Am J Transl Res 2018;10(6):1736-1749
www.ajtr.org /ISSN:1943-8141/AJTR0072039

Original Article

Immunization with glypican-3 nanovaccine containing TLR7 agonist prevents the development of carcinogen-induced precancerous hepatic lesions to cancer in a murine model

Kun Chen[1], Zhiyuan Wu[1], Mengya Zang[1], Ce Wang[2], Yanmei Wang[1], Dongmei Wang[1], Yifan Ma[2], Chunfeng Qu[1,3]

[1]Department of Immunology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China; [2]Guangdong Key Laboratory of Nanomedicine, Key Lab of Health Informatics of Chinese Academy of Sciences, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China; [3]State Key Laboratory of Molecular Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Received January 3, 2018; Accepted June 1, 2018; Epub June 15, 2018; Published June 30, 2018

Abstract: Background: Glypican-3 (GPC3) is one of the key tissue markers that could discriminate malignant precancerous lesions from benign hepatic lesions in cirrhotic patients. We aimed to develop a GPC3 cancer vaccine to induce specific T cells to intervene in hepatocellular carcinoma (HCC) development. Methods: Synthesizing mannosylated liposomes (LPMan) as vaccine delivery system, incorporating one Toll-like receptor (TLR)-7/8 agonist CL097 as adjuvant, we prepared a GPC3 nanovaccine, LPMan-GPC3/CL097. We injected 25 mg/kg diethylnitrosamine intraperitoneally to induce autochthonous HCC in HBV-transgenic mice, which persistently express hepatitis B surface antigen in hepatocytes. Starting from week 8 after diethylnitrosamine injection when malignant hepatocytes generated, we immunized the mice subcutaneously every 2 weeks 4 times with LPMan-GPC3/CL097 containing 5 μg of GPC3 plus 5 μg of CL097. Results: The vaccine efficiently targeted draining lymph nodes where naïve T cells reside and enhanced the expression of molecules involved in antigen presentation in migratory dendritic cells (DCs). Antigen was professionally processed in endoplasmic reticulum-Golgi system of DCs, subsequently priming both $CD4^+$ and $CD8^+$ T cells. The LPMan-GPC3/CL097 immunization generated significantly more GPC3-specific $CD4^+$ IFNγ- and $CD8^+$ IFNγ-producing T cells in mice spleens and livers, which specifically eliminated GPC3-expressing tumor cells. One week after last immunization (week 15 after diethylnitrosamine), 5/5 un-immunized, 5/5 sham (LPMan-CL097) and 1/5 LPMan-GPC3/CL097-immunized mice developed HCC. By week 20 after diethylnitrosamine, significantly less HCC developed in LPMan-GPC3/CL097-immunized mice than in sham-immunized mice ($P<0.01$). Conclusions: LPMan-GPC3/CL097 immunization induced *de novo* generation of specific T cells against tumor-associated antigen GPC3 that could prevent HCC development in cirrhotic liver.

Keywords: Cancer vaccine, murine model, T cells, tumor-associated antigen, hepatocellular carcinoma

Introduction

Preclinical and clinical evidences have demonstrated that early neoplastic cells (transformed cells that initiate cancer formation) express antigens that enable the immune system to distinguish them from normal cells. Cancer immunoprevention by modulating the host immune response was considered a better strategy to control the initiation or development of cancer. Cancer vaccine is one of the promising approaches [1, 2]. Hepatocellular carcinoma (HCC) arising from cirrhosis is usually preceded by the appearance of malignant precancerous lesion nodules [3]. At this stage, disease progression could be interfered if the malignant progenitors in cirrhosis were eliminated by HCC vaccine-elicited specific immunity.

Neoantigens resulting from coding gene mutations in tumors are considered as optimal personalized cancer vaccine targets because they

bypass thymic selection [4]. Disease progression was well controlled after immunization with personalized neoantigens in melanoma patients [5, 6]. However, we recently found that the mutations of coding genes and numbers of candidate neoantigens were much fewer in HCC, except for those of aflatoxin-related HCC [7]. Tumor-associated antigen, which is re-expressed in tumors and not/lowly expressed in normal tissues, might be an appropriate target [8]. Previous studies have documented that inducing specific T cells to alpha-fetoprotein (AFP), which is re-expressed in most HCC, led to tumor regression [8], and prevented carcinogen-induced murine autochthonous HCC [9]. However, significant hepatocyte damage was observed in the regenerating mouse liver [10].

Glypican-3 (GPC3) was identified as a new HCC-associated antigen [11]. Different from AFP, it is undetectable in the cirrhotic liver or even in benign hepatic lesions. Tissue expression of GPC3 was used to discriminate the nature of a <2 cm hepatocellular lesion lacking HCC radiological features detected in a cirrhotic patient [11]. Up to 60% of early HCC showed immunoreactivity to GPC3, either as membrane and/or cytoplasmic staining in the biopsy materials [12]. Therefore, we hypothesized that eliciting the host's own specific T-cell immunity against GPC3 could interfere with disease progression in cirrhosis patients.

Both $CD4^+$ and $CD8^+$ antigen-specific T cells are required to reject established tumor cells [13]. Normally, protein vaccines hardly gain access to the subcellular compartments of dendritic cells (DC) to be monitored by the MHC-I antigen presentation pathway, and hardly prime naïve $CD8^+$ T cells [14]. When vaccine protein was conjugated with some Toll-like receptor (TLR) agonists, mainly TLR7/8 agonists, the magnitude and quality of T-cell responses were significantly improved by activation of multiple DC subsets [15, 16]. In addition, the T-cell repertoire of tumor-associated antigens is limited and shows low-avidity because of the thymus selection [4]. Therefore, it is crucial to deliver the antigen signals into secondary lymphoid organs and activate DCs properly when tumor-associated antigen was used as a cancer vaccine [14, 17].

Polymeric nanoparticulate carriers may enhance antigen stability, immunogenicity and immunostimulatory effects with sustained and controlled release of the antigen to the target sites [18], which are efficient for vaccine system development, especially for prophylactic and therapeutic cancer vaccines. Using the model protein vaccine, we previously reported that mannosylated liposomes (LPMan) could be a good vaccine delivery system for lymph node targeting [19]. In addition, we found that, when the antigens were conjugated with chemically synthesized TLR7/8 agonists, such as CL097, they induced the generation of antigen-specific Th1 responses in an immune tolerant state [20, 21]. Here, we prepared a GPC3 nanovaccine, LPMan-GPC3/CL097, by synthesizing LPMan as vaccine delivery system, one TLR7/8 agonist CL097 as adjuvant. The results showed that LPMan-GPC3/CL097 nanovaccine could target draining lymph nodes *in vivo.* In the mice, LPMan-GPC3/CL097 immunization induced GPC3-specific immunity, which prevented the development of premalignant hepatic lesions to cancer.

Materials and methods

Supplementary Materials and Methods provide detailed information.

Analysis of molecule expressions in migratory DCs

Bone marrow-derived dendritic cells (BMDCs) were generated using standard laboratory protocols [22], stimulated with 5 μg/ml GPC3 (Sino-Biological, Beijing; endotoxin level <1 EU/μg protein) or 5 μg/ml GPC3 plus 0.5 μg/ml CL097 (Invivogen, CA) for 24 h. The molecular expression levels in BMDCs were determined using SYBR Premix Ex-Taq on an Applied Biosystems 7500 Real-Time PCR system, with GAPDH as the control and represented as $2^{-\Delta\Delta CT}$ [23]. Primers used are provided in Supplementary Table 1.

Preparation and characterization of the GPC3 nanovaccine containing CL097 using mannosylated liposomes

According to our previous report, DSPE-PEG-Man was synthesized, and LPMan was prepared by thin-film hydration method by mixing 5% DSPE-PEG-Man with 95% DOTAP [19]. To prepare LPMan-GPC3, we added different concentrations of purified GPC3 solution. To prepare LPMan-GPC3/CL097, different concentrations of CL097 were added into the LPMan-GPC3 solution.

The particle size and zeta potential were measured using Zetasizer Nano ZS (Malvern, Worcs, UK). The LPMan encapsulation efficiency of GPC3 and CL097 was determined by measuring percentage of liposome-bound components after removing free GPC3 and CL097 through dialysis (molecule weight cut-off: 100 kD). Liposome-bound components were then measured as we described previously [19, 21]. Presence of mannosylate residuals was determined using the concanavalin A agglutination assay [19].

Analysis of antigen uptake and lymph node trafficking in vivo

Alexa Flour-488 labeled BSA (AF-BSA, Invitrogen, CA) was used as a model antigen and was encapsulated with LPMan as performed for GPC3. BMDCs (3×10^6/ml) were stimulated with LPMan-BSA/CL097 containing 5 μg/ml AF-BSA plus 0.5 μg/ml or plus 5 μg/ml CL097 for 30 min. Treatment with same amount of LPMan-BSA or free AF-BSA was used as controls. FCM analyzed the antigen uptake efficacy. To analyze antigen trafficking into lymph nodes *in vivo*, 2.5 μg of AF-BSA in different formulations was injected into a mouse forepaw, and brachial and axillary lymph nodes were collected 2 days later and analyzed by FCM.

