Highly efficient and tumor-selective nanoparticles for dual-targeted immunogene therapy against cancer

Kuan-Wei Huang1*, Fu-Fei Hsu2*, Jiantai Timothy Qiu3,4,5*, Guann-Jen Chern1, Yi-An Lee1, Chih-Chun Chang1, Yu-Ting Huang6, Yun-Chieh Sung1, Cheng-Chin Chiang1, Rui-Lin Huang1, Chu-Chi Lin5, Trinh Kieu Dinh1, Hsi-Chien Huang1, Yu-Chuan Shih1, Donia Alson5, Chun-Yen Lin3,7, Yung-Chang Lin8, Po-Chiao Chang7, Shu-Yi Lin6†, Yunching Chen1†

While immunotherapy holds great promise for combating cancer, the limited efficacy due to an immunosuppressive tumor microenvironment and systemic toxicity hinder the broader application of cancer immunotherapy. Here, we report a combinatorial immunotherapy approach that uses a highly efficient and tumor-selective gene carrier to improve anticancer efficacy and circumvent the systemic toxicity. In this study, we engineered tumor-targeted lipid-dendrimer-calcium-phosphate (TT-LDCP) nanoparticles (NPs) with thymine-functionalized dendrimers that not only enhanced gene delivery capacity but also immune adjuvant properties by activating the stimulator of interferon genes (STING)–cGAS pathway. TT-LDCP NPs delivered siRNA against immune checkpoint ligand PD-L1 and immunostimulatory IL-2–encoding plasmid DNA to hepatocellular carcinoma (HCC), increased tumoral infiltration and activation of CD8+ T cells, augmented the efficacy of cancer vaccine immunotherapy, and suppressed HCC progression. Our work presents nanotechnology-enabled dual delivery of siRNA and plasmid DNA that selectively targets and reprograms the immunosuppressive tumor microenvironment to improve cancer immunotherapy.

INTRODUCTION

Immunotherapy is rapidly emerging as a promising therapeutic strategy against cancer. In contrast to conventional anticancer agents that are often directly cytotoxic toward cancer cells, immunotherapy activates immune cells to recognize and eradicate tumor cells. Recently developed cancer immunotherapies include vaccines, chimeric antigen receptor T cell therapy, immune checkpoint blockade, and cytokine therapy (1). For example, in the clinic, interleukin-2 (IL-2), a key cytokine that drives the proliferation and activation of T cells, is used as an effective immunotherapy against cancer (2). Nivolumab—the first anti–PD-1 (programmed cell death 1) antibody—has shown substantial clinical activity in various cancer types. Vaccines composed of genetically modified tumor cells secreting various cytokines (i.e., granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2) reportedly stimulate potent, specific, and long-lasting antitumor effects (3). However, immunotherapy-based stimulation of the immune system often induces adverse autoactive immune responses and related side effects (4). Confounding, an immunosuppressive tumor microenvironment (TME) limits the efficacy of immunotherapy. The structurally and functionally aberrant tumor vasculature leads to a highly heterogeneous and hypoxic TME that facilitates the release of immunosuppressive cytokines from both tumor and stromal cells, promotes the infiltration of immunosuppressive bone marrow–derived cells, and limits the tumor infiltration of T cells, leading to suppression of the antitumor immune response (4, 5). Thus, the application of immunotherapy to treat cancer remains challenging, and there is an urgent need to explore new strategies that are safe and effective (6).

Hepatocellular carcinoma (HCC), an aggressive primary liver cancer, develops from chronically damaged tissue (7). HCC is associated with a hypoxic and inflammatory TME that promotes tumor progression and causes resistance to therapy (8). Immunosuppression mechanisms, such as deficiency of immunostimulatory cytokines and induction of immunosuppressive cytokines and immunoregulatory cells caused by tumor hypoxia, are involved in the development of immunotherapy resistance and limit its therapeutic outcome (8). Although recently developed immune checkpoint inhibitors provided clinical benefit to patients with advanced HCC, targeting a single pathway may not be sufficient to achieve potent immunotherapeutic effects (9, 10). Thus, the development of combination strategies targeting multiple immune regulatory pathways may effectively shift the TME in HCC toward a proimmune state and achieve potent antitumor immunity with substantial therapeutic benefit (10, 11).

Here, we propose applying dual-targeted immunogene therapy using small interfering RNA (siRNA) against immune-suppressive factors and plasmid DNA (pDNA) encoding immunostimulating cytokines to modulate the TME and activate immune effector cells. Despite the potential of immunogene therapy in cancer treatment, the barriers against therapeutic siRNA/pDNA reaching their target cells and exerting efficient gene silencing/gene expression effects limit their potential. The short half-life in blood circulation, lack of tumor-specific cellular uptake, inefficient intracellular release of siRNA/pDNA, and poor nuclear entry of pDNA limit their efficacy in vivo (12). To overcome these challenges, nanoscale formulations were designed and used to encapsulate the therapeutic genetic cargoes with enhanced stability, controlled cargo release properties, and increased transfection activity (13, 14). In this study, we designed

