambridge, MA) were applied, followed by incubation at 4°C overnight. Staining was developed with peroxidase and 3,30-diaminobenzidine tetrahydrochloride (brown reaction product) followed by counterstaining with hematoxylin. Micrographs were manually obtained and evaluated with a computerized image analysis platform using Image-Pro Plus version 5.1. The expression of GPC3 in HCC tissues was scored as a staining index (total staining intensity per unit of tissue area). The Ki-67 positive staining was counted manually, and the results were shown as the total number of positive cells in five high power fields.

Flow cytometry

Cultured cells were harvested in cell dissociation solution (Invitrogen, Carlsbad, CA), washed, and resuspended in ice-cold flow buffer (PBS containing 5% BSA). One million cells were incubated on ice with an unconjugated primary Ab (32A9 and humanized YP7) or a recombinant protein which can be recognized by a fluorescent goat Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The fluorescence

of cells was measured using a FACS Calibur or FACS Verse system (BD Biosciences, San Diego, CA).

Western blotting and immunoprecipitation.

Cell lysates or culture supernatants were mixed with loading buffer, denatured at 95°C and subjected to SDS-PAGE. The immunoprecipitation of sGPC3 in culture supernatants was performed by incubation with a biotin-labeled anti-GPC3 monoclonal Ab (mAb) overnight, followed by mixing with streptavidin-agarose beads for 2 hours at 4°C. Immune complexes were washed and subjected to immunoblotting with an anti-GPC3 Ab.

Luciferase reporter assay.

HEK293 Super TOPFlash cells were seeded into a 48-well plate. When the cells grew to 70% confluence, the full-length GPC3 plasmid (0.1 μg/well) and Renilla luciferase plasmid (0.05 μg/well) were cotransfected with or without the sGPC3 plasmid (0.1 μg/well) into cells. Twenty-four hours later, 50% Wnt3a conditioned medium (CM) was added. In some cases, cells were pre-treated with recombinant sGPC3 for 30 min before Wnt3a CM treatment. Luciferase activity was measured 24 hours later with the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

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2. Supplementary Figures

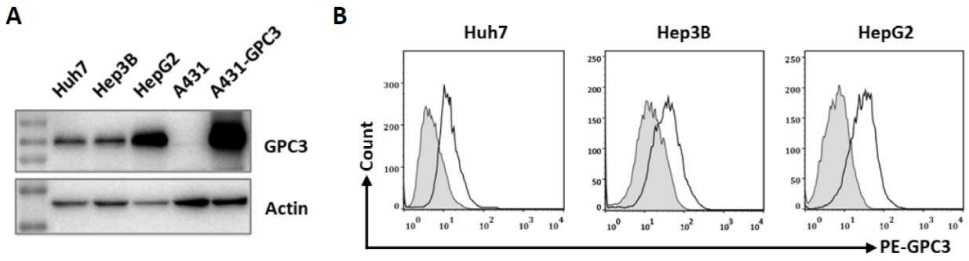

Fig. S1. GPC3 expression in HCC cells. (A) Measurement of GPC3 expression in tumor cell lines by western blotting. (B) Flow cytometry to measure the cell-surface expression of GPC3. Filled peak: isotype control. Open peak: GPC3.