Immunofluorescent microscopy

BMDCs were stimulated with LPMan-BSA/CL097 containing 5 μg/ml AF-BSA plus 0.5 μg/ml CL097, or the same amount LPMan-BSA or free AF-BSA for 3 h. The cells were then placed on poly-L-lysine-coated slides, fixed and permeabilized. After incubation 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, the cells were stained with Cy3-labeled donkey anti-goat IgG for 1 h. Image acquisitions and analysis were performed using a Leica microscope and software (Wentzler, Germany).

Flow cytometry (FCM) analysis

Immunofluorescent-labeled antibodies were all purchased from eBioscience (San Diego, CA). For intracellular staining, cells were firstly stained with antibodies against their surface markers, followed by fixation and permeabilization and stained with PE/Cy7-conjugated anti-mouse IFNγ. Data were acquired in LSR-II and analyzed using Flowjo software (Tree Star, OR).

Mice and murine autochthonous liver cancer model

Study protocols (NCC2015A011) involving mice were approved by the Institutional Animal Care and Use Committee at National Cancer Center, Chinese Academy of Medical Sciences (NCC-CAMS). All the mice were maintained under specific pathogen-free conditions at the Laboratory Animal Services Center of NCC-CAMS. C57BL/6 mice were purchased from Vital River Laboratory Animal Technology, Beijing. HBV transgenic (HBV-Tg) mice, C57BL/6J-TgN(Alb1-HBV)44Bri/J, which persistently express large and small HBV surface antigen within the hepatocytes and secrete small HBV surface antigen from hepatocytes [24], were purchased from Health Science Center, Peking University and used for breeding mice. For liver cancer induction, diethylnitrosamine (DEN; Sigma) was injected intraperitoneally at 25 mg/kg into male littermates at 2 weeks old [25].

Detection of serum alanine aminotransferase (ALT) and anti-GPC3 antibodies

Two days before each immunization, the serum ALT level was measured within 2 h after sample collection using the reagents from Biosino Bio-Technology (Beijing, China). To determine anti-GPC3 antibodies, the serum samples were stored at -20°C and detected at the same time using a method established in laboratory, which is provided in the Supplementary Materials and Methods.

Assay of antigen-specific cytotoxicity on liver cancer cells

Mouse liver was removed, and intrahepatic lymphocytes (IHL) were prepared as reported previously [26]. Syngeneic murine hepatoma cell line, GPC3-expressing Hepa/GPC3 was constructed (Supplementary Figure 2) and labeled with 2 μM CFSE (Invitrogen, CA). Two cell populations were co-cultured at a ratio of 2000 CFSE-labeled Hepa/GPC3 with 80,000 IHLs for 4 h, followed by FCM analysis [9].

Statistical analysis

Un-paired *t*-tests were used to compare differences between groups. A *P*-value less than 0.05 was considered to be statistically significant.

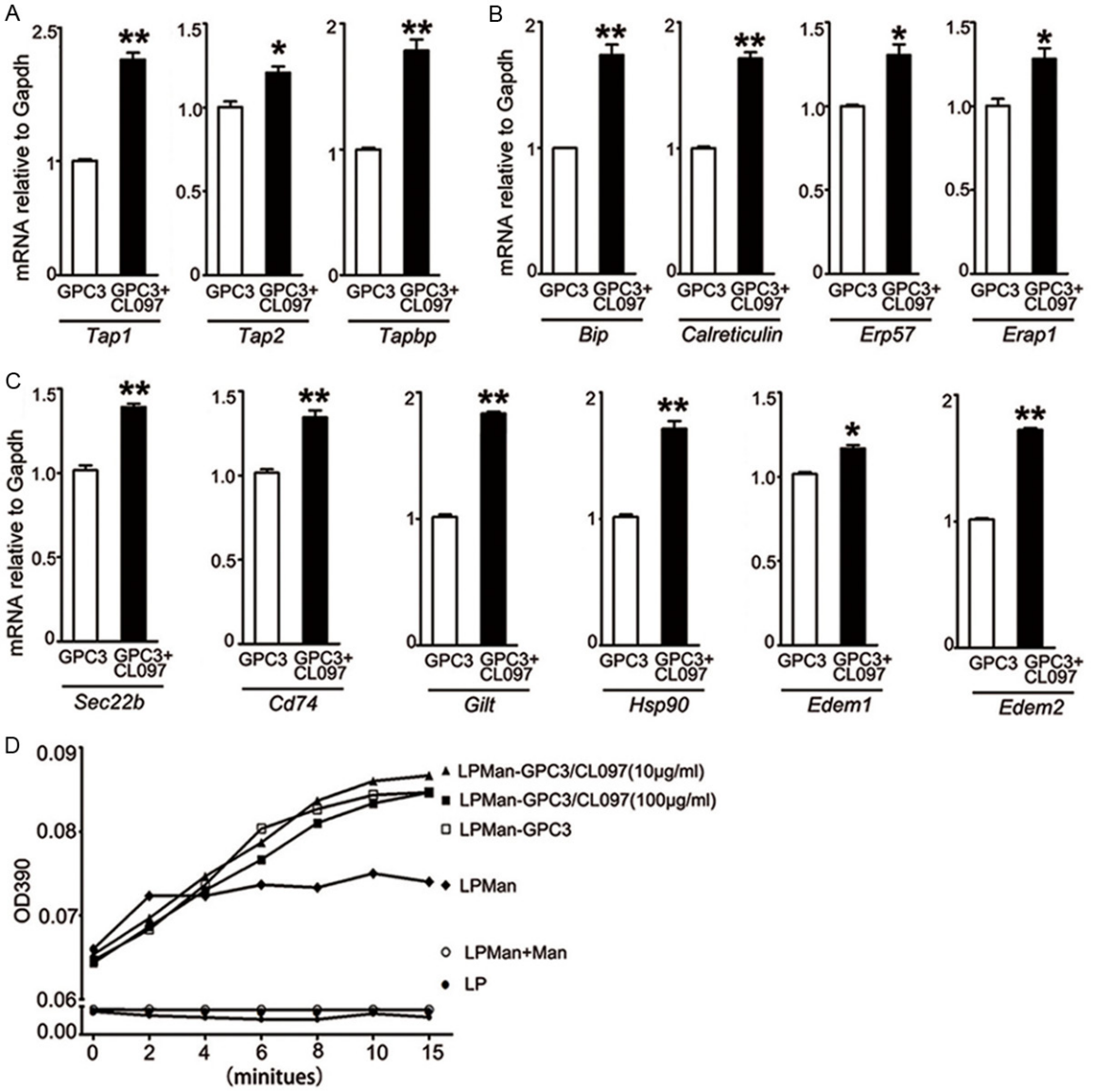


Figure 1. Effect of CL097 stimulation on molecules involved in the MHC-I antigen presentation pathway. BMDCs (3×10^6/ml) were stimulated with 5 μg/ml GPC3, or 5 μg/ml GPC3 plus 0.5 μg/ml CL097 for 24 h. A-C. Determined by qRT-PCR assay, the mRNA expression levels of TAP1, TAP2, TAPBP, Bip, calreticulin, ERp57, ERAP, Sec22b, CD74, GILT, HSP90, EDEM1 and EDEM2 were calculated based on GAPDH. Bars show fold changes, with the GPC3-treated sample arbitrarily set as 1 (n=3). Data are presented as mean ± SE, Paired *t*-test was conducted between two groups. **P*<0.05, ***P*<0.01. D. The concanavalin A agglutination assay was used to determine mannosylate residues (n=3).

