Supplementary Materials

A single local delivery of paclitaxel and nucleic acids via an immunoactive polymer eliminates solid tumors and induces antitumor immunity

Fanfei Meng, Jianping Wang, Yanying He, Gregory M. Cresswell, Nadia A. Lanman, L. Tiffany Lyle, Timothy L. Ratliff, Yoon Yeo

Detailed Materials and Methods
37 Supporting Figures
Detailed Materials and Methods

Materials

Linear polyethylenimine base form (PEI base form, MW: 2.5 kDa) and polyethylenimine hydrochloride (PEI salt form, MW 4 kDa equivalent to 2.5 kDa PEI base form) were purchased from Polysciences, Inc. (Warrington, PA). 1,1'-carbonyldiimidazole (CDI), lithocholic acid (LCA, ≥97%), and EtBr (10 mg/mL) were purchased from Sigma-Aldrich (St. Louis, MO). D-Luciferin potassium salt was purchased from Gold Biotechnology (St. Louis, MO, USA). siRNA specific for the mouse pdcd1lg1 mRNA (sense, 5′-CCCACAUAAAAAACAGUUGTT-3′; antisense, 5′-CAACUGUUUUUUAUGUGGGTT-3′), negative siRNA (sense, 5′-UGAAGUUAGCAUGUGACGACAdTdT-3′; antisense, 5′-GACUUAAGUUGCAACUUCAdTdT-3′) and Cy5-labeled negative siRNA were purchased from IDT (Coralville, Iowa, USA). iTAg Tetramer/APC–H-2 Kb TRP2 (SVYDFFVWL) was purchased from MBL International Corporation (Woburn, MA). Cyclic dinucleotide (CDN)-2′,3′-c-di-AM(PS)2 (Rp,Rp) was purchased from InvotroGen (San Diego, CA). Firefly luciferase-expressing plasmid DNA (pLuc) were replicated in DH5-α competent Escherichia coli as reported previously (1). CleanCap® EGFP mRNA (mRNA) was purchased from TriLink BioTechnologies (San Diego, CA).

2E’ synthesis and characterization

2E’ was synthesized as described in the previous report (2). Briefly, 8.7 mg (50 μmol) of CDI and 15.8 mg of LCA (40 μmol) were dissolved in 2.7 mL of chloroform under stirring. After 1 h, the mixture was slowly added to 10 mL chloroform solution containing 50 mg of PEI base (2.5 kDa) (20 μmol) at 60 °C and reacted for 24 h under stirring. The LCA-PEI conjugate (2E’) was purified by dialysis (molecular weight cut-off (MWCO): 1000 Da) against 95% ethanol, followed by acidified deionized (DI) water. For fluorescence labeling of 2E’ or PEI, 20 mg of 2E’ or PEI was dispersed in anhydrous ethanol (0.5 mL) containing sulfo-cy5-NHS (1 mg). The reaction solution was stirred in dark for 24 h and dialyzed against DI water using a dialysis bag with a molecular weight cut off of 1 kDa. 2E’ and PEI base were dissolved in DMSO and analyzed by a Bruker DRX500-2 NMR spectrometer equipped with a BBFO probe.

Measurement of the critical assembly concentration (CAC) of 2E’

2E’ was dissolved in DI water to 2 mg/mL and diluted stepwise by DI water to 1.5×10⁻⁵ mg/mL. To 0.5 mL of each solution, 5 μL of Nile red solution in acetone (2 mg/mL) was added to record the emission spectrum at 636 nm by the excitation at 552 nm.

Preparation and characterization of 2E’/PTX, 2E’/siPDL-1, and 2E’/PTX/siPD-L1

To prepare binary complexes of 2E’ and PTX (2E’/PTX), 2E’ and PTX were mixed at different weight ratios in a chloroform/ethanol mixture (3:1, v/v) in a round-bottom flask, dried by rotary evaporation to form a thin film on the wall of the flask, and hydrated in DI water by bath sonication for 5 min. 2E’/siPDL-1 binary complex was prepared by co-incubation of 2E’ and siPD-L1 in nuclease-free water varying the weight ratios for 30 min at room temperature. A ternary complex of 2E’, PTX, and siPD-L1 (2E’/PTX/siPD-L1) was prepared by incubating 2E’/PTX with siRNA for 30 min at room temperature. The formation of the ternary complex was confirmed by 1.1% agarose gel electrophoresis. The sizes and zeta potentials of all complexes were determined by the Malvern Zetasizer Nano ZS90.
Their morphology was examined by the FEI Tecnai T20 transmission electron microscope (Hillsboro, OR) after negative staining with 1% uranyl acetate.

**In vitro release of PTX from 2E’/PTX**

One milliliter of 2E’/PTX (2.5:1, w/w) or 2E’/PTX (5:1, w/w) equivalent to 200 μg of PTX was put in a dialysis cassette (MWCO: 10 kDa), placed in 15 mL of 0.2% Tween 80 aqueous solution, and incubated at 25 °C under constant agitation. At timed intervals, the entire release medium was replaced with fresh medium. The amount of PTX in the sampled medium at each time point was analyzed by the Agilent 1100 HPLC system (Palo Alto, CA) equipped with Ascentis C18 column (25 cm × 4.6 mm, particle size 5 mm). The mobile phase was composed of water and acetonitrile (50:50) and run at 1 mL/min. PTX was detected by a UV detector at wavelength of 227 nm.

