Supplementary materials and methods

Reagents and cell lines

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG-NH2) were purchased from Avanti Polar Lipids (Alabaster, USA).

Hepa1-6 cells were purchased from ATCC and cultured in RPMI medium. HepG2 cells were cultured in DMEM medium. LO2 cells were kindly provided by Professor Gangqiao Zhou at National Center for Protein Sciences, Beijing, China.

Analysis of molecular expression in migratory DCs

Bone marrow cells were collected from C57BL/6 mouse femur and tibia and cultured in RPMI 1640 medium containing 5% fetal calf serum, 50 mM 2-ME and 20 ng/ml recombinant mouse GM-CSF (Peprotech, USA) at 37°C for 6 days to generate BMDCs using standard laboratory protocols [1]. In total 3×10^6/ml BMDCs were stimulated with 5 μg/ml GPC3, or 5 μg/ml GPC3 plus 0.5 μg/ml CL097 (Invivogen, CA). After stimulation for 24 h, we collected the cells, prepared RNA using TRIzol (Invitrogen) and synthesized cDNA using PrimeScript RT Reagents (Takara, Dalian, China) following the manufacturer's protocols. Quantitative Real-Time PCR (qRT-PCR) was performed using SYBR Premix Ex Taq on an Applied Bio-systems 7500 Real-Time PCR system (Life Technologies) with the primers provided in Supplementary Table 1. The relative mRNA levels were determined with GAPDH as control and represented as $2^{-\Delta\Delta CT}$ based on report [2].

Characterization of GPC3 nanovaccine containing CL097 using mannosylated liposome

LPMan encapsulation efficiencies of GPC3 and CL097 were determined by measuring the percentage of liposome-bound components after removing free GPC3 and CL097 by dialysis (molecular weight cut-off: 100 kD). Liposome-bound components were then treated with 10% Triton X-100, followed by measurement of the protein levels using the Pierce BCA Protein Assay Kit (Thermo Scientific, MA) and CL097 level using UPLC-Q/TOF as we described previously [3, 4]. For optimal encapsulation efficiency, the mannose residues exposure on LPMan, LPMan-GPC3 and LPMan-GPC3/CL097 was determined by concanavalin A agglutination assay [3].

In total 100 μl of concanavalin A at the concentration of 0.1 mg/ml was mixed with 10 μl 0.1 mmol/ml different liposomes, and the absorbance at 390 nm was measured at different time points by Synergy H1 Hybrid Multi-Mode Reader. To determine the agglutination caused by mannose residues exposure on liposomes, concanavalin A pre-blocked with mannose was mixed with LPMan, and the absorbance at 390nm was measured. DOTAP (LP) was also measured.

Detection of anti-GPC3 antibodies

To determine anti-GPC3 antibodies, all serum samples were stored at -20°C and detected at the same time using a method established in our laboratory. Briefly, purified recombinant GPC3 protein was dissolved in carbonate bicarbonate buffer (pH 9.6) at 1 μg/ml and added to high-bound ELISA microtiter plates (100 μl/well). After being incubated at 4°C overnight, the plates were blocked with PBS containing 3% BSA for 2 h at room temperature. Diluted mouse serum (100 μl/well) was added, and normal mouse serum was used as the negative control, while mouse anti-GPC3 (BioMosaics, Burlington, VT) was used as the positive control. After incubation overnight at 4°C, the plates were washed and incubated with 1:10,000 diluted HRP-conjugated goat anti-mouse IgG (zsbio, Beijing, China) for 1 h at room temperature. The plates were colorized by adding tetramethylbenzidine for 30 min. The optical densities (OD) were measured at 450 nm using a spectrophotometer (Rayto, China). Cutoff value was set as the average OD450 nm calculated from 6 wells of normal serum plus a 3-fold standard deviation.