Results

CL097 stimulation augments molecular expression involved in MHC-I antigen presentation in migratory DCs

We stimulated BMDCs with purified GPC3 or GPC3 plus CL097 to validate the CL097 effect on molecules involved in MHC-I antigen presentation, which is required to prime naïve T cells for $CD8^+$ T cell generation [14]. Analysis by qRT-PCR showed that the mRNA expression levels of TAP1, TAP2 and TAPBP, which transport cytosolic antigenic peptides across the membrane of endoplasmic reticulum (ER) (**Figure 1A**), BiP, calreticulin and ERp57, which ensure correct folding of proteins in ER, and ERAP, which trim transported long peptides in ER to the right size

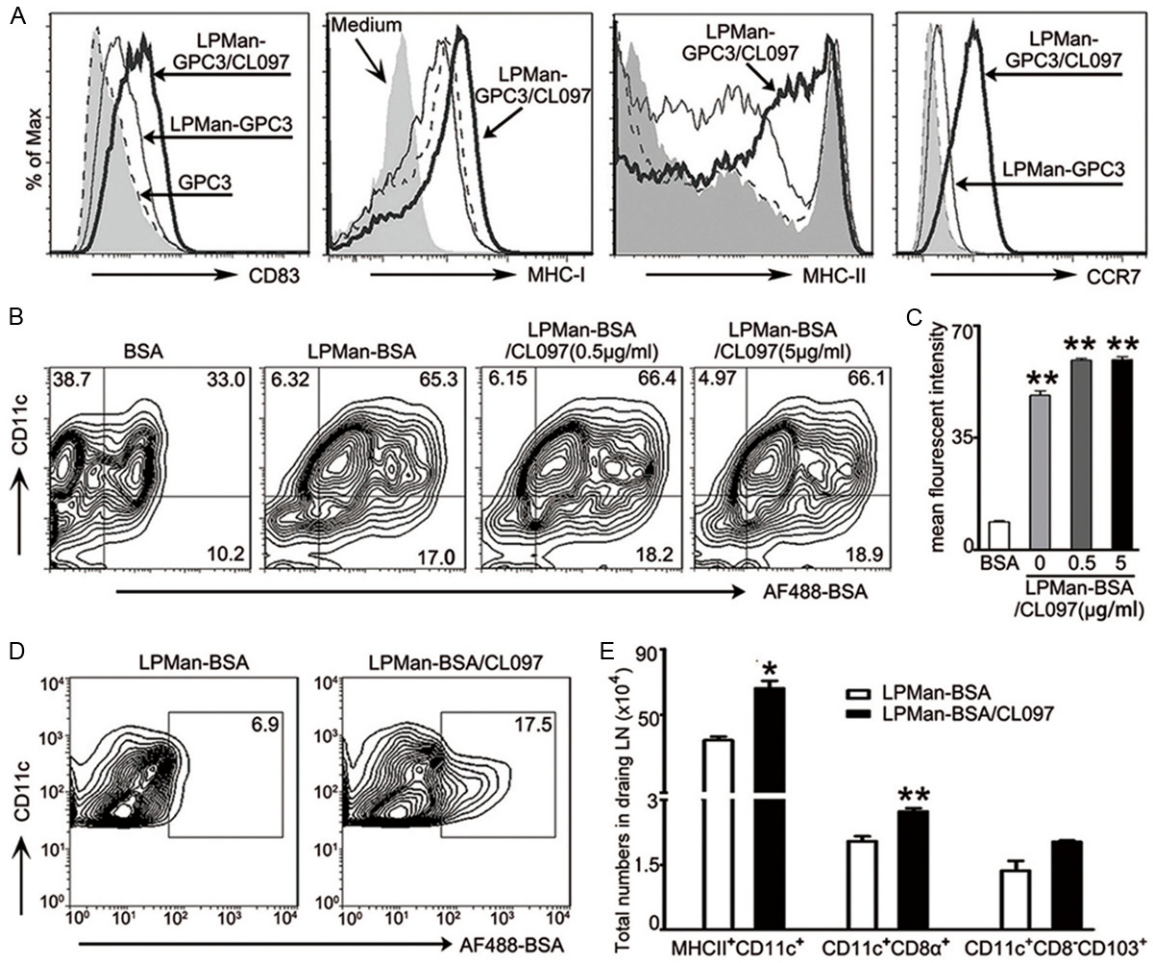


Figure 2. Effect of LPMan-GPC3/CL097 on migratory DCs and draining lymph nodes *in vivo*. A. FCM analysis of cell surface markers on BMDCs (3×10^6/ml) by staining with antibody to CD83, MHC-I, MHC-II and CCR7 after they were stimulated for 24 h with LPMan-GPC3/CL097 containing 5 μg/ml GPC3 plus 0.5 μg/ml CL097. The same amount of LPMan-GPC3 containing 5 μg/ml GPC3, or free GPC3 (5 μg/ml) were used as control. Profiles show representative of three independent experiments. B, C. AF488-labeled BSA (BSA) was used as the model antigen and encapsulated with LPMan as performed as GPC3. BMDCs (3×10^6/ml) were stimulated with LPMan-BSA/CL097 containing 5 μg/ml-BSA plus 0.5 μg/ml-CL097, or LPMan-BSA/CL097 containing 5 μg/ml BSA plus 5 μg/ml CL097, or LPMan-BSA containing 5 μg/ml BSA, or 5 μg/ml free BSA for 30 min. The percentage (B) and amount (C, as indicated by mean fluorescent intensity) of BSA in $CD11c^+$ DCs was determined. $^{**}P<0.01$ compared with the group treated with free BSA. D, E. Each mouse received 5 μg of AF-BSA in the form of LPMan-BSA (n=5) or received 5 μg of AF-BSA plus 5 μg of CL097 in the form of LPMan-BSA/CL097 via subcutaneous injection (n=5). raining lymph nodes were removed 48 h later. D. Profiles show the presence of AF-BSA in $CD11c^+$ cells of the draining lymph nodes. E. Bars show the total numbers of $MHCII^+CD11c^+$ cells, $CD11c^+CD8\alpha^+$ cells and $CD11c^+CD8\alpha^-CD103^+$ in draining lymph nodes of each mouse. Data are shown as mean ± SE, and un-paired *t* test was conducted between the two groups. $^*P<0.05$; $^{**}P<0.01$.

for MHC-I binding (**Figure 1B**), were all enhanced significantly after the cells were stimulated with GPC3 plus CL097. The stimulation also enhanced the expression levels of Sec22b, CD74, GILT, HSP90, and EDEM1, EDEM2, which are involved in recruiting ER proteins to phagosomes and translocating antigen from phagosomes into cytosol, accelerating ER-associated degradation of misfolded polypeptides (**Figure 1C**).

GPC3 nanovaccine containing CL097 promotes migratory DC antigen uptake, maturation and migration to draining lymph nodes

For better targeting lymph nodes where naïve T-cells reside, we synthesized positively charged cationic LPMan according to our previous report [19]. Using a previously defined molar ratio of 95% DOTAP and 5% DSPE-PEG-Man, we identified that the optimal encapsulation effi-

ciency was 1 μmol/ml LPMan with 100 μg/ml GPC3 (Supplementary Table 2). At this ratio, the maximal CL097 inclusion was 50-100 μg/ml. The particles showed no variation in the diameter but had decreased the surface charge with an increasing CL097 concentration (Supplementary Table 3). In the presence of the maximal concentration of mannosylate residues, no significant difference was found after CL097 was added into LPMan-encapsulated GPC3 (LPMan-GPC3/CL097) (**Figure 1D**). We prepared the GPC3 nanovaccine at the ratio of 1 μmol LPMan with 100 μg of GPC3 plus 10 μg of CL097 for *in vitro*, and 1 μmol LPMan with 100 μg of GPC3 plus 100 μg of CL097 for *in vivo* experiments.

We stimulated BMDCs with LPMan-GPC3/CL097 containing 5 μg/ml GPC3 plus 0.5 μg/ml CL097, or LPMan-GPC3 containing 5 μg/ml GPC3, or 5 μg/ml purified GPC3. FCM analysis showed that LPMan-GPC3/CL097 significantly increased the surface expression of CD83, MHC-I and MHC-II (**Figure 2A**). CCR7 expression, which is required for DC migration to draining lymph organs, was also significantly augmented (**Figure 2A**).

We used AF-BSA as a model antigen to confirm antigen uptake and delivery in lymph nodes. FCM analysis showed that 33% of $CD11c^+$ cells were AF-BSA positive when free AF-BSA added, but more than 65% of $CD11c^+$ cells were positive for AF-BSA when BMDCs were conditioned with LPMan-BSA containing 5 μg/ml BSA (**Figure 2B**). Adding different concentration of CL097 into LPMan-BSA did not alter the antigen-internalization capacity (**Figure 2C**). We injected 50 μl of LPMan-BSA/CL097 containing 5 μg of AF-BSA plus 5 μg of CL097, or the same amount of LPMan-BSA subcutaneously into mice. The encapsulated antigens were well drained to lymph nodes and taken up by $CD11c^+$ cells 2 days later (**Figure 2D**). In the presence of CL097, the total numbers of $MHCII^+CD11c^+$, particularly $CD11c^+CD8\alpha^+$ and $CD103^+$ DCs cells which were potent for priming $CD8^+$ T cells, increased significantly in the draining lymph nodes (**Figure 2E**).

GPC3 nanovaccines containing CL097 are efficiently processed in ER-Golgi system of migratory DCs for activating $CD4^+$ T and $CD8^+$ T cells

BMDCs were conditioned with LPMan-BSA/CL097 containing 5 μg/ml AF-BSA plus 0.5 μg/ml CL097, or LPMan-BSA containing 5 μg/ml AF-BSA or free AF-BSA for 3 h. Immunofluorescence microscopy analysis showed that the internalized antigen delivered by LPMan was mainly co-localized with early endosomes (EEA-1), inside the ER (Grp78) and Golgi apparatus (Giantin), particularly in the presence of CL097 (**Figure 3Aa-c**). Compared with free AF-BSA-treated BMDCs, internalized antigen delivered by LPMan was hardly detected within lysosomes (as indicated by LAMP-1) (**Figure 3Ad**).