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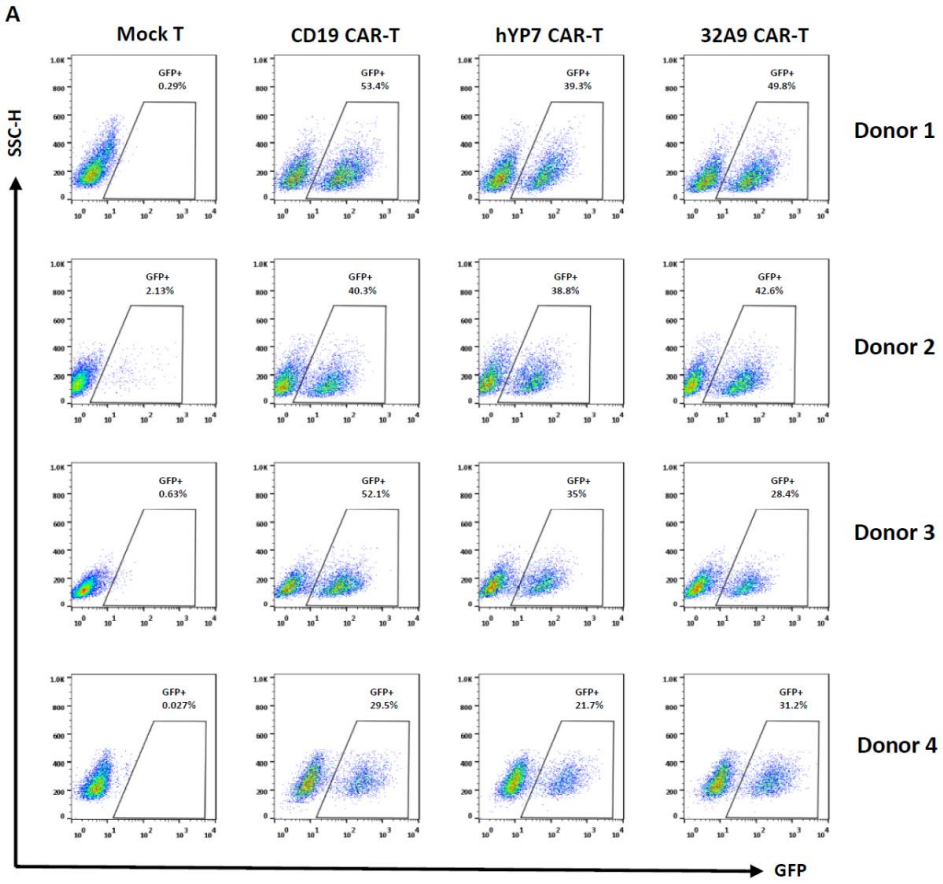

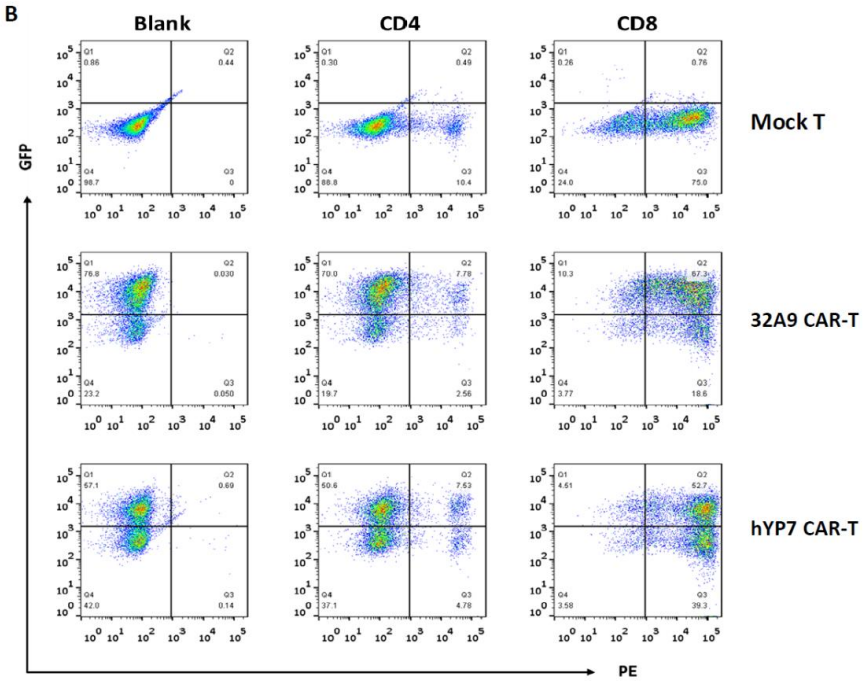

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Fig. S2. The transduction efficiency of CARs and the CD4/CD8 ration in the generated CAR T-cells. (A) Flow cytometry to show the transduction efficiency of anti-GPC3 CARs and anti-CD19 CAR in T cells from 4 different donors. GFP-positive cells were gated as CAR-T cells. (B) Flow cytometry to detect CD4 and CD8 proportions in the generated T cell population.