**CRT expression in tumor cells**

2×10⁵ CT26 or B16F10 cells were seeded in a 6-well plate overnight. The cell culture medium was replaced with a medium containing 2E’, PEI, PTX, 2E’/PTX or 2E’/PTX/siPD-L1 at varying concentrations. After 6 h or 24 h, the cells were collected, resuspended in staining buffer, incubated with anti-mouse CD16/32 antibody to block non-specific binding of the immunoglobulin to Fc receptors, and stained with Alexa Fluor 488-conjugated anti-CRT monoclonal antibody (ab196158, Abcam). The cells were incubated with 0.5 μg/mL propidium iodide for 1 min prior to the analysis with the BD Accuri C6 Flow Cytometer. For visualizing CRT exposure, 2×10⁵ CT26 or B16F10 cells were seeded in a confocal dish, incubated with different treatments for 24 h, washed, stained in the same manner as above, and fixed with 4% paraformaldehyde. Confocal images of the fixed cells were taken with the Nikon A1R confocal microscope (Nikon America Inc., Melville, NY) after brief staining with 2 μg/mL Hoechst 33342.

**BMDC and BMDM differentiation**

Bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMDMs) were differentiated from mouse bone marrow cells. The bone marrow was collected from the femur of female Balb/c or male C57BL/6 mice (7 weeks old), pipetted several times, and passed through a 100 and 40 μm cell strainer to obtain single-cell suspension. The cells were collected by centrifugation at 500 rcf for 8 min, treated with ACK lysis buffer, rinsed, and cultured in Alpha minimum essential medium (MEM-Alpha, ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM β-mercaptoethanol, 20 ng/mL murine GM-CSF and 20% fetal bovine serum. After 7 days, floating or loosely adherent cells were collected by centrifugation and identified as BMDC by APC anti-mouse CD11c antibody labeling. Adherent cells were identified as BMDM by FITC anti-mouse F4/80 antibody labeling.

**BMDC and BMDM stimulation**

BMDC or JAWSII DCs (ATCC) were plated at 2 x 10⁵ per well in a non-tissue culture treated 6-well plate and incubated with 2E’ or PEI at 3 μg/mL or 7 μg/mL, or LCA 5, 20, or 40 μg/mL, or a mixture of LCA 5 μg/mL and PEI 5 μg/mL. After 24 h incubation, the cells were collected, resuspended in staining buffer, incubated with Fc-blocking antibody for 15 min at 4 °C, labeled with anti-mouse CD11c, CD86, CD40, and MHC-II antibodies for 20 min at 4 °C, and analyzed by the BD Accuri C6 Flow Cytometer. For measuring cytokine production from the treated BMDC and BMDM, the cells were plated in 96 well plates at a density of 15,000 cells per well and treated with 2E, PEI, 2E’/PTX,
2E’/PTX/siNeg or 2E’/PTX/siPD-L1. After 24 h, the media were analyzed for IL-1β and TNF-α by ELISA (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols.

**TLR-4 and TLR-5 activation assays**

TLR-4 activation by 2E’ or PEI was evaluated with THP1-XBlue™-MD2-CD14 cells, TLR-4 reporter cells (Invitrogen, Carlsbad, CA). TLR-5 activation was tested with HEK-Blue™ mTLR5 Cells, TLR-5 reporter cells (Invitrogen, Carlsbad, CA). The TLR-4 reporter cells were plated in a 96 well plate at a density of 100,000 cells per well in RPMI medium. The TLR-5 reporter cells were plated in a 96 well plate with a density of 25,000 cells per well in HEK-Blue™ Detection medium. 2E’, PEI, LCA or lipopolysaccharide (LPS) was added to the cells at varying concentrations and incubated for 24 h at 37 °C in 5% CO₂. The supernatant was collected for secreted embryonic alkaline phosphatase (SEAP) assay. The absorbance of SEAP was measured at 620 nm.

**In vitro phagocytosis**

CT26 or B16F10 cancer cells were stained with Cell Tracker Green (Invitrogen, Carlsbad, CA) for 1 h and treated with 5 µg/mL PTX for 24 h. The PTX-treated cancer cells were collected, counted (2 ×10⁵), and co-cultured with Cell-Tracker Deep Red (Invitrogen, Carlsbad, CA)-stained BMDC, JAWSII DC or BMDM (2 ×10⁵) for another 24 h with or without 7 µg/mL of 2E’. The co-cultured cells were collected, resuspended in staining buffer, and analyzed by the BD Accuri C6 Flow Cytometer or BD LSRFortessa Flow Cytometer (San Jose, CA, USA). DCs taking up the PTX-treated cancer cells (cancer cell+ DC or macrophages) was quantified as the percentage of double-positive cells in Cell-Tracker Deep Red-stained BMDC, JAWSII DC or BMDM, and the phagocytized cancer cells as the percentage of double-positive cells in Cell Tracker Green-stained cancer cells. For visualizing in vitro cancer cell phagocytosis, the PTX-treated cancer cells (2 ×10⁵) were co-cultured with Cell-Tracker Deep Red-stained BMDC or BMDM (2 ×10⁵) for 24 h with or without 7 µg/mL of 2E’ and imaged with the Nikon A1R confocal microscope.

**Cytotoxicity**

CT26 cells, B16F10 cells, BMDC, or splenocytes were seeded in a 96 well plate at a density of 8,000 cells per well (CT26, B16F10) or 15,000 cells per well (BMBCs, splenocytes). After 24 h incubation, the cell culture medium was replaced with fresh complete medium containing 2E, PEI, 2E’/PTX or 2E’/PTX/siPD-L1 treatments in different concentrations. After incubation for another 24 h, the cell cytotoxicity was measured by the MTT assay (CT26, B16F10) or propidium iodide (PI) staining (BMDC, splenocytes). For the MTT assay, the treatments were replaced with 100 µL of fresh complete medium and 15 µL of 5 mg/mL MTT solution. After 4 h incubation, 100 µL of stop/solubilization solution was added to the cells and incubated overnight. The absorbance of dissolved formazan was read by the SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) at 560 nm. For PI staining, the treatments were removed and cells were rinsed with PBS, collected, and resuspended in 100 µL of cell staining buffer. Five microliters (40 ng) of PI staining solution was added to each sample immediately before the analysis by the BD Accuri C6 Flow Cytometer. The combination index (CI) was determined by Compusyn (Combosyn, Inc., Paramus, NJ). The values of CI < 1, CI = 1, and CI > 1 represent synergy, additivity, and antagonism, respectively (3).
Gel retardation assay