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1Institute of Biomedical Engineering and Frontier Research Center on Fundamental and Applied Sciences of Matters, National Tsing Hua University, Hsinchu 30013, Taiwan. 2Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. 3College of Medicine, Chang Gung University, Taoyuan, Taiwan. 4Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taoyuan, Taiwan. 5Department of Biomedical Sciences, School of Medicine, Chang Gung University, Taoyuan, Taiwan. 6Department of Biomedical Engineering and Nanomedicine, National Health Research Institutes, Miaoli County 35053, Taiwan. 7Division of Hepatology, Department of Gastroenterology and Hepatology, Linkou Medical Center; Chang Gung Memorial Hospital, Taoyuan, Taiwan. 8Division of Medical Oncology/Hematology, Chang Gung Memorial Hospital, Taoyuan, Taiwan. 9*These authors contributed equally to this work. †Corresponding author. Email: yunching@mx.nthu.edu.tw (Y.C.); shuuy@nhri.org.tw (S.-Y.L.)
nanoparticles (NPs) that contain an HCC-targeting peptide and a unique dendrimer–calcium phosphate (CaP) core that harbors nucleic acids for gene delivery. Figure 1 illustrates our design of a tumor-targeted NP that carries siRNA against the immune checkpoint PD-L1 and pDNA encoding the immunostimulating cytokine IL-2 to promote antitumor immunity and increase the efficacy of whole-cell cancer vaccines. This nanoscale immunogenotype therapy exhibits multifunctional characteristics, including (i) a tumor-targeting peptide (SP94) that enhances the tumor accumulation of NPs and increases the efficiency of intracellular delivery of the therapeutic pDNA/siRNA to HCC cells [(15, 16)]; (ii) a pH stimuli-responsive CaP core to achieve endosomal escape, along with enhanced release of the nucleic acid; (iii) thymine-capped polyamidoamine (PAMAM) dendrimers loaded in the CaP core to further enhance the endosomal escape and nuclear entry of pDNA, leading to promising gene transfection activity; and (iv) thymine-capped PAMAM dendrimers that can activate the stimulator of interferon genes (STING)–cyclic GMP-AMP synthase (cGAS) pathway and serve as immunotherapy adjuvants to promote cellular immunity. Efficient tumor-targeted delivery of therapeutic gene cocktails that achieve tumor-specific expression of immunostimulating cytokines and down-regulation of immune checkpoints holds promise for effective and safe immunotherapy regimens.

RESULTS

Preparation and characterization of tumor-targeted lipid/dendrimer/CaP NPs loaded with siRNA and pDNA

To overcome the challenges of current gene delivery carriers, we designed complex NPs with a core-shell structure composed of a dendrimer–CaP core loaded with nucleic acids that are coated with a synthetic lipid bilayer shell. The preparation and proposed structure of the siRNA/pDNA-loaded, HCC-targeted lipid-dendrimer–CaP (LDCP) NPs are shown in Fig. 1. While excessive nucleic acid condensation can impede the release of siRNA/pDNA, insufficient interaction of dendrimers with nucleic acid may limit the transfection efficiency. We found that unmodified, 10 and 50% thymine-modified PAMAM more readily condensed siRNA/pDNA than 90% thymine–modified PAMAM (Fig. 2A). Alternatively, both 50 and 90% thymine–modified PAMAM showed efficient dissociation of siRNA/pDNA in the presence of anionic materials, such as heparin (Fig. 2B). Of the tested thymine coverages, only the 50% thymine–modified PAMAM promoted both siRNA/pDNA condensation and efficient release of siRNA/pDNA, which is predictive of increased transfection activity. This finding is consistent with previous studies suggesting enhanced gene transfection mediated by the 50% thymine–capped PAMAM dendrimer/CaP complex (17). Thus, 50% thymine–capped PAMAM dendrimers were used to form the dendrimer/siRNA/pDNA complex at a weight ratio of approximately 2:1:1. Assembly of the dendrimer/siRNA/pDNA complex in 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA)–coated CaP cores was performed in a water-in-oil microemulsion containing Igepal-520 as a surfactant (18). To increase the in vivo stability and cellular uptake of NPs, outer leaflet lipids [1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol, and 1,2-distearyl-sn-glycero-3-phosphethanolamine-N-[methoxy(polyethylene glycol)]-2000 (DSPE-PEG2000)] were added to the DOPA-coated dendrimer/siRNA/pDNA/CaP cores. To achieve tumor-specific uptake of siRNA/pDNA, we further modified the surface of the NPs with the HCC-targeting peptide SP94.

To assess the impact of 50% thymine–capped PAMAM dendrimers on NP characteristics, transmission electron microscopy (TEM) was performed. Dendrimer incorporation did not significantly affect NP sphere formation as NPs with or without added dendrimers formed well-dispersed spheres (Fig. 2C). The average diameters of lipid-CaP (LCP) NPs without added dendrimers and LDCP NPs with 50% thymine–capped PAMAM dendrimers were 87.1 ± 3.6 and 110.5 ± 8.7 nm, respectively (Fig. 2D). NPs with or without added dendrimers showed a similar negative zeta potential of approximately −7 mV and a polydispersity index of 0.2 to 0.3 (Fig. 2D). While the percentage of pDNA encapsulated in NPs (greater than 95%) was not affected by dendrimers, the percentages of encapsulated siRNA increased from 64% in LCP NPs to 87% in LDCP NPs (Fig. 2D). To evaluate whether siRNA/pDNA would be efficiently released in acidic endosomes/lysosomes after entering cancer cells, TEM images were captured under acidic pH conditions. Structural alteration of the NPs due to decomposition of the CaP cores was observed (Fig. 2C). As expected, the release of genetic cargoes (FAM-labeled siRNA) from LDCP NPs under physiological conditions (pH 7.4) was much slower than that observed under acidic conditions (pH 5.5) (fig. S1), indicating pH-dependent decomposition of the CaP cores in NPs, which signifies the endosomal/lysosomal release of siRNA/pDNA from LDCP NPs.