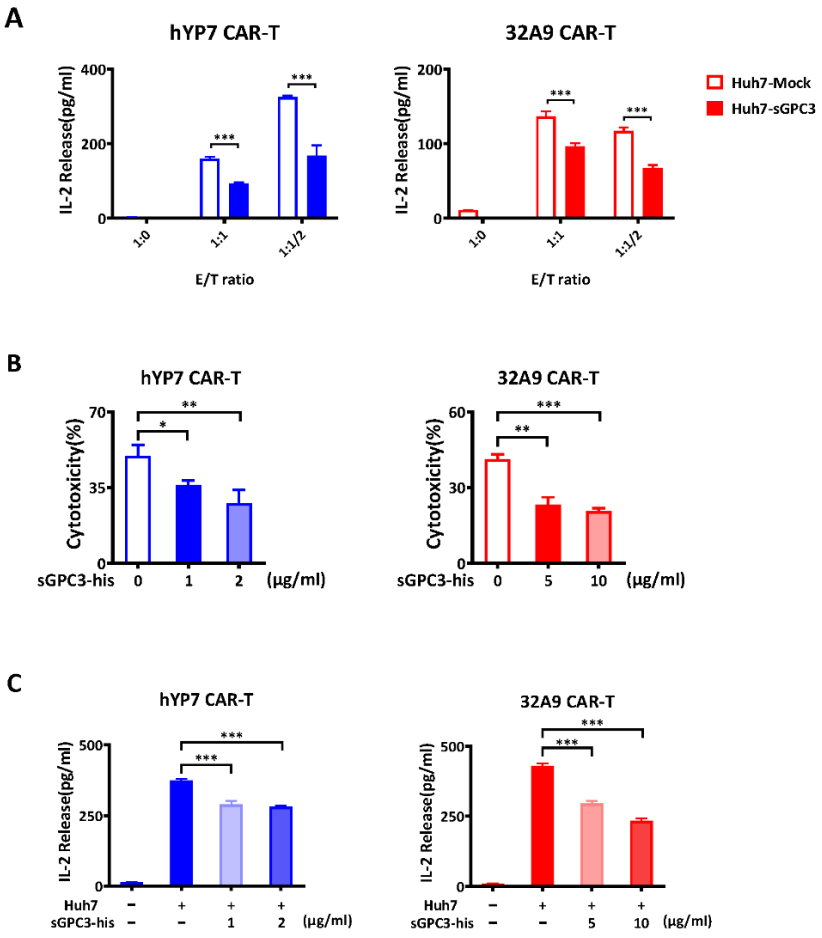

Fig. S3. sGPC3 inhibits the activation of GPC3-specific CAR-T cells *in vitro*. (A) Detection of IL-2 release in hYP7 CAR-T cells and 32A9 CAR-T cells targeting Huh7-Mock cells and Huh-7-sGPC3 cells for 12 hours at the indicated E/T ratio. GPC3-specific CAR-T cells targeted Huh-7 cells in the presence of the recombinant sGPC3

protein at an E/T ratio of 2:1 for 16 hours, then LDH assays to detect the cytotoxicity of GPC3-specific CAR-T cells (B) and ELISA to detect the secretion of IL-2 (C). Values represent the mean ± SD. ***p<0.001 (two-tailed Student's t-test).