The formation of 2E’/siPD-L1, 2E’/PTX/siPD-L1 or 2E’/PTX/pDNA complexes was tested by the agarose gel retardation assay. The complexes were prepared varying the weight ratios of 2E’ or 2E’/PTX to siPD-L1 or pDNA. The complexes were loaded in 1.1% agarose gel containing 0.5 μg/mL ethidium bromide in the amounts equivalent to 1 μg siRNA or pDNA and run in 1x TAE buffer at 60 V for 1 h. siRNA or pDNA bands were detected at 302 nm using Azure C300 (Dublin, CA, USA).

PD-L1 silencing

CT26 and B16F10 cells were plated in 6-well plates at a density of 10^5 cells per plate with 2 mL of culture medium and incubated for 24 h. PD-L1 expression was induced by IFN-γ. To determine the optimal incubation time for PD-L1 expression, the cells were collected at 0, 12, 24, 36 and 48 h after IFN-γ addition, resuspended in staining buffer, incubated with Fc-blocking antibody, stained with anti-mouse PD-L1 antibody, and analyzed by flow cytometry.

To evaluate the silencing effect of siPD-L1 complexes, the cells were incubated in the optimal condition for PD-L1 expression (B16F10 cells with 25 ng/mL of IFN-γ for 4 h and CT 26 cells with 100 ng/mL of IFN-γ for 12 h) and treated with 2E’/siPD-L1, 2E’/siNeg (siRNA irrelevant to PD-L1 silencing), 2E’/PTX/siPD-L1 or 2E’/PTX/siNeg equivalent to 2.66 μg/mL siRNA in serum-containing medium for 12 h. And then, treatments were replaced with fresh medium and further incubation for 36 h, PD-L1 expression was determined by western blot. The cells were lysed by cell lysis buffer (Invitrogen, Carlsbad, CA), and the lysates were centrifuged at 12,000 g for 20 min at 4 °C to separate a supernatant. The total protein content in the supernatant was quantified by the BCA assay, and the samples corresponding to 10 mg of protein were mixed with sodium dodecyl sulfate (SDS) gel-loading buffer and heated at 95 °C for 5 min. Samples were separated by 10% SDS-polyacrylamide gel electrophoresis (100 μg proteins per well) and transferred onto polyvinylidene fluoride membrane. The membrane was blocked at room temperature in TBST buffer containing 5% nonfat dried milk (pH 7.4, 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20). After 1 h, the membrane was incubated with anti-mouse PD-L1 and GAPDH antibodies for 24 h at 4 °C per the vendor’s recommendation. The membrane was washed three times and incubated with secondary anti-IgG-HRP antibody for 1 h at room temperature. After incubation with the secondary antibody, the membrane was washed three times, and protein bands were detected by Azure C300 (Dublin, CA).

Cellular uptake

To examine the cellular uptake of siPD-L1 complexes, 2E’/siPD-L1, Lipofectamine/siPD-L1 and 2E’/PTX/siPD-L1 were prepared with Cy3-labeled siPD-L1. CT26 cells were seeded in Nunc™ glass bottom dishes (Thermo Scientific) at a density of 2 ×10^5 and incubated for 24 h. 2E’/siPD-L1, Lipo/siPD-L1 or 2E’/PTX/siPD-L1, equivalent to 66 μg/mL siRNA, in serum-contained medium were incubated with the cells for 4 h or 6 h. The cells were washed, fixed in 4% paraformaldehyde, stained with 200 nM LysoTracker Green and 2 μg/mL Hoechst 33342, and imaged by the Nikon A1R confocal microscope.

Animal purchase and care

All animal procedures were approved by Purdue Animal Care and Use Committee, in conformity with the NIH guidelines for the care and use of laboratory animals. Female Balb/c mice (5-6 week old) and male C57BL/6 mice (5-6 week old) were purchased from Envigo (Indianapolis, IN, USA) and acclimatized for 1 week prior to the procedure.
Tumor retention of 2E’-Cy7 and PEI-Cy7

The retention of intratumorally-injected 2E’-Cy7 and PEI-Cy7 was evaluated in the CT26 model. CT26 tumor cells (5 × 10^5) were subcutaneously inoculated in the upper flank of the right hind limb of Balb/c mice. When tumors grew to 100 mm^3 on the average, the mice were intratumorally injected with 40 µL of 75 µg/mL 2E’-Cy7 or PEI-Cy7. The fluorescence intensity of 2E’-Cy7 and PEI-Cy7 was monitored by the Spectral Ami Optical Imaging System (Spectral Instruments, Tucson, AZ).

Antitumor effects in Balb/c mice with bilateral CT26 tumors

The antitumor effect of 2E’/PTX/siPD-L1 was evaluated in Balb/c mice bearing bilateral CT26 tumors in the flank. Tumors were established in both flanks simultaneously by subcutaneous inoculation. 1×10^6 of CT26 cells were inoculated in the flank of the right hind limb, and 3×10^5 of CT26 cells in the left flank of the same mouse. When the tumor on the right side reached 30-50 mm^3 on the average, the mice were randomly assigned to different groups to receive 5% dextrose (D5W), 2E’, 2E’/PTX, 2E’/PTX/siNeg or 2E’/PTX/siPD-L1 in the tumor on the right side by intratumoral injection. The sizes of the treated tumor and the non-treated tumor on the left side were measured with a digital caliper every other day, and tumor volumes were calculated as (width^2 × length)/2.