To verify the effect of LPMan-GPC3/CL097-conditioned migratory DCs in priming naïve T cells for the generation of GPC3-specific T cells, we treated BMDCs with 5 μg/ml of LPMan-GPC3/CL097, or LPMan-GPC3, or free GPC3 for 24 h. Negatively selected T cells were labeled with CFSE and co-cultured with the above conditioned BMDCs for 5 days. T-cell proliferation (both $CD8^+$ T and $CD4^+$ T cells) was only observed when the cells were stimulated with BMDCs, which were conditioned with LPMan-GPC3/CL097 (**Figure 3B**), indicating the antigen in LPMan-GPC3/CL097 could be processed professionally.

LPMan-GPC3/CL097 immunization prevents DEN-induced premalignant hepatocytes to tumor development in an autochthonous liver cancer murine model

A single postnatal injection of DEN induces hepatocyte DNA damage and results in mouse liver cancer, the development course of which is similar to human HCC [25]. The tumor progenitors have been identified to highly express GPC3, AFP and other malignant markers 2-3 months after DEN and develop to an HCC mass in the inflamed liver [27]. Because HCC mainly develops in an HBV background [28], we adopted the method to induce murine autochthonous liver cancer by injecting one dose of DEN to an HBV-transgenic mouse at 2 weeks old (**Figure 4A**). By wk6 after DEN, clusters of premalignant hepatocytes were observed with GPC3 over-expression (**Figure 4B**). Liver cancer nodules were firstly observed by wk14, and many cancer nodules developed by wk20 after DEN, with more than 90% of the tumor cells expressing GPC3 (**Figure 4B**).

Starting at wk8 after DEN, when GPC3 over-expressing premalignant hepatocyte clusters

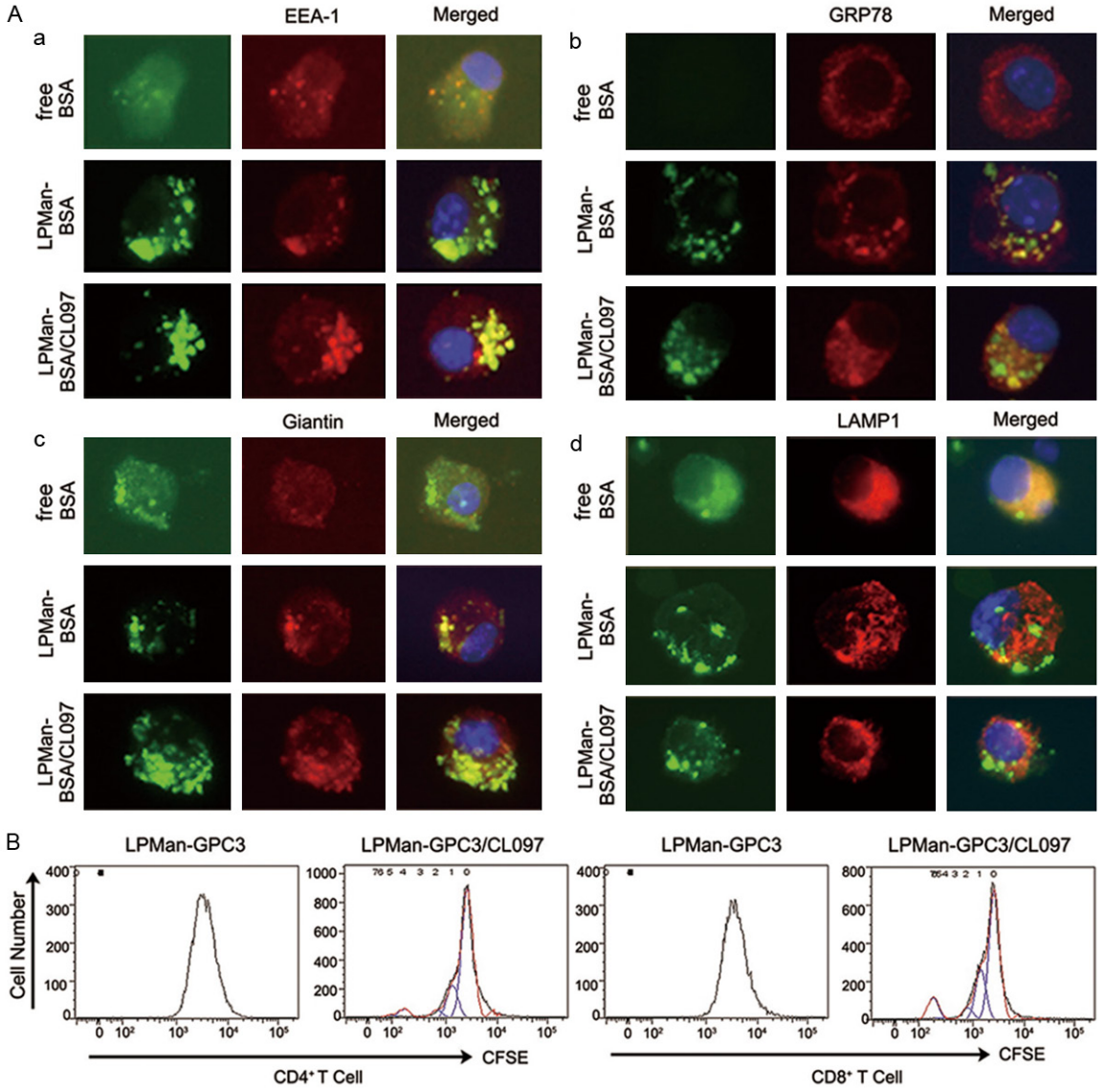


Figure 3. Fate of internalized antigens in DCs delivered by mannosylated liposomes. A. AF488-labeled BSA (BSA, green) was used a model antigen. BMDCs were treated for 3 h with 5 μg/ml BSA alone, or LPMan-BSA containing 5 μg/ml BSA, or LPMan-BSA/CL097 containing 5 μg/ml-BSA plus 0.5 μg/ml-CL097. The cells were washed and placed on poly-L-lysine-coated slides and then were fixed with 2% paraformaldehyde for 10 min. The cells were stained with Cy3-labeled (red) antibodies of anti-EEA-1 (a), anti-Grp78 (b), anti-Giantin (c) or anti-LAMP1 (d). One representative experiment of five is shown. B. BMDCs were treated with LPMan-GPC3/CL097 containing 5 μg/ml GPC3 plus 0.5 μg/ml CL097, or same amount of LPMan-GPC3 containing 5 μg/ml GPC3 for 24 h. Untouched T cells were isolated from naïve mice and labeled with 5 μM CFSE. The two cell populations were co-cultured at the ratio of 1-BMDC to 20-T cells for 5 days. $CD4^+$ T cell (left panel) and $CD8^+$ T cell (right panel) proliferation levels were determined by FCM (one representative experiment out of three).

generated, we immunized the DEN-treated HBV-transgenic mice 4 times with LPMan-GPC3/CL097 every 2 weeks (**Figure 4A**). At the indicated time point, each mouse received 2.5 μg, 5 μg, or 50 μg of GPC3 with 5 μg of CL097. Sham-immunized mice were given LPMan/CL097 containing 5 μg of CL097 in the same way. Compared with un-immunized and sham-immunized mice, the elevation of serum ALT, which reflects the status of hepatocyte damage related to the accumulation of HBV surface antigens [24], was alleviated, particularly in the group of mice immunized with 5 μg of GPC3 plus 5 μg of CL097 (**Figure 4C**). Anti-GPC3 antibody began to be detectable after immunization for 2 doses (**Figure 4D**).