TT-LDCP NPs efficiently deliver siRNA and pDNA into HCC cells with potent transfection efficiency in vitro and in vivo

We next examined how the thymine-capped PAMAM dendrimer and HCC-targeted SP94 peptide affected uptake of NPs in vitro and in vivo. We examined the cellular uptake of siRNA/pDNA loaded in LDCP NPs in two HCC cell lines: human Hep3B and murine HCA-1. The cellular uptake of fluorescently labeled siRNA/pDNA in both cell lines was higher when the siRNA/pDNA was loaded in tumor-targeted LDCP (TT-LDCP) NPs than when it was loaded in non-targeted LDCP NPs or in TT-LCP (TT-LCP) NPs without dendrimers (Fig. 3, A and B). In addition, enhanced nuclear uptake of pDNA was observed for pDNA delivered by TT-LDCP NPs containing 50% thymine–capped PAMAM, compared with pDNA delivered by TT-LCP NPs or by TT-LDCP NPs containing unmodified PAMAM (Fig. 3, A and B, and figs. S2 and S3).

We further evaluated the biodistribution of TT-LDCP NPs in murine orthotopic HCC (HCA-1) models after intravenous administration of NPs. FAM-labeled siRNA was used to track the biodistribution of TT-LDCP NPs (fig. S4). Increased uptakes of TT-LDCP NPs in tumor, liver, spleen, and lung were observed compared with free siRNA 2 hours after intravenous administration. More significantly, most TT-LDCP NPs were accumulating to a larger extent in tumors and liver as compared with other organs (fig. S4). We then studied the uptake of fluorescently labeled siRNA/pDNA in murine orthotopic HCC (HCA-1) models after intravenous administration of LDCP NPs. Higher siRNA/pDNA delivery was observed in HCC tumor tissue of mice that received TT-LDCP NPs compared with mice that received nontargeted NPs (Fig. 3C and fig. S5). Delivery was mostly cytosolic with a heterogeneous distribution within the HCC tumor tissues (Fig. 3C).

Next, we studied how the thymine-capped PAMAM dendrimer and HCC-targeted SP94 peptide affected the transfection efficiency of pDNA encoding firefly luciferase (luc pDNA) in human (Hep3B, JHH-7) and murine (HCA-1) HCC cells. At higher concentrations of pDNA, all cell lines exhibited significantly increased luciferase expression when luc pDNA was delivered by TT-LDCP NPs compared
to delivery by LDCP NPs or TT-LCP NPs (Fig. 3D and fig. S6). In addition, enhanced transfection efficacy was observed when luc pDNA was delivered by TT-LDCP NPs containing 50% thymine-capped PAMAM, compared to delivery by NPs containing unmodified, 10 or 90% modified PAMAM. Our results indicate that 50% thymine coverage is optimal for dendrimer-mediated delivery of pDNA through the nuclear barrier to achieve effective gene transfection (fig. S7).

We further evaluated the transfection efficiency of pDNA in orthotopic HCC (HCA-1) models by measuring luciferase expression in different organs 48 hours after systemic administration of luc pDNA in different formulations. Luciferase expression in liver tumors was significantly higher in mice that received TT-LDCP NPs, compared to that in mice that received LDCP NPs, TT-LCP NPs, or free pDNA. No significant difference in luciferase expression was observed in the liver, heart, spleen, kidney, and lung (Fig. 3E). Our results using TT-LDCP NPs demonstrate ligand (HCC-targeted SP94 peptide)– and dendrimer-dependent cellular uptake and transfection efficiency in HCC in vitro and in vivo.

Codelivery of PD-L1 siRNA and IL-2 pDNA by TT-LDCP NPs silences PD-L1 expression and increases IL-2 production in HCC cells in vitro and in vivo

To enhance antitumor immunity, siRNA against the immune checkpoint ligand PD-L1 and pDNA encoding the immunostimulatory cytokine IL-2 were loaded into TT-LDCP NPs, and their effect on target gene expression was examined in HCA-1 HCC cells. Compared with delivery by nontargeted NPs, IL-2 pDNA delivered by TT-LDCP NPs significantly increased IL-2 secretion from HCA-1 cells (Fig. 4A). In addition, PD-L1 siRNA loaded in TT-LDCP NPs efficiently silenced PD-L1 expression in HCA-1 cells, whereas PD-L1 siRNA formulated in nontargeted LDCP NPs did not affect PD-L1 expression (Fig. 4B). Neither the control siRNA nor the control pDNA (pEGFP pDNA) delivered by TT-LDCP NPs altered IL-2 or PD-L1 expression in HCA-1 cells (Fig. 4, A and B).

We next evaluated the effects of PD-L1 siRNA and IL-2 pDNA codelivered by TT-LDCP NPs on the expression of PD-L1 and IL-2 in an orthotopic HCC (HCA-1) model (Fig. 4, C and D). Systemic co-administration of PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs significantly decreased PD-L1 and increased IL-2 expression in liver tumors (Fig. 4, C and D). PD-L1 siRNA and IL-2 pDNA codelivered by LDCP NPs or TT-LCP NPs showed minimal impact on the expression of PD-L1 or IL-2 in HCA-1 tumors (Fig. 4, C and D). Our results indicate that delivery of PD-L1 siRNA and IL-2 pDNA by TT-LDCP NPs is efficient and can decrease the expression of the immune checkpoint ligand PD-L1 and increase the production of the immunostimulatory cytokine IL-2 in HCC.