Antitumor effects in Balb/c mice with CT26 tumors and delayed rechallenge(s) of live cells

CT26 tumor cells (5 × 10^5) were subcutaneously inoculated in the upper flank of the right hind limb of Balb/c mice. When tumor size reached 30-50 mm^3 on the average, the mice were treated with an intratumoral injection of D5W, 2E’, 2E’/PTX, 2E’/PTX/siNeg or 2E’/PTX/siPD-L1. Tumor growth was monitored as described above. To test whether antitumor immune memory was developed, the mice surviving with complete tumor regression by 30 days from the treatment were rechallenged with 1×10^5 live CT26 cells on the contralateral flank. The mice resistant to the first rechallenge were challenged again with 2 × 10^6 live CT26 cells on 60 days from the treatment. In two separate experiments, surviving Balb/c mice were rechallenged once on 6 or 17 days after the treatment.

2E’/PTX/CDN was tested in Balb/c mice with CT26 tumors. When the tumor grew to 50-100 mm^3 on the average, D5W, paclitaxel nanocrystals and free CDN mixture, and 2E’/PTX, or 2E’/PTX/CDN were administered by intratumoral injection, and tumor growth was monitored over 80 days. Tumor-free mice were rechallenged with 1 × 10^5 live CT26 cells or 4T1 cells on the contralateral injection on 82 days or 140 days after the treatment.

Antitumor effects in B16F10 tumors of C57BL/6 mice

B16F10 tumor cells (1 × 10^6) were subcutaneously inoculated in the upper flank of the right hind limb of C57BL/6 mice. When tumor size reached ~150 mm^3, the mice were treated with an intratumoral injection of D5W, 2E’, 2E’/PTX, 2E’/PTX/siNeg or 2E’/PTX/siPD-L1. Tumor growth was monitored by measuring the size. Tumor-free mice were rechallenged with 1 × 10^5 live B16F10 cells or 4T1 cells on the contralateral injection on 82 days or 140 days after the treatment.

Antitumor effects in C57BL/6 mice with bilateral B16F10 tumors

1 × 10^6 and 1 × 10^5 B16F10 tumor cells were subcutaneously inoculated in the upper flank of the right and left hind limb, respectively. When the tumor on the right side grew to 65-100 mm^3, it was treated with single intratumoral injection of D5W (n=6), 2E’/siPD-L1+ PTX NC (n=8), 2E’/PTX/siNeg (n=9) and 2E’/PTX/siPD-L1 (n=9). The growth of treated and untreated tumors was monitored by measuring the size.
Antitumor effects in 4T1 orthotopic tumors of Balb/c mice

4T1-Luc cell line was a gift from Prof. Michael Wendt at Purdue University. 4T1-Luc $2.5 \times 10^4$ were inoculated in the mammary fat pad of female Balb/c mice. When tumor size reached ~50 mm$^3$, DSW, 2E'/PTX/siNeg, or 2E'/PTX/siPD-L1 were administered by intratumoral injection, or the tumor was removed by partial or complete surgical resection. Tumor growth was monitored by measuring the size. In addition, whole-body imaging was performed by the Spectral Ami Optical Imaging System (Spectral Instruments, Tucson, AZ) to determine the luciferase expression in lieu of tumor growth. Tumor-free mice were rechallenged with $2.5 \times 10^3$ live 4T1-Luc cells on the contralateral mammary gland.

Isolation of splenocytes

The spleen was collected from healthy or tumor-bearing mice to isolate splenocytes. The collected spleens were cut into pieces and filtered through 70 μm and 40 μm cell strainers sequentially to obtain a single-cell suspension. The cell suspension was incubated with 1 mL ammonium-chloride-potassium (ACK) lysis buffer for 3 min to remove red blood cells.

Tumor-specific immunity

The single cell suspension of splenocytes was stained with zombie dye, incubated with anti-mouse CD16/32 antibody to block non-specific binding of the immunoglobulin to Fc receptors, and then labeled with fluorochrome-conjugated antibodies: iTAg Tetramer/APC– H-2 Kb Trp2 (SVYDFFVWL), FITC anti-mouse CD8 antibody (KT15), and PE anti-mouse CD3 antibody (17A2). The labeled cells were analyzed by BD LSRFortessa Flow Cytometer.

Splenocytes collected from B16F10 tumor-bearing mice were challenged with MHC-I-restricted peptide antigen Trp2180-188 (SVYDFFVWL) to determine the response. Splenocytes were suspended in MEM-alpha medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM β-mercaptoethanol, 20 ng/mL murine GM-CSF, and 20% fetal bovine serum were seeded at $1 \times 10^6$ cells per well in a 96 well plate and stimulated with 1-5 μg/mL of Trp2 peptides. After 48 h incubation, the cells were centrifuged at 500 rcf for 8 min to collect the supernatant. The IFN-γ concentration in each supernatant was measured by ELISA (Biolegend, San Diego, CA, USA) and compared with that of the non-challenged cells collected from the same mouse.