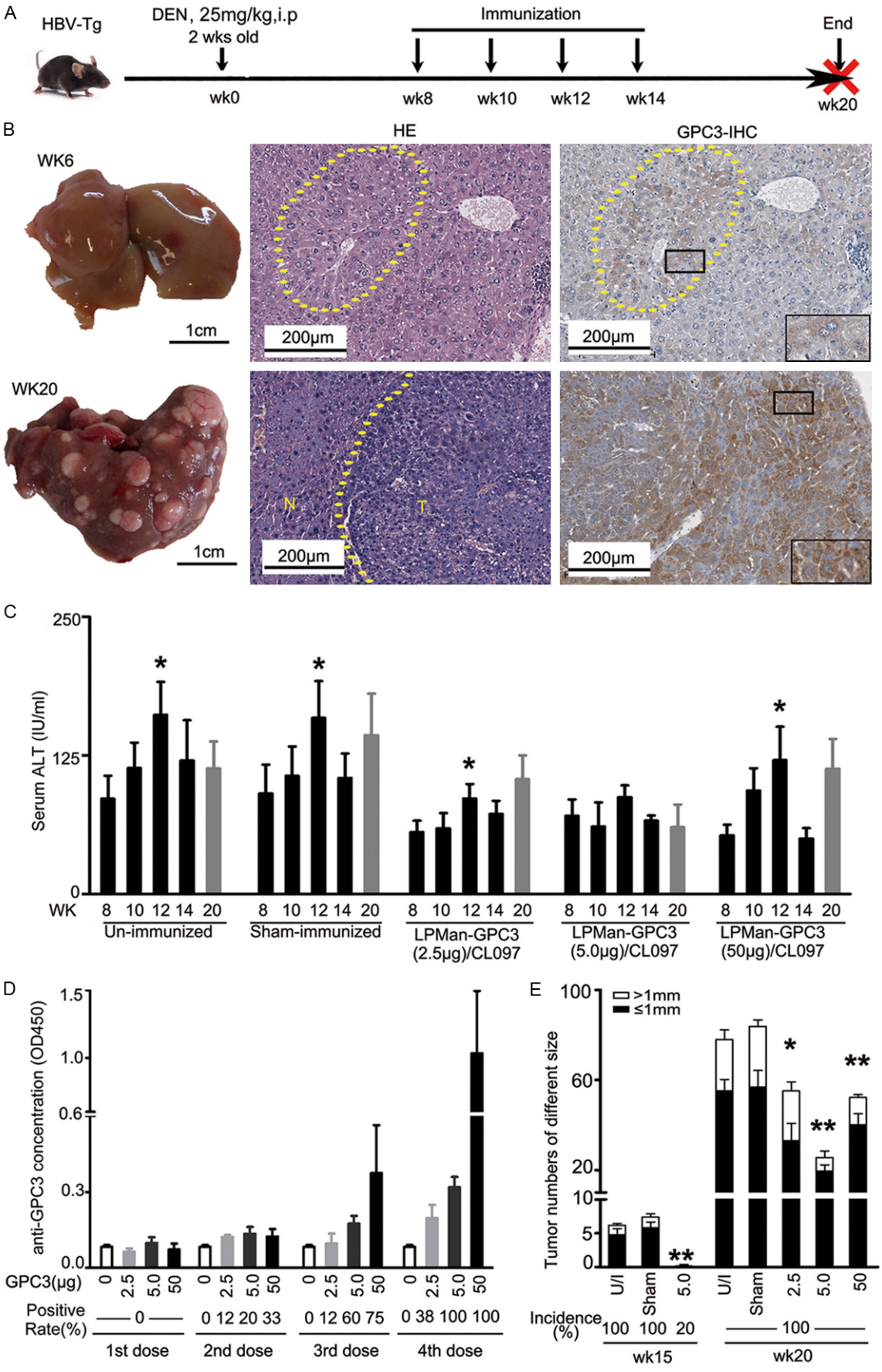

A
HBV-Tg
DEN, 25mg/kg,i.p
2 wks old
Immunization
End
wk0
wk8
wk10
wk12
wk14
wk20
B
HE
GPC3-IHC
WK6
1cm
200μm
WK20
N
T
C
Serum ALT (IU/ml)
250
125
0
WK
8 10 12 14 20
Un-immunized
Sham-immunized
LPMan-GPC3 (2.5μg)/CL097
LPMan-GPC3 (5.0μg)/CL097
LPMan-GPC3 (50μg)/CL097
D
anti-GPC3 concentration (OD450)
1.5
1.0
0.6
0.3
0.0
GPC3(μg)
0 2.5 5.0 50
Positive Rate(%)
0
0 12 20 33
0 12 60 75
0 38 100 100
1st dose
2nd dose
3rd dose
4th dose
E
Tumor numbers of different size
>1mm
≤1mm
100
60
20
10
5
0
U/I
Sham
5.0
2.5
50
Incidence (%)
100 100 20
100
wk15
wk20

Figure 4. Effect of LPMan-GPC3/CL097 immunization on liver cancer development in a DEN-induced murine autochthonous liver cancer model. A. Experimental scheme. Male HBV-transgenic mice (HBV-Tg) received 25 mg/kg of DEN intraperitoneally at 2 weeks old. They were immunized 4 times with LPMan-GPC3/CL097. At each time point, each mouse received 2.5 μg (n=5), 5 μg (n=15) or 50 μg (n=5) of GPC3 plus 5 μg of CL097 starting 8 weeks after DEN injection (wk8). Sham-immunized mice received 5 μg of CL097/mouse in the form of LPMan-CL097 at the same time. The livers of DEN-administered mice were sampled at wk6, wk8, wk14, wk16 and wk20 after DEN. B. H&E staining, and anti-GPC3 immunohistochemistry staining of the mouse liver sampled at wk6 and wk20 after DEN administration. C. Variation of serum ALT determined at different time points, $^{**}P< 0.05$ compared with serum ALT determined at wk8. D. Serum anti-GPC3 antibody concentration at different time points. E. Tumor numbers of different sizes counted at wk15 and wk20 in the mice immunized with different dose of GPC3. Un-paired *t* test was conducted. $^{*}P<0.05$ and $^{**}P<0.01$ compared with sham-immunized mice.

By wk20 after DEN all mice were sacrificed and all the mice developed liver cancer. However, compared with sham-immunized mice, the tumor burden in LPMan-GPC3/CL097 immunized mice was reduced significantly, with a maximal decrease in the mice that received 5 μg of GPC3 plus 5 μg of CL097 (**Figure 4E**). We repeated our experiments in another batch of DEN-treated HBV-transgenic mice to confirm the effect by administration 4 doses of LPMan-GPC3/CL097 containing 5 μg of GPC3 plus 5 μg of CL097. In each group, 5 mice were sacrificed 1 week after the last immunization (wk15 after DEN). All unimmunized (5/5) and sham-immunized (5/5) mice developed liver tumor nodules. Only 1/5 mice immunized with LPMan-GPC3/CL097 developed one small-volume tumor nodule (**Figure 4E**). Consistent with the previous observation, LPMan-GPC3/CL097-immunized mice developed significantly fewer tumor numbers than sham-immunized mice by wk20. No significant difference was observed in the tumor burden between sham group and LPMan-GPC3 or GPC3 immunized mice (Supplementary Figure 1).

LPMan-GPC3/CL097 immunization-induced GPC3-specific T cells eliminate GPC3-expressing tumor cells

Mouse splenocytes were collected 1 week after last immunization (wk15) and re-stimulated with 5 μg/ml purified GPC3 protein. The numbers of GPC3-specific $CD4^+$ IFNγ-producing and $CD8^+$ IFNγ-producing cells were all significantly increased in LPMan-GPC3/CL097-immunized than in sham-immunized mice. Among spleen $CD3^+$ T cells, ~1.5% were GPC3-specific $CD4^+$ IFNγ-producing cells, and ~0.25% were GPC3-specific $CD8^+$ IFNγ-producing cells (**Figure 5A**). After the cells were stimulated with GPC3, IFNγ was increased by ~2.5 fold and Granzyme B was increased by ~1.6 fold in the LPMan-GPC3/CL097-immunized mice (**Figure 5B**).

To confirm the T-cell activity in eradicating GPC3-expressing tumor cells, we over-expressed GPC3 in Hepa1-6 cells (Hepa/GPC3) (Supplementary Figure 2) derived from C57-BL/6 mice [8] and confirmed the $H\text{-}2K^b$/$H2\text{-}D^b$ expression in the cells (**Figure 5C**). IHLs were isolated and co-cultured with Hepa/GPC3 for 4 h. The antigen-specific cytotoxicity was increased significantly in the IHLs from LPMan-GPC3/CL097-immunized compare with that in sham-immunized mice (**Figure 5D**).

We collected liver interstitial fluid and quantified IFNγ and Granzyme B produced locally 6 weeks after the last immunization. Significantly higher amounts of these two cytokines were presented in LPMan-GPC3/CL097-immunized mice compared with that in sham-immunized mice (**Figure 5E**). After the IHLs were stimulated with GPC3, ~0.4% of $CD4^+$ T cells and ~0.7% $CD8^+$ T cells were GPC3-specific IFNγ-producing cells (**Figure 5E**).

Discussion

By synthesizing LPMan as a vaccine delivery system and incorporating TLR7/8 agonist CL097 as the adjuvant, we prepared the GPC3 nanovaccine, LPMan-GPC3/CL097. The vaccine efficiently targeted draining lymph nodes where naïve T cells reside, promoting DC maturation with increased surface expressions of MHC-I and MHC-II, and CCR7. The delivered antigen in DCs escaped from lysosomes and was processed professionally into the MHC-I antigen presentation pathway. Consequently, naïve T cells were primed and GPC3-specific $CD4^+$ T and $CD8^+$ T cells were *de novo* generated that could eradicate GPC3-expressing tumor cells. Immunization with 4 doses of LPMan-GPC3/CL097 containing 5 μg of GPC3 plus 5 μg of CL097 could prevent carcinogen-induced premalignant/malignant hepatic lesion development to cancer in HBV-transgenic mice.