Generation of GPC3-expressing murine hepatoma cell line

A GPC3-expressing plasmid was constructed by sub-cloning the human GPC3 DNA fragment (73 to 600) in frame into the p3xFLAG-cmv-14 vector (Sigma) to generate Pcmv-GPC3 (Supplementary Figure 2). The Pcmv-GPC3 plasmid contains the amino acids of GPC3 from 25 to 200 in the N-terminus. A murine hepatoma cell line, Hepa1-6, with the C57BL background was transfected with Pcmv-GPC3. Stable transfected cells (Hepa/GPC3) were selected in the presence of 1 mg/ml of G418 for 4 weeks. To confirm GPC3 expression, cell lysates were prepared from Hepa1-6 and Hepa/GPC3 cells. Immunoblotting with the anti-human GPC3 antibody (Biomocaics, VT) and anti-human β-actin antibody (Sigma) was used. HRP-conjugated secondary antibody was incubated with the membranes, and the target protein bands were detected using ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

Quantification of cytokines in liver tissues and in cell culture supernatant

To quantify Granzyme B and IFNγ in liver tissues, interstitial liquid was prepared as reported previously [5]. Each 100 mg tissue sample was cut into small pieces in 400 μl of ice cold normal saline and incubated on ice for 15 min. The Granzyme B and IFNγ concentrations in the liver interstitial fluid and cell culture supernatant were measured using commercialized ELISA kits (eBioscience) according to the manufacturer's instructions.

Immunofluorescent staining

BMDCs were then stimulated with LPMan-BSA/CL097 containing 5 μg/ml AF-BSA plus 0.5 μg/ml CL097, or the same amount of LPMan-BSA, or free AF-BSA for 3 h at 37°C in an atmosphere of 5% CO_2. After extensive washing twice with PBS, the cells were placed on poly-L-lysine-coated slides using Cytospin (WESCO), and fixed with 2% (wt/vol) paraformaldehyde for 10 min at room temperature. Cells were permeabilized in PBS containing 0.1% (wt/vol) saponin for 10 min at room temperature, washed, and incubated with goat anti-EEA1 (N-19), or anti-Erp78 (N-20), or anti-Giantin (N-18, Santa Cruz, CA) or anti-LAMP1 (Sigma-Aldrich, MO) overnight at 4°C and then stained with Cy3-labeled donkey anti-goat IgG antibodies for 1 h at room temperature. After extensive washing, the glass slides were mounted with Vectashield. Image acquisitions and analysis were performed using a Leica microscope and software (Wentzler, Germany).

Flow cytometry (FCM) analysis

The following antibodies were all purchased from eBioscience: anti-mouse CD45 (30-F11), CD3 (145-2C11), CD4 (GK1.5), CD8α (53-6.7), IFN-γ (XMG1.2), CD103 (2E7), CD11c (N418), and CCR7 (4B12). Anti-mouse H-2K^b/H-2D^b (28-8-6), MHCII (m5/114.15.2), and CD83 (MICHEL-19) were purchased from Biolegend (San Diego, CA). Flow cytometry was performed using standard laboratory protocols. Briefly, fluorescence conjugated antibody was added directly to the cell suspension for 30 min in the dark at 4°C for cell surface antigens staining. To detect the presence of GPC3-specific T cell, splenocytes were collected and ex vivo stimulated immediately with the human GPC3 proteins (10 μg/ml) for 90 h. Cells were collected, stained with surface markers and then fixed with 100 μl of intracellular Fixation Buffer (eBioscience) for 30 min on dark at 4°C. After twice washing with 1× Permeabilization Buffer (eBioscience), cells were re-suspended with PE/Cy7-conjugated anti-mouse IFN-γ antibody diluted in 100 μl of 1× Permeabilization Buffer (eBioscience) and incubated in the dark at room temperature for 45 min. After twice washing, cells were re-suspended in PBS containing 0.1% BSA and 1 mM EDTA. Data were acquired in an LSR-II sytem (Becton Dickinson, San Diego, CA) and analyzed using Flowjo software (Tree Star Inc, Asland, OR).

Antigen-specific cytotoxicity assay

Liver tissues were harvested and intrahepatic lymphocytes (IHL) were collected as previous reported [6]. Hepa1-6 tumor cells were labeled with 2 μM CFSE at 37°C for 10 min. After washing twice with PBS, 2000 CFSE-labeled Hepa/GPC3 cells were co-cultured with 8,000 IHLs for 4 h. The killing of target Hepa/GPC3 cells was analyzed by flow cytometry. The specific killing activity was measured using the following formula: (1-ratio of CFSE in the absence of IHL/ratio of CFSE in the presence of IHL) ×100.