Immunophenotyping of tumors and lymphoid organs

Tumors were collected from C57BL/6 mice with B16F10 tumors on 7 days after the treatment, treated with 2 mg/mL collagenase type IV, 0.2 mg/mL DNase I, and 0.2 mg/mL hyaluronidase for 2 h at 37 °C, and ground with the rubber end of a syringe plunger. The cell suspension was filtered through 70 μm and 40 μm cell strainers sequentially and centrifuged at 500 ×g for 8 min. Red blood cells were removed with ACK lysis buffer. The single cell suspension was stained with zombie dye and incubated with anti-mouse CD16/32 antibody to block non-specific binding of the immunoglobulin to Fc receptors and then labeled with fluorochrome-conjugated antibodies: FITC anti-mouse CD3 antibody (17A2), PE anti-mouse CD4 antibody (RM4-5), APC anti-mouse CD8a antibody (53-6.7), FITC anti-mouse CD11c antibody (N418), APC anti-mouse CD86 antibody (GL-1), APC anti-mouse CD40 antibody (3/23), APC anti-mouse MHC-II antibody (M5/114.15.2), FITC anti-mouse F4/80 antibody (BM8), PE anti-mouse CD40 antibody (FGK45), FITC anti-mouse CD45 antibody (I3/2.3), PE anti-mouse CD11c antibody (N418), PerCP anti-mouse CD86 antibody (GL-1), PE/Cy7 anti-mouse CD40 antibody (3/23), PerCP anti-mouse CD45 antibody (30-F11), PE/Cy7 anti-mouse CD11b antibody (M1/70), FITC anti-mouse Ly6C antibody (HK1.4), PE anti-mouse Ly6G antibody (1A8), BV785 anti-mouse PD-L1 antibody.
(10F.9G2), BV605 anti-mouse F4/80 antibody (BM8), PE/Cy7 anti-mouse CD206 antibody (C068C2), APC anti-mouse CD80 antibody (16-10A1), or AlexaFluor700 anti-mouse Foxp3 antibody (MF-14). The labeled cells were analyzed by the BD Accuri C6 Flow Cytometer or BD LSRFortessa Flow Cytometer.

**RNA sequencing assay**

Generation of libraries and sequencing: When B16F10 tumors in C57BL/6 mice grew to ~150 mm³, the mice received an intratumoral injection of D5W, 2E'/PTX/siNeg or 2E'/PTX/siPD-L1 (day 0). Tumors were collected from the untreated animals on day 0 or from the treated animals on day 7 and digested to a single cell suspension. Cell pellets were lysed in 2 mL of TRK lysis buffer and stored frozen until all samples had been collected. Each TRK lysate 700 µL was moved forward to RNA isolation using the RNeasy extraction kit (Qiagen). RNA concentration was determined by Nanodrop. One microgram of RNA was used to generate libraries with the Universal Plus mRNA-Seq kit (Tecan) per manufacturer instructions. A single Illumina NovaSeq 6000 S4 300 cycle, v1.5 chemistry, lane was clustered with a pool of the libraries to produce paired-end 2x150 base reads.

Adapter and Quality Trimming of Reads: The program fastp v.0.12.5 was used to further trim reads based on quality score and to remove adapter sequences (4). The minimum quality score was set to 30, and reads shorter than 50 bases or that were unpaired after trimming were discarded. STAR v. 2.5.4b was used to align reads to the Ensembl Mus musculus genome database version GRCm38.p6 using --twopassMode Basic, modifying the tag HI in the BAM alignment file to start at 0, and removing noncanonical splice junctions (5). The Subread v.2.0.2 software module featureCounts on stranded mode was used to tabulate reads mapping to genes into a gene count matrix using Ensembl Mus musculus genome annotations (6).

Statistical analyses: The Bioconductor package edgeR v.3.24.3 was used to fit a quasi-likelihood negative binomial generalized log-linear model to the count data, followed by genewise statistical tests to identify differentially expressed genes (7, 8). The Benjamini-Hochberg false discovery rate correction is used to correct p-values for multiple testing (9). The Bioconductor packages BiomaRt v. 2.38.0 (10) and ClusterProfiler v 3.10.1 (11) were used in the annotation of genes and in performing pathway and gene ontology enrichment analyses on the differentially expressed genes (results were deemed significant if the adjusted p-value<0.05).

**Histology**

Organs (heart, liver, spleen, lung, kidney and tumors) from C57BL/6 mice with B16F10 tumors were collected on 7 days after the treatment, fixed in 10% neutral buffered formalin and sectioned at a thickness of 4 µm. Heart, liver, spleen, lung and kidney sections were stained with H&E, and tumor sections were stained with rat anti-mouse CD8a monoclonal antibody (eBioscience, clone 4SM15) followed by goat anti-rat secondary antibody (Vector Labs, MP-5444) or with rabbit anti-mouse PD-L1 antibody (Novus biologicals, clone 2096A) followed by horse anti-rabbit secondary antibody (Vector Labs, MP-5401). Slides were rinsed twice in tris buffered saline with Tween® 20, applied with vector immPACT red (Vector Labs, SK-5105) for 20 min and transferred to a Leica Autostainer XL for hematoxylin counterstain, dehydration and coverslipping. Images were taken using a Leica Versa8 whole-slide scanner.
Statistical analysis

All statistical analyses were performed with GraphPad Prism 9 (La Jolla, CA). All data were analyzed with one-way or two-way ANOVA test to determine the statistical difference of means among various groups, followed by the recommended multiple comparisons tests. A value of \( p < 0.05 \) was considered statistically significant.

References

1. M. Feng et al., Stabilization of a hyaluronate-associated gene delivery system using calcium ions. *Biomaterials Science* 2, 936-942 (2014).
2. J. Wang, F. Meng, B.-K. Kim, X. Ke, Y. Yeo, In-vitro and in-vivo difference in gene delivery by lithocholic acid-polyethyleneimine conjugate. *Biomaterials* 217, 119296 (2019).
3. T. C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 58, 621-681 (2006).
4. S. Chen, Y. Zhou, Y. Chen, J. Gu, fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884-i890 (2018).
5. A. Dobin et al., STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15-21 (2012).
6. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923-930 (2013).
7. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139-140 (2009).
8. D. J. McCarthy, Y. Chen, G. K. Smyth, Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research* 40, 4288-4297 (2012).
9. Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 57, 289-300 (1995).
10. S. Durinck et al., BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics* 21, 3439-3440 (2005).
11. G. Yu, L.-G. Wang, Y. Han, Q.-Y. He, clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS: A Journal of Integrative Biology* 16, 284-287 (2012).
Fig. S1. a. Schematic of LCA-PEI conjugate (2E’) synthesis. b. H-NMR spectra of LCA, PEI and 2E’.
Fig. S2. Critical assembly concentration of 2E’ in water.
Fig. S3. In vitro cytotoxicity of 2E’ and PEI in CT26 cells and splenocytes. n=3 replicates of a representative batch, mean ± SD.
Fig. S4. 2E’ has a better safety profile and a longer retention in tumor than PEI. 