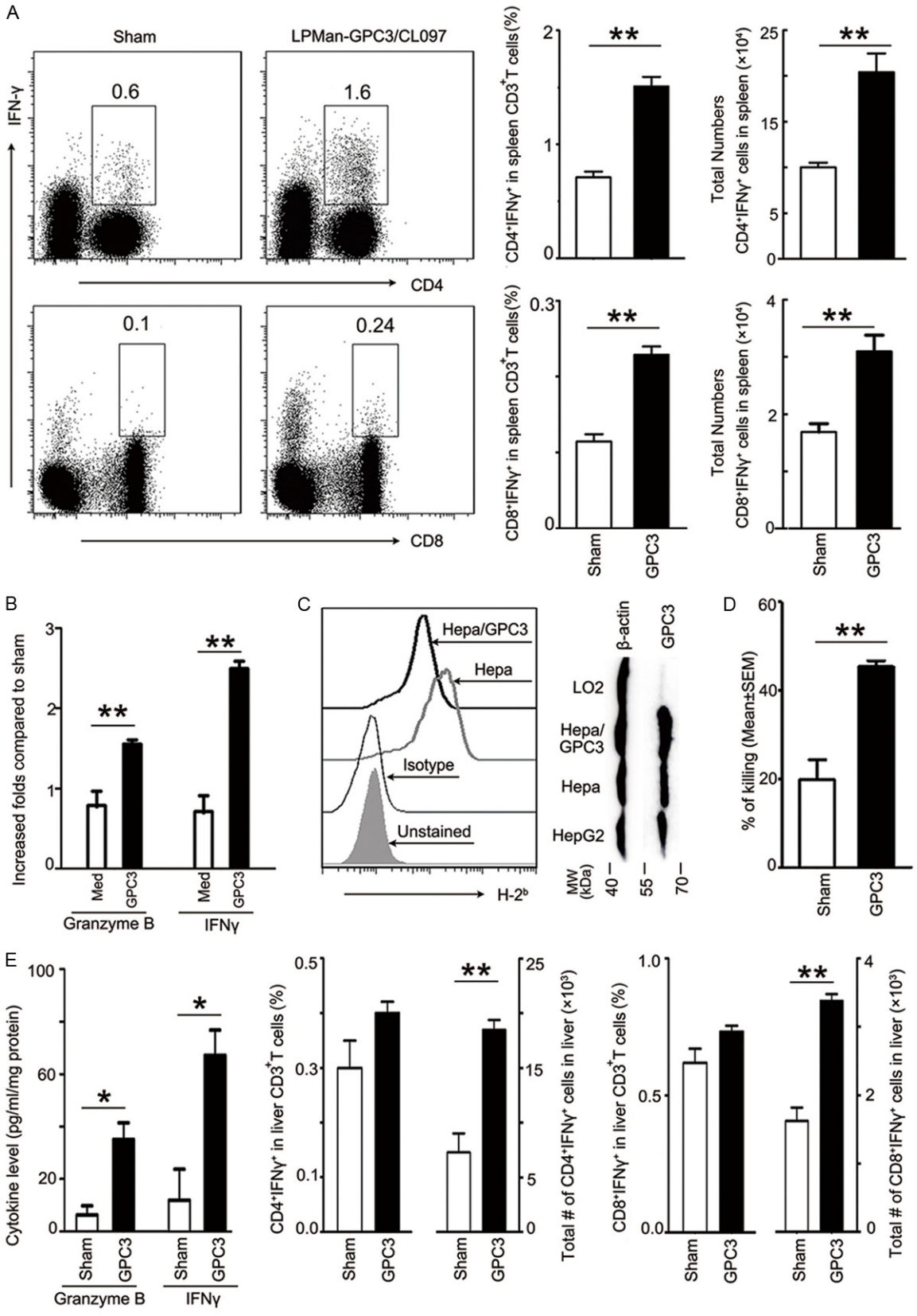


Figure 5. Immunization of LPMan-GPC3/CL097 induces potent immunity, eliminating GPC3-expressed tumor cells. A, B. Splenocytes (5×10^6/ml) were isolated from sham- or LPMan-GPC3/CL097-immunized mice and stimulated

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. The increased folds of IFNγ and Granzyme B in the GPC3-stimulated splenocyte supernatants from LPMan-GPC3/CL097-immunized mice. C. Confirmation of GPC3 expression by western blotting and MHC-I surface expression by FCM using PE-labeled anti-$H2K^b$/$H2D^b$-specific antibody on Hepa and Hepa/GPC3 cells which were constructed by transfecting Hepa1-6 with plasmid (Supplementary Figure 2). D. IHLs (as effector) were isolated from LPMan-GPC3/CL097-immunized or sham-immunized mice and co-cultured with CFSE-labeled Hepa/GPC3 cells (as target) for 4 h at the ratio of 40 effectors to 1 target. Percentage of double positivity for CFSE and PI cells was calculated. E. All mice were sacrificed at wk20 after DEN administration. The concentration of IFNγ and Granzyme B in the livers of sham- or LPMan-GPC3/CL097-immunized mice was measured using commercialized ELISA kits. Percentage and total numbers of IFN-γ^+CD4^+ T cells or IFN-γ^+CD8^+ T cells in the livers of sham- or LPMan-GPC3/CL097-immunized mice are presented. Un-paired *t* test was conducted. **P*<0.05. ***P*<0.01.

GPC3-specific T cells generated after immunization could eliminate GPC3-expressing tumor cells to prevent HCC. The current study provided a possible approach for cirrhotic patients with malignant precancerous lesion nodules.

DCs have been found to play fundamental roles in cell-based vaccination immunotherapy [29, 30]. Our current results displayed that many key molecules involved in MHC-I presentation were significantly augmented when BMDCs were stimulated with GPC3 plus CL097. These results provided the molecular basis for TLR7/8 agonists in activating migratory DCs to induce antigenic peptide presentation on MHC-I molecules to prime naïve $CD8^+$ T cells. Because naïve T cells do not directly patrol peripheral tissues, it is crucial to deliver antigen to draining lymph nodes to generate antigen-specific $CD4^+$ and $CD8^+$ T cells, which are both required to reject established tumor cells [13]. Previous studies have reported that mannosylated antigens without the addition of adjuvants could be processed in both MHC-I- and MHC-II-presenting pathways and induce the generation of antigen-specific $CD4^+$ T and $CD8^+$ T cells [19, 31]. The addition of some TLR agonists is helpful to cross-present protein vaccines that are in a cell's external environment to prime naïve $CD8^+$ T cells [14]. Our functional assay validated that only the DCs conditioned with LPMan-GPC3/CL097, not LPMan-GPC3, could stimulate T-cell proliferation. Notably, the vaccine with CL097 increased the numbers of DCs in draining lymph nodes, particularly cells that have potent capacity of antigen cross-presentation i.e., $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-CD103^+$ cells [14]. The current study indicated that the addition of CL097 in the vaccine formula was necessary to induce the *de novo* generation of GPC3-specific $CD4^+$ and $CD8^+$ T cells.

Promising results from clinical trials were observed in advanced HCC patients receiving GPC3-peptide vaccines [32] but not in anti-GPC3 specific antibody therapy [33]. Peptide vaccine needs the exact MHC-I molecule to induce monoclonal or oligoclonal T-cell populations. In addition, some of the synthetic short peptides predicted by computer may not be presented by the targeted malignant cells. The whole GPC3-protein vaccine could induce multiple clones of T cells when the protein is processed and presented by potent DCs in appropriate ways. Our results showed that LPMan-encapsulated GPC3 containing CL097 was processed professionally in migratory DCs, subsequently inducing the GPC3-specific $CD4^+$ T and $CD8^+$ T cell generation.

We injected DEN into HBV-transgenic mice to possibly simulate HCC development clinically in the HBV background. Dysplastic hepatocytes and GPC3-positive cell clusters were detected 6 weeks after DEN injection, which was recently confirmed as liver cancer progenitors [27]. Our results showed that immunization beginning at the stage when premalignant/malignant hepatocytes generated could prevent liver cancer development. Although the mice immunized with the GPC3 nanovaccine developed liver cancer, the tumor burden reduced significantly in the GPC3-immunized mice compared with sham-immunized mice. The HCC-associated antigen GPC3 proves as a useful target. Still better strategy is required to be developed in order to eradicate the established tumors, including induction of more potent GPC3-specific $CD4^+$ T and $CD8^+$ T cells, or in combination with the immune check-point inhibitors.

In summary, the GPC3 nanovaccine LPMan-GPC3/CL097 efficiently targeted draining lymph nodes where naïve T cells reside. When precancerous hepatocytes generated, immunization with LPMan-GPC3/CL097 induced specific T-cell immunity, eliminating GPC3-expressing tumor progenitors or tumor cells. Hence,

when the malignant precancerous lesion nodules were presented in the cirrhotic liver, immunization with LPMan-GPC3/CL097 could prevent HCC development. The benefit of the vaccine must be evaluated in high-risk patients.