Codelivery of PD-L1 siRNA and IL-2 pDNA by TT-LDCP NPs significantly increases tumoral infiltration of CD8^+ T cells, suppresses primary HCC growth, and inhibits distal metastasis

We next assessed whether TT-LDCP NPs that codeliver PD-L1 siRNA/IL-2 pDNA could effectively shape antitumoral effector immunity...
in HCC. Treatment of mice bearing orthotopic HCC tumors with PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs significantly facilitated tumoral infiltration of CD8$^+$ T cells, compared with that seen upon treatment with PD-L1 siRNA or IL-2 pDNA alone in TT-LDCP NPs (Fig. 4E). No effect on CD8$^+$ T cell numbers was observed in the absence of dendrimers (Fig. 4E), and none of the combinations tested affected the number of tumor-infiltrating CD4$^+$ T cells (Fig. 4F).

Accordingly, we evaluated whether the increase in tumor-infiltrating T cells in HCC cells upon codelivery of PD-L1 siRNA and IL-2 pDNA led to suppression of tumor growth. Compared with treatment with PD-L1 siRNA or IL-2 pDNA alone in TT-LDCP NPs, codelivery of PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs significantly suppressed tumor growth in the HCC orthotopic model (Fig. 4G). Codelivery of PD-L1 siRNA and IL-2 pDNA in nontargeted NPs or in TT-LCP NPs without dendrimers showed only a moderate
inhibitory effect on tumor growth (Fig. 4G). Because mice bearing orthotopic HCA-1 tumors develop metastases in the lungs 4 weeks after HCC implantation (19), we also evaluated the effect of PD-L1 siRNA and IL-2 pDNA codelivery on metastasis. We observed that codelivery of PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs not only inhibited primary HCC growth but also suppressed distal lung metastasis (Fig. 4, H and I). The combination of PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs was well tolerated by mice, as demonstrated by unchanged hepatic enzyme levels [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline
Fig. 4. Delivery of IL-2 pDNA and PD-L1 siRNA in TT-LDCP NPs modulated TME and suppressed tumor progression in a murine orthotopic HCC model. HCA-1 cells were treated with TT-LDCP NPs containing IL-2 pDNA or PD-L1 siRNA, followed by enzyme-linked immunosorbent assay (ELISA)–based estimation of IL-2 secretion (A) and Western blotting for PD-L1 expression (B). (A) Samples from supernatants were collected 48 hours after treatment and tested for the presence of IL-2 by ELISA. Data are means ± SEM (n = 6), *P < 0.05. (B) PD-L1 inhibition of HCA-1 cells was investigated by Western blotting. HCA-1 cells were incubated with PD-L1 siRNA (4 μg/ml) loaded in different formulations for 48 hours. (C and D) On the 14th day after HCC tumor implantation, tumor-bearing mice were treated with TT-LDCP NPs containing IL-2 pDNA and PD-L1 siRNA with two consecutive intravenous administrations (1.2 mg/kg per dose), and euthanized 24 hours after the last treatment for immunostaining of tumors. The expression of IL-2 and PD-L1 in murine orthotopic HCC tumors was imaged and quantified with a Zeiss LSM 780 confocal microscope. Scale bars, 50 μm. Data are means ± SEM (control, TT-LCP, TT-LDCP, n = 5; LDCP, n = 7); **P < 0.01 and ***P < 0.001. (E and F) Orthotopic HCA-1 tumor–bearing mice were intravenously injected with different formulations containing IL-2 pDNA and PD-L1 siRNA (1.2 mg/kg per dose, 3 doses per week) starting on the 10th day after tumor implantation. The mice were euthanized 2 weeks after the first treatment for further analysis. Recruitment of CD8+ (E) and CD4+ (F) T cells in tumors subjected to different treatments was measured by flow cytometry (control, TT-LCP, TT-LDCP, n = 5; LDCP, n = 7); **P < 0.01 and ***P < 0.001. (G and H) Size of tumors in orthotopic tumor-bearing mice was significantly reduced upon treatment with IL-2 pDNA and PD-L1 siRNA loaded in TT-LDCP NPs (control, n = 12; TT-LCP, n = 5; LDCP, n = 5; TT-LDCP, n = 12; TT-LDCP/IL-2 pDNA, n = 8; TT-LDCP/PD-L1 siRNA, n = 7). Data are means ± SEM. (I) The number of lung metastatic nodules was significantly reduced in mice treated with IL-2 pDNA and PD-L1 siRNA in TT-LDCP NPs. (J) Hematoxylin and eosin staining images showing distant metastasis tumor nodules in the lung. Scale bars, 200 μm. Data are means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
phosphatase (ALP), and gamma-glutamyl transferase (γ-GT)) when compared with untreated mice (fig. S8). Evaluation of systemic toxicities by hematoxylin and eosin staining showed no histopathological changes in the major organs after treatment of PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs (fig. S8).