References

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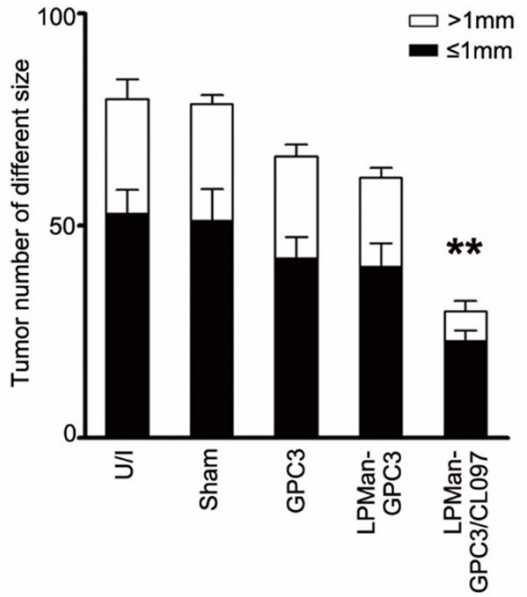
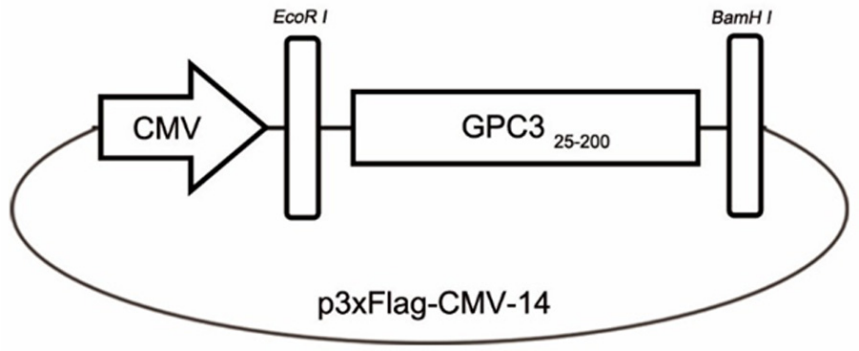
[6] Wu J, Du J, Liu L, Li Q, Rong W, Wang L, Wang Y, Zang M, Wu Z, Zhang Y and Qu C. Elevated pretherapy serum IL17 in primary hepatocellular carcinoma patients correlate to increased risk of early recurrence after curative hepatectomy. PLoS One 2012; 7: e50035.

Supplementary Table 1. List of mouse quantitative RT-PCR primers

Genes	Accession Number	Sequence (5' to 3')	Location	F/R	Product Size
Bip	NM_001163434	GCATCACGCCGTCGTATGT	182-200	F	134 bp
		ATTCCAAGTGCGTCCGATGAG	315-295	R	
Calreticulin	NM_007591	AAGATGCCCGATTTTACGCAC	209-229	F	110 bp
		CCCACAGTCGATATTCTGCTC	318-298	R	
Cd74	NM_001042605	AGATGCGGATGGCTACTCC	266-284	F	96 bp
		TCATGTTGCCGTACTTGGTAAC	361-340	R	
Edem1	NM_138677	TCCAGAAGGCAGTCAAGTTAGT	590-611	F	89 bp
		CAGAACCCTTATCGTAGCTTCG	678-657	R	
Edem2	NM_145537	ACCACGCCTACGACAGTTAC	131-150	F	113 bp
		GGCATCAATTAGCGTCAGAGAA	243-222	R	
Erap1	NM_030711	TAATGGAGACTCATTCCCTTGGA	93-115	F	102 bp
		AAAGTCAGAGTGCTGAGGTTTG	194-173	R	
Erp57	NM_007952	CATGCTAGTCGAGTTCTTCGC	141-161	F	119 bp
		CAGTGCAATCCACCTTTGCTAA	259-238	R	
Gapdh	NM_008084	AGGTCGGTGTGAACGGATTTG	8-28	F	123 bp
		TGTAGACCATGTAGTTGAGGTCA	130-108	R	
Gilt	NM_023065	CCTGGTCTCCGATCCTACCAT	5-25	F	118 bp
		TTGCAGGTGGTTGTGCCTT	122-104	R	
Hsp90	NM_010480	TGTTGCGGTACTACACATCTGC	1388-1409	F	116 bp
		GTCCTTGGTCTCACCTGTGATA	1503-1482	R	
Sec22b	NM_011342	CTGACGATGATCGCCCGTG	10-28	F	107 bp
		TGCTTAGCCTGACTCTGATACTG	116-94	R	
Tap1	NM_013683	GGACTTGCCTTGTTCCGAGAG	253-273	F	116 bp
		GCTGCCACATAACTGATAGCGA	368-347	R	
Tap2	NM_011530	CTGGCGGACATGGCTTTACTT	40-60	F	130 bp
		CTCCCACTTTTAGCAGTCCCC	169-149	R	
Tapbp	NM_001025313	GGCCTGTCTAAGAAACCTGCC	118-138	F	97 bp
		CCACCTTGAAGTATAGCTTTGGG	214-192	R	