Acknowledgements

Current address for Yifan Ma: HRYZ BIO TECH Co., Shenzhen 518057, China. This work was supported by National Natural Science Foundation of China (No. 81172888, 81161120495 to CQ), CAMS Innovative Medicine (No. 2016-I2M-1-007 to CQ); State Key Projects Specialized for Infectious Diseases (No. 2012ZX10002008-001 to CQ), PUMC Innovation Fund for PhD students (No. 2014-1001-1014, 3332015061 to KC) and Shenzhen Science and Technology program (No. CYZZ20170331150956189 to YM). The sponsors had no role in the study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Disclosure of conflict of interest

None.

Abbreviations

AF-BSA, alexa flour-488 labeled bovine serum albumin; AFP, alpha-fetoprotein; BMDC, bone marrow-derived dendritic cells; BSA, bovine serum albumin; CFSE, 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cells; DEN, diethylnitrosamine; ER, endoplasmic reticulum; FCM, Flow cytometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPC3, Glypican-3; IHL, intrahepatic lymphocytes; IFNγ, interferon gamma; LPMan, mannosylated liposomes; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; TAPBP, TAP binding protein.

Address correspondence to: Dr. Chunfeng Qu, Department of Immunology, Cancer Hospital, Chinese Academy of Medical Sciences, No 17 Panjiayuan Nanli, Chaoyang District, Beijing 100021, China. Tel: +86-10-87783103; Fax: +86-10-67715925; E-mail: quchf@cicams.ac.cn

References

[1] Umar A. Cancer immunoprevention: a new approach to intercept cancer early. Cancer Prev Res (Phila) 2014; 7: 1067-1071.

[2] Finn OJ and Beatty PL. Cancer immunoprevention. Curr Opin Immunol 2016; 39: 52-58.

[3] Di Tommaso L, Sangiovanni A, Borzio M, Park YN, Farinati F and Roncalli M. Advanced precancerous lesions in the liver. Best Pract Res Clin Gastroenterol 2013; 27: 269-284.

[4] Starr TK, Jameson SC and Hogquist KA. Positive and negative selection of T cells. Annu Rev Immunol 2003; 21: 139-176.

[5] Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, Zhang W, Luoma A, Giobbie-Hurder A, Peter L, Chen C, Olive O, Carter TA, Li S, Lieb DJ, Eisenhaure T, Gjini E, Stevens J, Lane WJ, Javeri I, Nellaiappan K, Salazar AM, Daley H, Seaman M, Buchbinder EI, Yoon CH, Harden M, Lennon N, Gabriel S, Rodig SJ, Barouch DH, Aster JC, Getz G, Wucherpfennig K, Neuberg D, Ritz J, Lander ES, Fritsch EF, Hacohen N and Wu CJ. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature 2017; 547: 217-221.

[6] Sahin U, Derhovanessian E, Miller M, Kloke BP, Simon P, Lower M, Bukur V, Tadmor AD, Luxemburger U, Schrors B, Omokoko T, Vormehr M, Albrecht C, Paruzynski A, Kuhn AN, Buck J, Heesch S, Schreeb KH, Muller F, Ortseifer I, Vogler I, Godehardt E, Attig S, Rae R, Breitkreuz A, Tolliver C, Suchan M, Martic G, Hohberger A, Sorn P, Diekmann J, Ciesla J, Waksmann O, Bruck AK, Witt M, Zillgen M, Rothermel A, Kasemann B, Langer D, Bolte S, Diken M, Kreiter S, Nemecek R, Gebhardt C, Grabbe S, Holler C, Utikal J, Huber C, Loquai C and Tureci O. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature 2017; 547: 222-226.

[7] Zhang W, He H, Zang M, Wu Q, Zhao H, Lu LI, Ma P, Zheng H, Wang N, Zhang Y, He S, Chen X, Wu Z, Wang X, Cai J, Liu Z, Sun Z, Zeng YX, Qu C and Jiao Y. Genetic features of aflatoxin-associated hepatocellular carcinoma. Gastroenterology 2017; 153: 249-262, e242.

[8] Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, Eisele S, Encke J, Blum HE and Geissler M. Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology 2000; 119: 1104-1112.

[9] Hong Y, Peng Y, Guo ZS, Guevara-Patino J, Pang J, Butterfield LH, Mivechi NF, Munn DH, Bartlett DL and He Y. Epitope-optimized alpha-fetoprotein genetic vaccines prevent carcinogen-induced murine autochthonous hepatocellular carcinoma. Hepatology 2014; 59: 1448-1458.

[10] Geissler M, Mohr L, Weth R, Kohler G, Grimm CF, Krohne TU, von Weizsacker F and Blum HE. Immunotherapy directed against alpha-feto-

protein results in autoimmune liver disease during liver regeneration in mice. Gastroenterology 2001; 121: 931-939.
[11] Filmus J and Capurro M. Glypican-3: a marker and a therapeutic target in hepatocellular carcinoma. FEBS J 2013; 280: 2471-2476.
[12] Di Tommaso L, Destro A, Seok JY, Balladore E, Terracciano L, Sangiovanni A, Iavarone M, Colombo M, Jang JJ, Yu E, Jin SY, Morenghi E, Park YN and Roncalli M. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. J Hepatol 2009; 50: 746-754.
[13] Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ and Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001; 410: 1107-1111.
[14] Cruz FM, Colbert JD, Merino E, Kriegsman BA and Rock KL. The biology and underlying mechanisms of cross-presentation of exogenous antigens on MHC-I molecules. Annu Rev Immunol 2017; 35: 149-176.
[15] Wille-Reece U, Flynn BJ, Lore K, Koup RA, Miles AP, Saul A, Kedl RM, Mattapallil JJ, Weiss WR, Roederer M and Seder RA. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. J Exp Med 2006; 203: 1249-1258.
[16] Kastenmuller K, Wille-Reece U, Lindsay RW, Trager LR, Darrah PA, Flynn BJ, Becker MR, Udey MC, Clausen BE, Igyarto BZ, Kaplan DH, Kastenmuller W, Germain RN and Seder RA. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. J Clin Invest 2011; 121: 1782-1796.
[17] Romero P, Banchereau J, Bhardwaj N, Cockett M, Disis ML, Dranoff G, Gilboa E, Hammond SA, Hershberg R, Korman AJ, Kvistborg P, Melief C, Mellman I, Palucka AK, Redchenko I, Robins H, Sallusto F, Schenkelberg T, Schoenberger S, Sosman J, Tureci O, Van den Eynde B, Koff W and Coukos G. The human vaccines project: a roadmap for cancer vaccine development. Sci Transl Med 2016; 8: 334ps9.
[18] Kalam MA, Khan AA and Alshamsan A. Non-invasive administration of biodegradable nanocarrier vaccines. Am J Transl Res 2017; 9: 15-35.
[19] Wang C, Liu P, Zhuang Y, Li P, Jiang B, Pan H, Liu L, Cai L and Ma Y. Lymphatic-targeted cationic liposomes: a robust vaccine adjuvant for promoting long-term immunological memory. Vaccine 2014; 32: 5475-5483.
[20] Qu C, Nguyen V, Merad M and Randolph G. MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells. J Immunol 2009; 182: 3650-3659.
[21] Wang Y, Chen K, Wu Z, Liu Y, Liu S, Zou Z, Chen SH and Qu C. Immunizations with hepatitis B viral antigens and a TLR7/8 agonist adjuvant induce antigen-specific immune responses in HBV-transgenic mice. Int J Infect Dis 2014; 29: 31-36.
[22] Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S and Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992; 176: 1693-1702.
[23] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
[24] Chisari FV, Pinkert CA, Milich DR, Filippi P, McLachlan A, Palmiter RD and Brinster RL. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. Science 1985; 230: 1157-1160.
[25] Maeda S, Kamata H, Luo JL, Leffert H and Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 2005; 121: 977-990.
[26] 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.
[27] He G, Dhar D, Nakagawa H, Font-Burgada J, Ogata H, Jiang Y, Shalapour S, Seki E, Yost SE, Jepsen K, Frazer KA, Harismendy O, Hatziapostolou M, Iliopoulos D, Suetsugu A, Hoffman RM, Tateishi R, Koike K and Karin M. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell 2013; 155: 384-396.
[28] El-Serag HB and Kanwal F. Epidemiology of hepatocellular carcinoma in the United States: Where are we? Where do we go? Hepatology 2014; 60: 1767-1775.
[29] Palucka K and Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. Immunity 2013; 39: 38-48.
[30] Chen Q, Cui XX, Liang PF, Dou JX, Liu ZY and Sun WW. Immunotherapy with dendritic cells and cytokine-induced killer cells for MDA-MB-231 breast cancer stem cells in nude mice. Am J Transl Res 2016; 8: 2947-2955.
[31] Sheng KC, Kalkanidis M, Pouniotis DS, Esparon S, Tang CK, Apostolopoulos V and Pietersz GA. Delivery of antigen using a novel man-

nosylated dendrimer potentiates immunogenicity in vitro and in vivo. Eur J Immunol 2008; 38: 424-436.