**PD-L1 siRNA and IL-2 pDNA codelivered by TT-LDCP NPs reprogram the TME and sensitize HCC to a cancer vaccine**

The immunosuppressive TME is a major cause for failure of cancer vaccine immunotherapy (20). Therefore, we next examined whether codelivery of PD-L1 siRNA and IL-2 pDNA could modulate the immunosuppressive TME to restore the efficacy of a tumor vaccine in HCC. To establish an immunostimulating whole-cell hepatoma vaccine, HCA-1 cells were transfected with the codon-optimized GM-CSF (cGM-CSF) as an adjuvant to promoting dendritic cell (DC) recruitment and maturation and augment vaccine efficacy by facilitating the development of both humoral and cellular immunity (3, 21). Mice implanted with HCA-1 HCC tumors were vaccinated with the GM-CSF–secreting HCA-1 cells in combination with our TT-LDCP–based immunogene therapy (Fig. 5A). Vaccination alone only moderately increased the T cell infiltration in tumors compared with untreated mice (Fig. 5B). Treatment with PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs significantly enhanced the intratumoral infiltration of CD8+ T cells in mice vaccinated with cGM-CSF–transduced HCA-1 cells: The number of tumor-infiltrating CD8+ T cells in the combined immunogene therapy and vaccine group was threefold higher than that of the control group and twofold higher than that of the vaccine group (Fig. 5B). Although the combination of TT-LDCP–based immunogene therapy and HCC vaccination only slightly increased the number of tumor-infiltrating T cells compared with that of TT-LDCP–based immunogene therapy alone (Fig. 5B), the combination significantly increased the activation of tumor-infiltrating CD8+ T cells, as indicated by granzyme B (Fig. 5, C and D) and interferon-γ (IFN-γ) expression (Fig. 5E), compared with that of TT-LDCP–based immunogene therapy or vaccine treatment alone (Fig. 5, C to E).

We further assessed whether a combination of TT-LDCP NPs containing PD-L1 siRNA/IL-2 pDNA and vaccine treatment could effectively suppress HCC progression. Vaccination alone led to moderate tumor growth inhibition, suggesting that the immunosuppressive TME limited the anticancer efficacy of the HCC vaccine (Fig. 5F). The combined administration of the HCC vaccine together with PD-L1 siRNA and IL-2 pDNA loaded in TT-LDCP NPs significantly suppressed cancer progression and increased the overall survival of mice, compared with mice that received immunogene therapy or vaccination alone (Fig. 5, F and G).

Our findings demonstrating a role for CD8+ T cells in immunogene-mediated tumor growth suppression prompted us to assess the role of splenic CD8+ T cells in response to combined immunogene and HCC vaccine therapy. To further verify the tumor-specific T cell response induced by the combination of immunogene therapy and HCC vaccination, lymphocytes were collected from the spleens of HCA-1 tumor–bearing mice after treatment with PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs and/or HCC vaccine. The splenic lymphocytes were cocultured with mitomycin C–treated HCA-1 cells, followed by estimation of IFN-γ secretion. Splenic lymphocytes from mice treated with a combination of TT-LDCP–based immunogene therapy and HCC vaccine secreted higher levels of IFN-γ than those from mice treated with TT-LDCP–based immunogene therapy or vaccine treatment alone, indicating increased activity (Fig. 5H). In addition, the splenic lymphocytes were cocultured with HCA-1 cells for 48 hours, followed by the staining with 7-AAD (7-aminactinomycin D) to assess the cell-mediated cytotoxicity. Splenic lymphocytes from mice treated with a combination of TT-LDCP–based immunogene therapy and HCC vaccination showed enhanced cell death in HCA-1 cells compared with the controls (fig. S9). Moreover, splenic CD8+ T cells isolated from HCA-1 tumor–bearing mice after immunogene and/or HCC vaccine treatments were adaptively transferred into mice that had received intrahepatic inoculations of HCA-1 HCC cells (Fig. 5I). Splenic CD8+ T cells from mice treated with the combination of immunogene therapy and HCC vaccination significantly suppressed tumor growth after being adaptively transferred into HCA-1 tumor–bearing mice (Fig. 5I). These results verified that PD-L1 siRNA and IL-2 pDNA in TT-LDCP together with the HCC vaccine efficiently activate tumor-specific immune responses to suppress cancer progression.

To examine whether immunogene therapy is able to regulate antigen-specific immune response, we investigated the effect of immunogene therapy in hemagglutinin (HA)–expressing BNL HCC models. The murine HCC BNL-HA cells were generated by stable transfection of BNL-HCC cells with the HA gene and orthotopically implanted in the livers of 6- to 8-week-old male BALB/c mice. Consistently, treatment with PD-L1 siRNA and IL-2 pDNA in TT-LDCP NPs significantly increased activation of CD8+ T cells and suppressed HCC progression in the orthotopic BNL-HA HCC model (fig. S10). To obtain direct evidence that immunogene therapy led to the accumulation of antigen-specific cytotoxic T cells in tumors, an HA tetramer assay was performed in mice treated with immunogene therapy. The proportion of HA peptide–specific CTLs among intratumor CD8+ T cells was increased after treatment of immunogene therapy compared with the control group (fig. S10).