Supplementary Figure 1. Tumor numbers of different sizes determined at wk20 after DEN administration in differently treated mice. Male HBV-transgenic mice (HBV-Tg) received 25 mg/kg of DEN intraperitoneally at 2 weeks old (wk0). They then received different treatments starting from wk8 after DEN administration for 4 doses, as shown in **Figure 4A**. Un-paired *t*-test was conducted. $^{**}P<0.01$ compared with sham-immunization. U/I: Mice did not receive any treatment after DEN administration. Sham: Mice were immunized with LPMan-CL097, each dose containing 5 μg of CL097, n=5. GPC3: Mice were immunized with 5 μg of purified GPC3, n=5. LPMan-GPC3: Mice were immunized with LPman-GPC3, each dose containing 5 μg of GPC3. LPMan-GPC3/CL097: Mice were immunized with LPMan-GPC3/CL097, each dose containing 5 μg of GPC3 plus 5 μg of CL097.



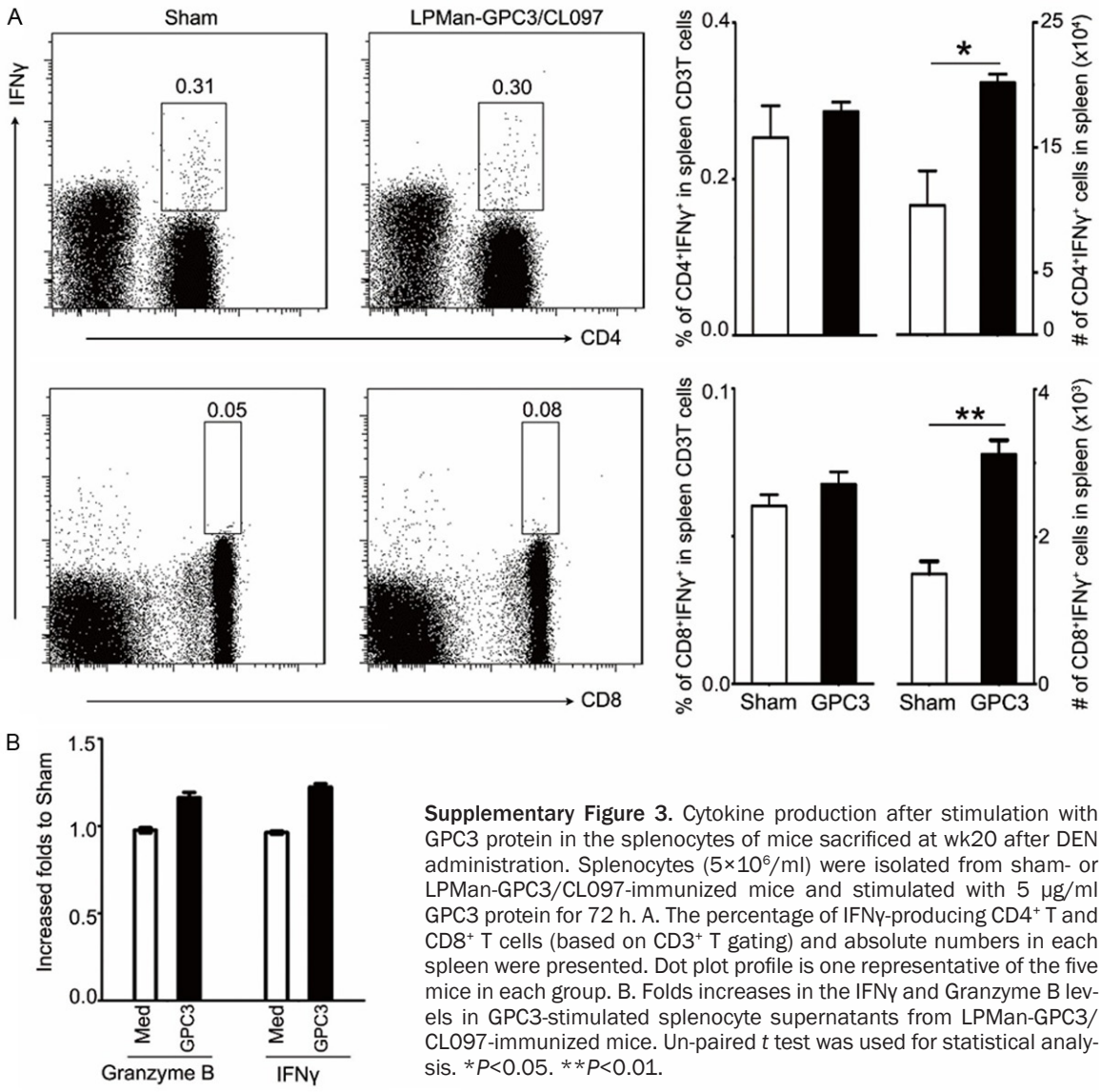
Supplementary Figure 2. Diagram of the GPC3-expressing plasmid pCMV-GPC3. The human GPC3 DNA fragment (73 to 600) was created by subcloning into the p3xFLAG-cmv-14 vector (Sigma), which contains the amino acids from 25 to 200 in the N-terminus.