[32] Sayem MA, Tomita Y, Yuno A, Hirayama M, Irie A, Tsukamoto H, Senju S, Yuba E, Yoshikawa T, Kono K, Nakatsura T and Nishimura Y. Identification of glypican-3-derived long peptides activating both CD8+ and CD4+ T cells; prolonged overall survival in cancer patients with Th cell response. Oncoimmunology 2016; 5: e1062209.

[33] Abou-Alfa GK, Puig O, Daniele B, Kudo M, Merle P, Park JW, Ross P, Peron JM, Ebert O, Chan S, Poon TP, Colombo M, Okusaka T, Ryoo BY, Minguez B, Tanaka T, Ohtomo T, Ukrainskyj S, Boisserie F, Rutman O, Chen YC, Xu C, Shochat E, Jukofsky L, Reis B, Chen G, Di Laurenzio L, Lee R and Yen CJ. Randomized phase II placebo controlled study of codrituzumab in previously treated patients with advanced hepatocellular carcinoma. J Hepatol 2016; 65: 289-295.

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

[1] Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S and Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992; 176: 1693-1702.

[2] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[3] Wang C, Liu P, Zhuang Y, Li P, Jiang B, Pan H, Liu L, Cai L and Ma Y. Lymphatic-targeted cationic liposomes: a robust vaccine adjuvant for promoting long-term immunological memory. Vaccine 2014; 32: 5475-5483.

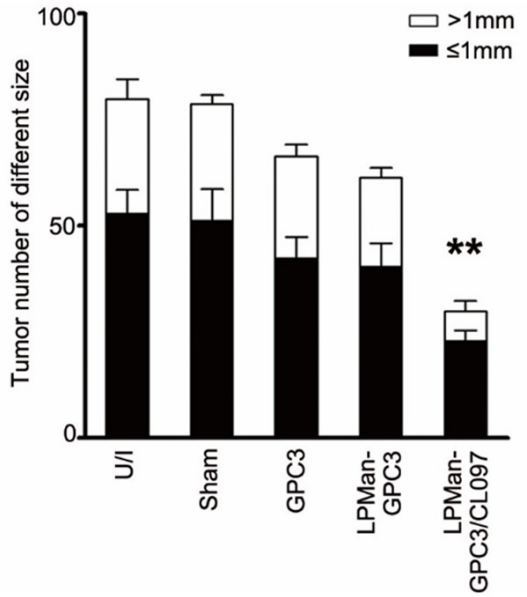
[4] Wang Y, Chen K, Wu Z, Liu Y, Liu S, Zou Z, Chen SH and Qu C. Immunizations with hepatitis B viral antigens and a TLR7/8 agonist adjuvant induce antigen-specific immune responses in HBV-transgenic mice. Int J Infect Dis 2014; 29: 31-36.

[5] Baronzio G, Schwartz L, Kiselevsky M, Guais A, Sanders E, Milanesi G, Baronzio M and Freitas I. Tumor interstitial fluid as modulator of cancer inflammation, thrombosis, immunity and angiogenesis. Anticancer Res 2012; 32: 405-414.

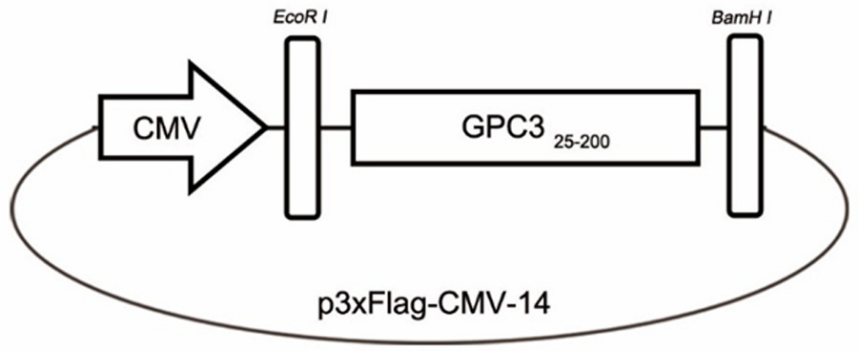
[6] Wu J, Du J, Liu L, Li Q, Rong W, Wang L, Wang Y, Zang M, Wu Z, Zhang Y and Qu C. Elevated pretherapy serum IL17 in primary hepatocellular carcinoma patients correlate to increased risk of early recurrence after curative hepatectomy. PLoS One 2012; 7: e50035.

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.



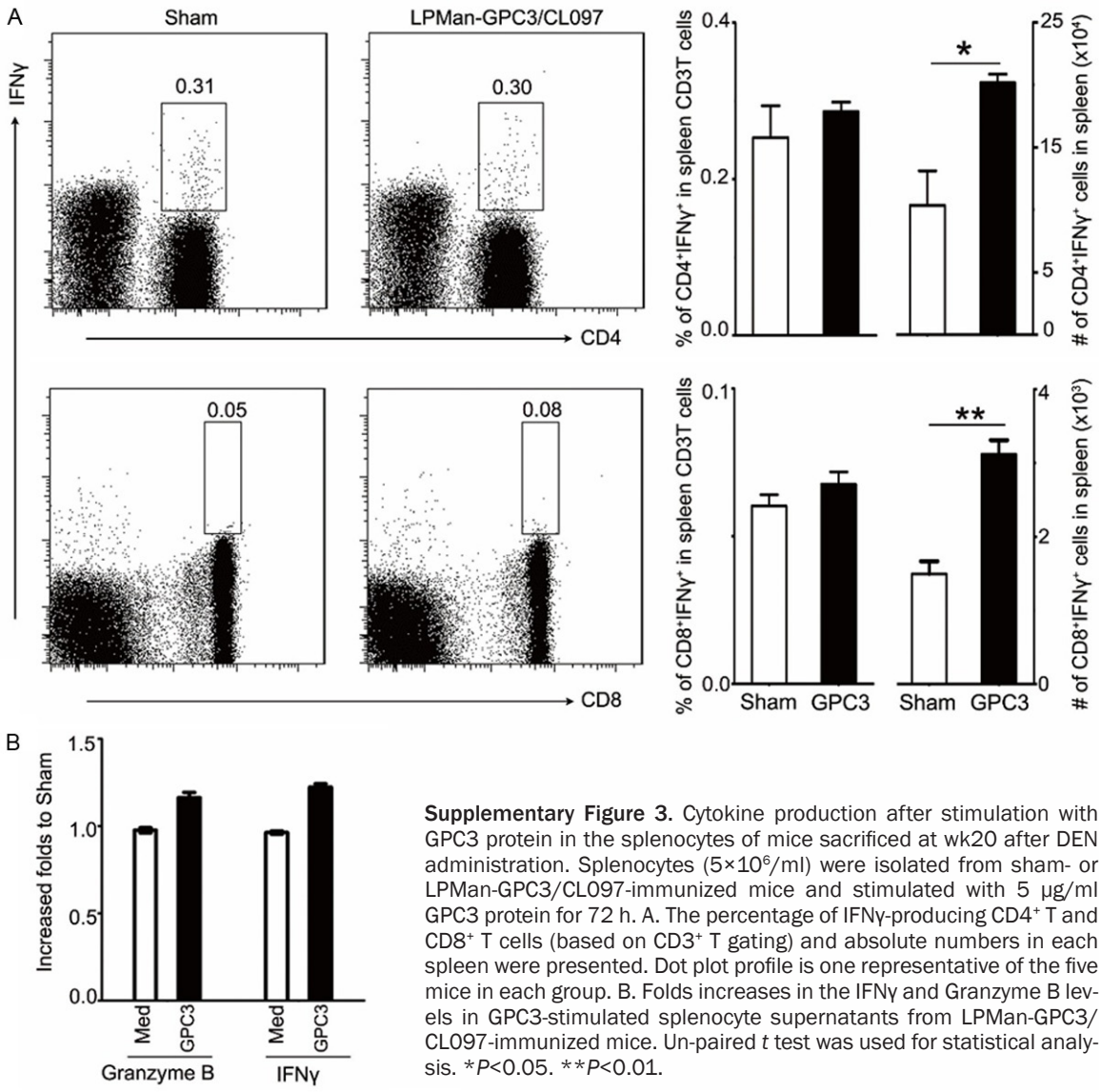
Supplementary Figure 2. Diagram of the GPC3-expressing plasmid pCMV-GPC3. The human GPC3 DNA fragment (73 to 600) was created by subcloning into the p3xFLAG-cmv-14 vector (Sigma), which contains the amino acids from 25 to 200 in the N-terminus.

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.