**Thymine-capped PAMAM dendrimers in TT-LDCP NPs stimulate the STING pathway and promote the maturation of tumor-infiltrating DCs**

Although production of IL-2 and down-regulation of immune checkpoint molecules such as PD-L1 may help recruit and activate cytotoxic T cells, the antitumor immune response in the TME may still be limited due to tumor-induced functional deficiency of tumor-associated antigen-presenting cells (APCs), such as intratumoral DCs (22). Cationic macromolecules (i.e., chitosan or nanoformulations (i.e., NPs containing DNA) can promote the maturation of DCs and stimulate innate immunity through activation of the STING pathway (23). We, therefore, examined the possible adjuvant mechanisms underlying enhanced tumor immunity seen with TT-LDCP NPs. We examined whether thymine-capped dendrimers loaded in TT-NPs could activate the STING pathway in HCC cells and indirectly trigger the maturation of intratumoral DCs. TT-LDCP NPs loaded with control siRNA and pDNA (pEGFP pDNA) significantly induced STING-dependent TBK1 and IRF3 activation (Fig. 6A) and increased the expression of STING-triggered proinflammatory cytokines, CCL5, CXCL10, and IFN-β, in HCA-1 cells (Fig. 6B). Naked dendrimer/siRNA/pDNA complex (Fig. 6A, left, lane 3) or siRNA/pDNA loaded in TT-LCP without dendrimers (Fig. 6A, right, lane 2) only moderately stimulated the STING-TBK1-IRF3 pathway. Dendrimer or siRNA/pDNA alone did not significantly activate the STING pathway. Our results indicate that the incorporation of
Fig. 5. IL-2 pDNA and PD-L1 siRNA in TT-LDCP NPs enhanced cytotoxic T cell activation and suppressed tumor progression in combination with a whole-cancer cell vaccine in a murine orthotopic HCA-1 HCC model. (A) Three days after the implantation of HCA-1 cells, mice were injected intraperitoneally five times (at 2- to 3-day intervals) with the HCC vaccine. For the combination groups, mice treated with the HCC vaccine received intravenous immunogene therapy (1.2 mg siRNA and pDNA/kg per dose) on days 10, 12, 14, 17, 19, and 21. Immunogene therapy: IL-2 pDNA and PD-L1 siRNA in TT-LDCP; vaccine: 5 × 10^6 mitomycin C–treated cGM-CSF–overexpressing HCA-1 cells. (B) Combination of immunogene therapy and the vaccine increased the number of CD8^+ T cells in tumors, as measured by flow cytometry (control, n = 18; immunogene therapy, n = 10; vaccine, n = 6; combination group, n = 6). Data are means ± SEM. (C and D) The percentage of granzyme B–positive CD8^+ T cells in HCA-1 tumors was imaged and quantified 24 days after implantation for the treatment with immunogene therapy or the HCC vaccine. DAPI, blue; granzyme B, green; CD8^+ T cells, red (control, n = 8; immunogene therapy, n = 6; vaccine, n = 6; combination group, n = 7). (E) IFN-γ intracellular staining in tumor-infiltrating CD8^+ T cells measured by flow cytometry (n = 9). (F and G) The combination of immunogene therapy and vaccine treatment significantly reduced tumor sizes (control, n = 12; immunogene therapy, n = 12; vaccine, n = 12; combination group, n = 24) and distal lung metastatic nodules (F) and increased the overall survival (G) (n = 5, **P < 0.01 compared with control; P < 0.05 compared with vaccine treatment) in an orthotopic HCC model. (H) IFN-γ production by splenocytes from HCA-1 tumor–bearing mice that were subjected to different treatments. Splenocytes from HCA-1 tumor–bearing mice were incubated with or without mitomycin C–treated HCA-1 cells for 48 hours at 37°C. Samples from supernatants were collected and analyzed for IFN-γ secretion by ELISA (n = 6). Data are means ± SEM. (I) Mice with established orthotopic HCA-1 tumors received adoptive transfer of CD8^+ T cells from HCA-1 tumor–bearing mice treated with different treatments. The adoptive transfer of tumor-specific effector T cells from mice that received the combined immunogene therapy and vaccine treatment resulted in a significant reduction in the size of primary tumors and the number of distal lung metastatic nodules (n = 6). Data are means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 6. STING activation and DC maturation after treatment with TT-LDCP NPs containing siRNA/pDNA. (A) Protein extracts from HCA-1 cells treated with different formulations including dendrimers alone, pEGFP pDNA/control siRNA, dendrimers/pEGFP pDNA/control siRNA complex, or pEGFP pDNA/control siRNA loaded in TT-LCP or TT-LDCP for 6 hours were resolved by SDS–polyacrylamide gel electrophoresis, and phosphorylation of the indicated proteins was detected by Western blot. (B) Quantitative polymerase chain reaction analysis of Ifnb, Ccl5, and Cxcl10 mRNA expression by HCA-1 cells stimulated for 6 hours with different formulations (n = 4 to 7). Data are means ± SEM. *P < 0.05 and ***P < 0.001. (C) Thymine-capped PAMAM dendrimers/pEGFP pDNA/control siRNA loaded in TT-LDCP NPs induced intratumoral DC maturation. Orthotopic HCA-1 tumor–bearing mice were intravenously injected with different formulations containing pEGFP pDNA/control siRNA (1.2 mg/kg per dose, three doses per week) on the 10th day after tumor implantation. The mice were euthanized 2 weeks after the first treatment for flow cytometry analysis. The number of MHCII⁺ CD8⁺ mature intratumoral DCs increased after treatment with pDNA/siRNA in TT-LDCP NPs, D50, dendrimer modified with 50% thymine group. n = 10. Data are means ± SEM. ****P < 0.001. (D) Efficient tumor-targeted codelivery of PD-L1 siRNA and IL-2 pDNA by TT-LDCP NPs achieves tumor-specific expression of IL-2 and down-regulation of PD-L1, activates TIDCs and increases infiltration and activation of CD8⁺ T cells in HCC, and induces a strong tumor-suppressive effect in HCC in synergy with a vaccine.
In this study, we showed that delivery of PD-L1 siRNA into HCC cells not only augmented anticancer immunity, as indicated by increased tumor-infiltrating CD8+ T cells, but also significantly suppressed distal metastasis. In addition to the immunosuppressive effect of PD-L1 via engagement of its receptor PD-1 on T cells, recent studies have reported that cancer cell–intrinsic PD-L1 reprograms metabolic activities and promotes metastasis-related phenotypes (32). Down-regulating PD-L1 reverses epithelial-to-mesenchymal transition in cancer cells and inhibits their invasion and migration activity, suggesting that PD-L1 may serve as a potential target for suppressing metastasis (33). In addition to PD-L1, several other immunosuppressive molecules, such as indoleamine 2,3-dioxygenase (IDO), and cytokines [i.e., transforming growth factor–β (TGF-β), IL-10, and prostaglandin E2] produced by cancer cells or stromal cells also play important roles in modulating immune cell phenotypes in the TME, inhibiting T cell activation and promoting immune tolerance, resulting in suppression of anticancer immunity (34–36). We envision that in the future, the TT-LDCP NPs developed in this study can serve as efficient tools to deliver siRNA cocktails against multiple key immunosuppressive factors and reprogram immune cells residing in the TME, leading to reduced tumor progression.