Supplementary Table 2. Encapsulation efficiency and character with different concentrations of GPC3

Component			Particle character		
DOTAP	DSPE-PEG-Man	GPC3	Encapsulation	Diameter	Surface Charge
(molar ratio, 1 μmol/ml)		(μg/ml)	Efficacy (%)	Z-Ave (nm)	Zeta P (mV)
95%	5%	0	0	158.3±9.8	48.6±8.1
		50	62.54	111.4±3.0	38.8±1.8
		75	41.72	100.6±2.9	37.2±1.8
		100	96.02	105.4±7.4	42.2±1.2
		125	80.76	137.7±2.1	49.9±2.8
		150	65.9	144.5±2.9	48.6±7.6
		175	73.16	136±12.3	46.6±8.0
		200	76.92	143.2±3.5	52.6±1.5

Supplementary Table 3. Encapsulation efficiency and character with different concentrations of CL097

Component				Particle character		
DOTAP	DSPE-PEG-Man	GPC3	CL097	Encapsulation	Diameter	Voltage
(molar ratio, μmol/ml)		(μg/ml)	μg/ml	Efficacy (%)	Z-Ave (nm)	Zeta P (mV)
95%	5%	100	0	96.02	105.4±7.4	42.2±1.2
			1	95.96	105.1±3.8	42.0±1.5
			10	95.93	104.8±4.7	40.9±1.1
			50	95.89	106.4±3.0	38.8±1.8
			100	95.65	101.4±1.4	29.5±1.0
			150	80.76	117.7±2.1	19.9±2.8
			200	65.9	114.5±2.9	18.6±7.6



Supplementary Figure 3. Cytokine production after stimulation with GPC3 protein in the splenocytes of mice sacrificed at wk20 after DEN administration. Splenocytes (5×10^6/ml) were isolated from sham- or LPMan-GPC3/CL097-immunized mice and stimulated with 5 μg/ml GPC3 protein for 72 h. A. The percentage of IFNγ-producing $CD4^+$ T and $CD8^+$ T cells (based on $CD3^+$ T gating) and absolute numbers in each spleen were presented. Dot plot profile is one representative of the five mice in each group. B. Folds increases in the IFNγ and Granzyme B levels in GPC3-stimulated splenocyte supernatants from LPMan-GPC3/CL097-immunized mice. Un-paired *t* test was used for statistical analysis. **P*<0.05. ***P*<0.01.