a. Mouse survival over time after one-time intratumoral (IT) injection of 2.74 mg PEI or 2E’ (n=7).

b. Fluorescence intensity of sulfo-cyanine7 NHS ester labeled PEI and 2E’ in DI water.

c. Tumor inoculation (CT26 in female Balb/c mice) and treatment schedule, and fluorescence intensity of IT-injected 2E’-Cy7 and PEI-Cy7, indicating their retention in tumors (n=3 per group, mean ± SD).
Fig. S5. Phenotyping of BMDCs and BMDMs. Bone marrow cells were collected and differentiated into BMDCs and BMDMs by incubation in 20 ng/mL GM-CSF or 30 ng/mL M-CSF for 7 days, respectively. The formation of BMDCs and BMDMs was confirmed by flow cytometry based on the expression of CD11c and F4/80, respectively.
Fig. S6. Representative flow cytometry plots of DC maturation marker CD86, MHC-II and CD40 expression on JAWSII DCs after incubation with 2E’ and PEI (7 μg/mL) for 24 h.
Fig. S7. Immunostimulatory effects of 2E’. a. 2E’ induced CD86 and MHC-II expression on JAWSII DCs in a concentration dependent manner. b. 2E’ and PEI (3 μg/mL) induced maturation marker expression on BMDCs (n=3 replicates of a representative batch, mean ± SD). c. 2E’ induced TNF-α secretion from BMDCs and BMDMs in a concentration-dependent manner (n=4 replicates of a representative batch, mean ± SD). d. 2E’ and PEI induced TNF-α and IL-1β secretion from BMDMs (n=3 or 4 replicates of a representative batch, mean ± SD). p-values were calculated by Tukey’s multiple comparisons test following ordinary one-way ANOVA (b) or Šídák's multiple comparisons test following two-way ANOVA (d).
Fig. S8. TLR-4 reporter cell activation by PEI and 2E’ as indicated by SEAP production. LPS 10 ng/mL was used as a positive control.
Fig. S9. **a.** Expression of maturation marker CD86 on BMDC after incubation with different concentrations of LCA for 24 h. **b.** Expression of maturation marker CD86 on BMDC after incubation with LCA (5 μg/mL) and/or PEI (5 μg/mL) for 24 h. n=3 replicates of a representative batch, mean ± SD. p-values were calculated by Dunnett's multiple comparisons test following ordinary one-way ANOVA.
Fig. S10. Immunostimulatory effects of PTX. 

**a.** PTX-induced TNF-α production by BMDCs and BMDMs in a concentration-dependent manner. 

**b.** PTX-induced IL-1β secretion from BMDCs and BMDMs. (n=3 or 4 replicates of a representative batch, mean ± SD).

**c.** PTX (200 ng/mL)-induced CRT exposure on CT26 and B16F10 cells (n=3 replicates of a representative batch, mean ± SD). p-values were calculated by Šídák's multiple comparisons test following two-way ANOVA.
Fig. S11. a. Schedule of phagocytosis assay. b. Flow cytometric analysis of the phagocytosis of CT26 cells by BMDMs without (-) or with (+) 2E’ (7 μg/mL). % CT26+ BMDMs: fraction of BMDMs taking up CT26 cells; % phagocytosed CT26: fraction of CT26 cells taken up by BMDMs (n=3 or 6 replicates of a representative batch, mean ± SD). For calculation of the two fractions, see Fig. S12. c. Representative confocal images of CT26 cells incubated with BMDMs without (-) or with (+) 2E’ (7 μg/mL) (Left) and the calculated Pearson’s correlation coefficient, indicating colocalization of CT26 cells and BMDMs (Right). CT26 cells were stained with Celltracker Green (green) and BMDM cells with CellTracker DeepRed (red). Scale bars: 50 μm. Arrow heads indicate colocalization of CT26 cells and BMDMs. d. Flow cytometric analysis of the phagocytosis of CT26 cells by JAWSII DCs without (-) or with (+) 2E’ (7 μg/mL) (n=3 or 6 replicates of a representative batch, mean ± SD). e. Flow cytometric analysis of the phagocytosis of B16F10 cells by JAWSII DCs without (-) or with (+) 2E’ (7 μg/mL) (n=3 or 6 replicates of a representative batch, mean ± SD). P-values were calculated by two-tailed unpaired t-test.
Fig. S12. Representative flow cytometry plots of cancer cells taken up by BMDCs, BMDMs, or JAWSII DCs without (-) or with (+) 2E'. % CT26+ BMDCs: fraction of BMDCs taking up CT26 cells, Q2/(Q2+Q3); % phagocytosed CT26: fraction of CT26 cells taken up by BMDCs, Q2/(Q1+Q2).
**Fig. S13.** Representative confocal images of CT26 cells incubated with Lipofectamine2000 (L2k)/siPD-L1 or 2E'/PTX/siPD-L1 for 4 h at a dose equivalent to 2.66 μg/mL siPD-L1 (blue: nuclei, green: lysosome, red: siPD-L1). Scale bars: 10 μm.
Fig. S14. A single intratumoral injection of 2E’ or 2E’/PTX induces quick regression of tumor and antitumor immune responses in CT26@Balb/c model with bilateral tumors. a. Schedule of CT26 tumor inoculation in Balb/c mice and treatment injection. b. Individual growth curves of tumors treated with D5W, 2E’ (0.5 mg), 2E’ (1 mg) or 2E’/PTX (1 mg:0.2 mg) and the size of treated tumors on day 10 post-treatment (n=5 per group). CR: complete regression. c. Individual growth curves of untreated distant tumors and the size of distant tumors on day 10 post-treatment of primary tumors (n=5 per group). Mean ± SD; p-values were calculated by Dunn’s multiple comparisons test following Kruskal-Wallis one-way ANOVA.
Fig. S15. A single intratumoral injection of 2E’ or 2E’/PTX induces quick regression of tumor and antitumor immune responses in CT26@Balb/c model with a delayed 2nd tumor challenge. 