IL-2 has long been recognized as a key cytokine that drives T cell proliferation and enhances effector T cell activity, and IL-2 was the first effective immunotherapy for human cancer (2). However, repetitive systemic administration of IL-2, which is required to obtain sufficient exposure at tumor tissues and achieve tumor suppression, causes adverse events in patients (37). In addition, recent studies have shown that treatment with low-dose IL-2 induces immune tolerance...
and promotes the development of regulatory T cells that may impair antitumor immunity and limit therapeutic efficacy (37). Our findings, on the other hand, showed that TT-LDCP NP–mediated delivery of IL-2 pDNA to tumor cells resulted in high levels of secreted IL-2 in the TME and enhanced tumor immunity by increasing the proliferation of tumor-specific cytotoxic T cells and facilitating infiltration of activated T cells into tumors when combined with PD-L1 siRNA. Furthermore, tumor-targeted delivery of IL-2 pDNA with NPs may further decrease the systemic side effects observed with systemic administration of IL-2 for treatment.

Although TT-LDCP NPs can selectively and efficiently deliver siRNA/pDNA into tumors, their transient transfection efficacy may limit clinical application. To prolong pDNA/siRNA expression and the antitumor response, cutting-edge technologies, such as the Sleeping Beauty transposon and CRISPR-based genome editing systems, can be exploited. If the death of tumor cells results in reduced expression of IL-2 that limits immune stimulation, stromal cells can be targeted instead of cancer cells. For example, targeted delivery of pDNA into tumor-associated fibroblasts causes the stromal cells to sustainably produce immunostimulating cytokines, modulate the immunosuppressive TME, and achieve long-term antitumor immunity (38). More studies are needed to overcome limitations and achieve successful clinical translation of cancer gene therapy.

In summary, nanotechnology-enabled delivery of siRNA against immunosuppressive factors and pDNA encoding immunostimulating cytokines is safe, highly efficient, selectively targeted to the TME, synergistic in action with a cancer vaccine, and suitable as a next-generation cancer immunotherapy strategy. This system has the potential for new immunotherapy cocktails to be used in the clinic.

**MATERIALS AND METHODS**

**Study design**

This was a preclinical study to assess the efficacy and safety of a PD-L1 siRNA and IL-2 pDNA delivered by a tumor-targeted nanocarrier to silence PD-L1 expression and increase IL-2 production in HCC cells. We hypothesized that down-regulation of PD-L1 and production of IL-2 in HCC would stimulate antitumor immunity and suppress HCC progression, as well as improve antitumor efficacy when combined with cancer vaccine treatment. This hypothesis was tested through an established orthotopic murine HCC model. The numbers of mice used for the in vivo experiments are specified in the figure legends.