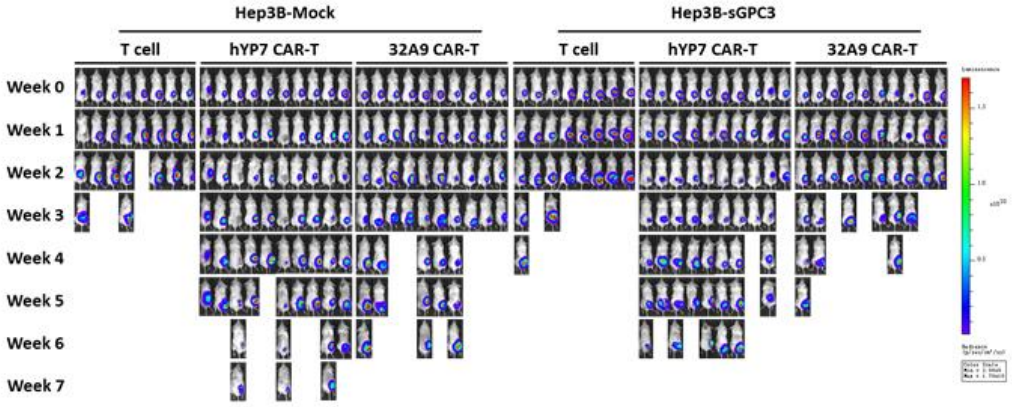

Fig. S4. Weekly bioluminescence imaging of mice treated with hYP7 CAR-T cells and 32A9 CAR-T cells. NCG mice with subcutaneous luciferase-expressing Hep3B-Mock or Hep3B-sGPC3 xenograft tumors were i.v. injected with 2 doses of CAR-T cells when the tumor size reached approximately 400 mm^3.

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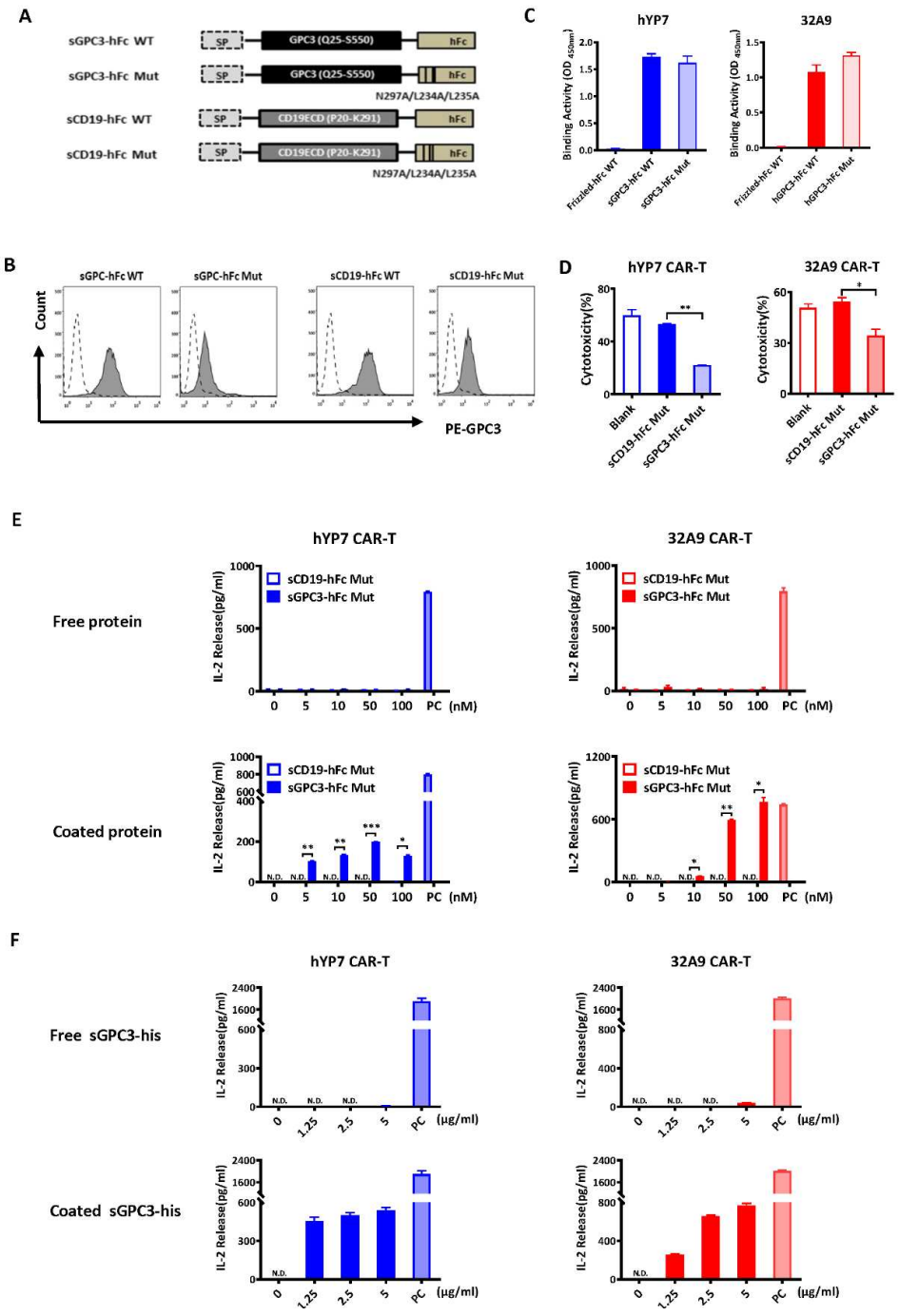