a. Schedule of CT26 tumor inoculation in Balb/c mice and treatment injection.

b. Individual growth curves of tumors treated with D5W (n=4), 2E’ (2.7 mg) (n=7) or 2E’/PTX (2.7 mg:0.4 mg) (n=7) and the size of treated tumors on day 22 post-treatment. Mean ± SD; p-value was calculated by Dunn's multiple comparisons test following Kruskal-Wallis one-way ANOVA. 

c. Individual growth curves of untreated distant tumors. CR: complete regression.
Fig. S16. Antitumor effects of 2E'/PTX on CT26 tumors. a. Schedule of CT26 tumor inoculation in Balb/c mice, treatment injection, and rechallenge. b. Individual growth curves of tumors after a single IT injection of D5W, 2E’ (1.4 mg), or 2E'/PTX (1.4 mg:0.2 mg) and survival curve of treated mice (n=5 per group). p-values: vs. D5W by Log-rank (Mantel-Cox) test. c. Individual growth curves of rechallenged tumors in tumor-free mice after single treatment and percentage of tumor-free mice after re-challenge. CR: complete regression.
Fig. S17. A single intratumoral injection of 2E'/PTX reduces the recurrence of tumors and lung metastasis after incomplete surgical removal of primary tumors in orthotopic 4T1@Balb/c model. 

a. Schedule of 4T1 tumor inoculation in Balb/c mice, surgical removal, and treatment injection. 

b. *In vivo* bioluminescence imaging of 4T1 tumors after incomplete surgical removal of the primary tumor and IT injection of 2E'/PTX (0.2 mg:0.2 mg) (n=5 per group). Red arrow heads (day 17) indicate lung metastasis. 

c. Survival curves of treated mice.
Fig. S18. Combination index (CI) vs. effect levels (Fa) plots of 2E'/PTX, 2E'/PTX/siNeg, and 2E'/PTX/siPD-L1. 2E’ and PTX show strong synergistic effects at all the tested concentrations with CI values of < 0.5.
**Fig. S19.** Characterization and immunostimulatory activities of 2E'/PTX/siPD-L1. 

a. Confocal image of CT26 cells after 4 h incubation with 2E'/PTX/siPD-L1 consisting of 4 μg/mL 2E’, 0.8 μg/mL PTX and 2.66 μg/mL siPD-L1 (blue: nuclei, green: lysosome, red: siPD-L1), scale bar: 10 μm.

b. A representative western blot of PD-L1 expression in IFN-γ-activated CT26 and B16F10 cells after treatment with 2E'/PTX/siNeg or 2E'/PTX/siPD-L1 at a dose of 6 μg/mL 2E’, 1.2 μg/mL PTX and 4 μg/mL siRNA.

c. TNF-α and IL-1β secretion by BMDCs and BMDMs after incubation with 2E'/PTX/siNeg or 2E'/PTX/siPD-L1 (w/w/w, 5:1:3.33) for 24 h (n = 3 or 4 replicates of a representative batch, mean ± SD).