**Cells and materials**

The murine HCC cell line HCA-1 and the human HCC cell lines Hep3B and JHH-7 were provided by D. Duda, MGH Boston. Hep3B cells were maintained in minimum essential medium (MEM)–α-medium, JHH-7 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium, and HCA-1 cells were maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO). All media were supplemented with 10% fetal bovine serum and were purchased from Invitrogen (Carlsbad, CA). The SP94 peptide (NH2-CGGSFSIIHTPILPL-COOH) with 10% fetal bovine serum and were purchased from Invitrogen medium (Sigma-Aldrich, St. Louis, MO). All media were supplemented with 10% fetal bovine serum. JHH-7 cells were provided by D. Duda, MGH Boston. Hep3B cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM–α-medium, and JHH-7 were provided by D. Duda, MGH Boston. Hep3B cells were maintained in minimum essential medium (MEM)–α-medium, JHH-7 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium, and HCA-1 cells were maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO). All media were supplemented with 10% fetal bovine serum and were purchased from Invitrogen (Carlsbad, CA). The SP94 peptide (NH2-CGGSFSIIHTPILPL-COOH) was synthesized and purified (95% purity) by Kelowna International Scientific Inc. (Taipei, Taiwan). Dimethyl sulfoxide and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol (EtOH) was obtained from Alfa Aesar (Ward Hill, MA). DOPA, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DOTAP, DSPE-PG2000, and DSPE-PG2000-maleimide were purchased from Avanti Polar Lipids (Alabaster, AL). Luciferin was purchased from Promega Corp. (Madison, WI). Plasmids encoding firefly Luc driven by the cytomegalovirus (CMV) promoter were gifts from C.-W. J. Chang, National Tsing Hua University, Taiwan. pCMV-Luc plasmid was cloned by inserting luciferase DNA (from pGL3, Promega) into the pDNA3 plasmid. pEGFP-C3 plasmids were purchased from Addgene. Control siRNA with the sequence 5′-AAUUCCCGAAGCUUCACGUTT-3′, PD-L1_01 siRNA with the sequence 5′-GAUAUUUG-CUGGCAUAUAUU-3′, and PD-L1_02 siRNA with the sequence 5′-GAGGUAAUCUGGCAACAAACAUU-3′ were purchased from Dharmacon (Lafayette, CO, USA).

The synthesis of thymine-capped PAMAM dendrimers

The thymine was converted to (bromobutyl)thymine according to a previous report (39). Upon mixing (bromobutyl)thymine (47.8 mg) with G4NH2 (Sigma-Aldrich, 1 ml, 5.7 μmol, 10 weight % methanol solution) in deionized water (4 ml), dendrimers capped with thymine molecules of 50% coverage were successfully synthesized by microwaving the relevant mixture at 300 W and 120°C for 30 min (CEM, Discover LabMate System). For each synthesis, the resulting crude mixture was extracted by dichloromethane to remove excess (bromobutyl)thymine, and the compound was freeze dried from the aqueous solution.

Preparation of LDCP NPs loaded with siRNA and pDNA

LDCP NPs were prepared using a modified protocol (16, 40). Two separate microemulsions (3 ml each) were prepared. To prepare the calcium-loaded microemulsion, pDNA (6.5 μg) and siRNA (6.5 μg), dendrimer (26 μg), and 40 μl of 500 mM CaCl2 (pH 7) were added to the oil phase of cyclohexane and Igepal-520 (7:3, v/v). To prepare the phosphate buffer–loaded microemulsion, Na2HPO4 solution (74 μl, 100 mM, pH 9) and DOPA (74 μl, 35 mM) were added into the oil phase of cyclohexane and Igepal-520 (7:3, v/v). Two separate microemulsions were stirred for 10 min at room temperature. The emulsions were then mixed for 20 min to form the condensed cores of CaP/pDNA/siRNA/dendrimer. Later, 6 ml of 100% EtOH was added to disrupt the emulsion, and the mixture was centrifuged at 10,000g for 20 min. After removing the supernatant solution, the CaP/pDNA/siRNA/dendrimer cores were collected and washed twice with 100% EtOH to remove residual organic solvents. The CaP/pDNA/siRNA/dendrimer cores suspended in chloroform, vortexed, and sonicated. A mixture of free lipids (DOPC:DOTAP:DSPE-PG2000:DSPE-PG2000-MAL:cholesterol = 1:1:1.8:0.2:4 molar ratio) was added into the CaP/pDNA/siRNA/dendrimer cores (2.5:1 ratio of total free lipids to DOPA) and then dried under N2. After evaporating the chloroform, 160 μl of water was added to form LDCP NPs. For peptide conjugation, peptide was reduced using immobilized TCEP disulfide-reducing gel (Thermo Fisher Scientific) according to the manufacturer’s recommendations. SP94 peptides were added into LDCP NPs where they reacted with DSPE-PG2000-maleimide on the surface of the NPs. Four hours later, the unreacted maleimide groups were quenched by adding i-cysteine.

Characterization of NPs

The LDCP or LCP NPs were formulated as described above and were resuspended in deionized water. The morphology of the NPs was characterized by TEM (H-7500, Hitachi High-Tech, Tokyo, Japan). The NPs were dropped on dried Formvar-coated 100-mesh copper grids at room temperature. All grids were further dried for 2 days.
The tumor volume was evaluated 2 weeks after the first treatment. Mice with orthotopic HCC beginning 10 days after the implantation. siRNA and IL-2 pDNA loaded in different formulations (1.2 mg/kg per dose, three doses per week) were intravenously administered to mice with orthotopic HCC beginning 10 days after the implantation. The tumor volume was evaluated 2 weeks after the first treatment. The tumor tissue was collected for further analysis. Tumor size was measured using a caliper and estimated using the formula: tumor volume = length × width^2/2.

Quantitative reverse transcription polymerase chain reaction
To evaluate gene expression by quantitative reverse transcription polymerase chain reaction (PCR), the cells were seeded in 12-well plates and allowed to grow for 12 hours. Later, the cells were exposed to different formulations. The cells were washed in PBS and lysed by TRIzol reagent (Thermo Fisher, USA) at 6 hours after treatment. Then, 1 μg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) on a Piko Thermal Cycler (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. Quantitative PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, USA) on a 7500 Real-Time PCR System (Applied Biosystems, USA). The gene expression levels were evaluated using Ct in all tested samples and normalized to β-actin as a reference gene. The primer sequences are listed in Table S1.

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