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Fig. S5. The effect of recombinant sGPC3 proteins on CAR-T cell cytotoxicity and activation. (A) Schematic constructs of the recombinant sGPC3 protein and sCD19 protein with wild-type and mutant human Fc fragments. SP, signal peptide. (B) Flow cytometry to detect the binding properties of the recombinant proteins described in (A) (20 nM) with IFN-γ-primed THP-1 monocytes. (C) ELISA for the comparison of the binding activities of hYP7 and 32A9 to sGPC3-hFc Mut or sGPC3-hFc WT. (D) LDH assays to detect the cytotoxicity of GPC3-specific CAR-T cells targeting Hep3B cells in the presence of 20 μg/ml sGPC3-hFc Mut protein at an E/T ratio of 2.5:1 for 12 hours. (E-F) ELISA to measure the secretion of IL-2 in the culture supernatants of hYP7 CAR-T or 32A9 CAR-T cells treated with free or pre-coated recombinant sGPC3-hFc Mut protein (E) or sGPC3-his protein (F). CAR-T cells cocultured with Hep3B cells were used as positive controls. Values represent the mean ± SD. *p<0.05; **p<0.01; ***p<0.001 (two-tailed Student's t-test).

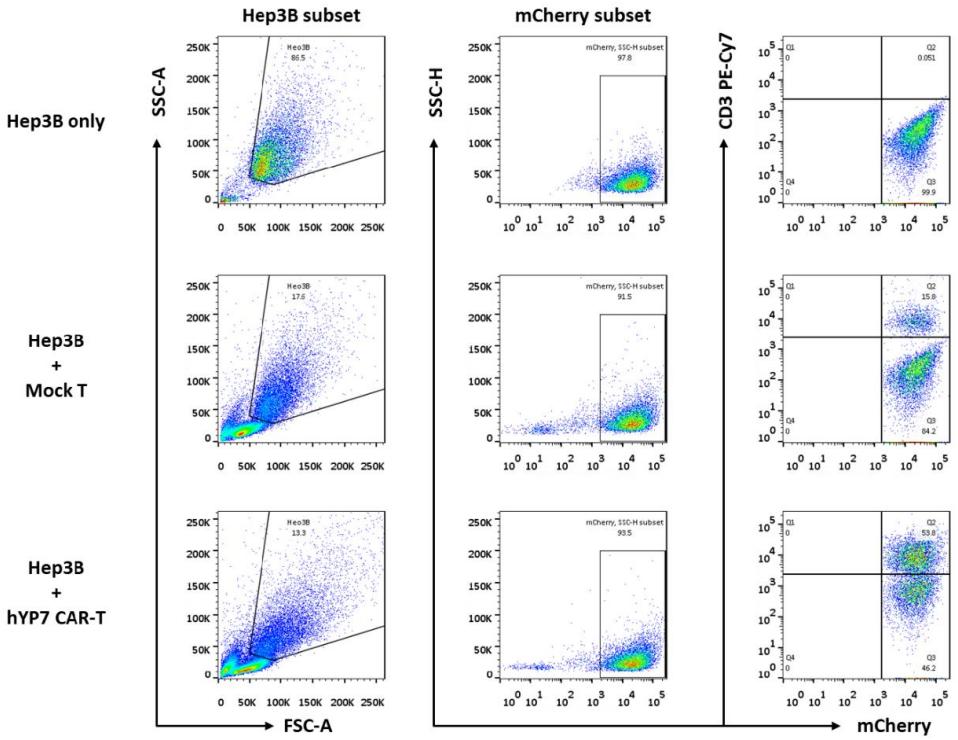

Fig. S6. Gating strategy for analyzing the binding between GPC3-specific CAR-T cells and Hep3B cells.