d. CRT exposure on CT26 and B16F10 cells after treatment with 2E'/PTX/siPD-L1 at a dose of 1 μg/mL 2E’, 0.2 μg/mL PTX, and 0.67 μg/mL siPD-L1 for 6 or 24 h (n = 3 replicates of a representative batch, mean ± SD). p-values were calculated by Šídák's multiple comparisons test following two-way ANOVA.
Fig. S20. Representative confocal image of CT26 cells incubated with L2k/siPD-L1 or 2E'/PTX/siPD-L1 at a dose equivalent to 2.66 µg/m siPD-L1 for 4 h (blue: nuclei, green: lysosome, red: siPD-L1), Scale bars: 10 µm.
Fig. S21. Representative confocal image of CRT exposure on CT26 and B16F10 cells treated with 2E'/PTX/siPD-L1 at a dose equivalent to 1 μg/mL 2E', 0.2 μg/mL PTX and 0.67 μg/mL siPD-L1 for 24 h. Scale bar: 100 μm.
Fig. S22. A single intratumoral injection of 2E'/PTX/siPD-L1 induces tumor regression and immunophenotype change in TDLNs of Balb/c mice with CT26 tumors. a. Schedule of CT26 tumor inoculation in Balb/c mice and treatment injection. b. Individual growth curves of tumors treated with D5W (n=4), 2E' (n=4), 2E'/PTX (n=4), 2E'/PTX/siNeg (n=4) or 2E'/PTX/siPD-L1 (n=5) consisting of 1 mg 2E', 0.2 mg/mL PTX and 0.67 mg siRNA and body weight change after treatment. c. T cell and DC cell populations in TDLNs on day 7 post-treatment. Mean ± SD; p-values were calculated by Dunnett's multiple comparisons test following one-way ANOVA.
Fig. S23. Gating strategies for flow cytometric analysis of lymphocytes in TDLNs of CT26@Balb/c mouse model (Fig. S22). Single cells were selected from the total TDLN cell population. CD3+ T cells or CD11c+ DCs were selected from the single cell population. Subsequently, CD8+ or CD4+ T cells were selected from the CD3+ T cell population, and matured CD86+ DCs were selected from the CD11c+ DC population.
Fig. S24. Photomicrographs of H&E-stained heart, liver, spleen, lung and kidney sections of
B16F10@C57BL/6 model on day 7 after treatment (Fig. 3). In all treatment groups, no significant
lesions were observed in the examined organs. Scale bars: 400 µm.
Fig. S25. Gating strategies for flow cytometric analysis of lymphocytes in B16F10 tumors. Live cells were selected from total tumor cell population. CD3\(^+\), CD8\(^+\), CD4\(^+\) T cells, CD45\(^+\) or CD45\(^-\) cells were selected from the live cell population. Subsequently, Foxp3\(^+\) Treg cells were gated from CD4\(^+\) T cells, and PD-L1\(^+\) cells were gated from CD45\(^+\) and CD45\(^-\) cells.
**Fig. S26.** Gating strategies for flow cytometric analysis of DCs in B16F10 tumors. Live cells were first selected from total tumor cell population. Subsequently, CD11c⁺ DCs were gated from live cells. Mature CD86⁺ DCs and CD40⁺ DCs were then gated from CD11c⁺ DCs.
Fig. S27. Gating strategies for flow cytometric analysis of macrophages in B16F10 tumors. Live cells were first selected from total tumor cell population. Subsequently, F4/80+ macrophages were gated from live cells. CD80+ macrophages (M1-like) and CD206+ macrophages (M2-like) were then gated from F4/80+ macrophages.
**Fig. S28.** Gating strategies for flow cytometric analysis of MDSC in B16F10 tumors. CD11b⁺ cells were first selected from total tumor cell population. Subsequently, Ly6C⁺ monocyctic cells and Ly6G⁺ neutrophilic cells were gated from CD11b⁺ cells. PD-L1⁺ cells were then gated from CD11b⁺Ly6C⁺ and CD11b⁺Ly6G⁺ cells.
Fig. S29. Representative flow cytometry plots of CD8⁺, CD4⁺ and Treg⁺ cells in B16F10 tumors administered with treatments in Fig. 3.
**Fig. S30.** Immunohistochemistry (IHC) staining of CD$8^+$ T cells (top row) and PD-L1 expression (bottom row) in B16F10 tumors from mice treated with test particles described in Fig. 3. Scale bar: 100 µm. Red chromogen signal indicates CD$8^+$ T cells or PD-L1$^+$ cells.
**Fig. S31.** a. Schedule of B16F10 tumor inoculation in C57BL/6 mice and treatment injection. b. RNA sequencing of cytokine/chemokine expression in tumors at day 0 (before treatment) and day 7 (after treatment). The heat map shows z-scores of binary logarithms of counts per millions (log2CPM) for each gene.
Fig. S32. Systemic antitumor immune response in B16F10@C57BL/6 model. Melanoma-specific antigen Trp2 peptide-induced IFN-γ secretion by splenocytes collected from B16F10@C57BL/6 mice treated with D5W (n=3), 2E'/PTX/siNeg (n=4) or 2E'/PTX/siNeg (n=4). Mean ± SD. The complexes consisted of 1 mg 2E’, 0.2 mg PTX and 0.67 mg siRNA. Spleens were collected on day 7 after treatment.
Fig. S33. Systemic antitumor immune response in B16F10@C57BL/6 model. a. Representative flow cytometry plots of the frequency of TRP2-specific CD8+ T cells in the spleen. p-values were calculated by Tukey's multiple comparisons test following one-way ANOVA. b. The frequency of Trp2 (SVYDFVWL)-specific CD8+ T cells from the spleen of B16F10@C57BL/6 mice treated with D5W (n=3), 2E'/PTX/siNeg (n=4), or 2E'/PTX/siPD-L1 (n=4). Mean ± SD. The complexes consisted of 1 mg 2E’, 0.2 mg PTX and 0.67 mg siRNA. Spleens were collected on day 7 post-treatment and analyzed by flow cytometry.
Fig. S34. Antitumor effects of 2E’/PTX/siPD-L1 in B16F10@C57BL/6 mice with bilateral tumors. a. Schedule of bilateral B16F10 tumor inoculation in C57BL/6 mice and treatment injection. b and c. Individual growth curves of treated (b) and untreated tumors (c). d. Survival curves and body weight change (mean ± SD) after a single treatment of the following: D5W (n=6); 2E’/siPD-L1 + PTX NC (n=8); 2E’/PTX/siNeg (n=9); or 2E’/PTX/siPD-L1 (n=9). Note that some of the 2E’/PTX/siPD-L1-treated mice were euthanized due to tumor ulceration despite the small tumor size.
Fig. S35. Tumor disappearance and skin recovery after treatment with 2E'/PTX/siPD-L1 or 2E'/PTX/CDN in CT26@Balb/c, B16F10@C57BL/6 or 4T1-Luc@Balb/c models.
Fig. S36. **2E’ as a versatile carrier of hydrophobic drugs.** 2E’ forms spherical particles upon assembly with various hydrophobic compounds, such as ICD inducers [carfilzomib (CFZ, selective proteasome inhibitor) and camptothecin (CPT, DNA topoisomerase inhibitor)]; hydrophobic fluorescence dyes: DiR (DiIC18(7); 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindotricarbocyanine iodide); niflumic acid (a drug used for joint and muscular pain); probucol (anti-hyperlipidemic drug).
Fig. S37. 2E’ as a carrier of nucleic acids. a. Gel electrophoresis of 2E’/PTX/pDNA complexes at various weight ratios of 2E’/PTX to pDNA. b. TEM images of 2E’/PTX/pDNA (1:0.4:0.7). Scale bars: 200 nm. c. Gel electrophoresis of 2E’/mRNA complexes at various weight ratios of 2E’ to mRNA. All lanes contain complexes equivalent to 1 µg pDNA or mRNA.