Electronic supplementary data for "**Engineering biodegradable guanidyl-decorated PEG-PCL nanoparticles as robust exogenous activators of DCs and antigen cross-presentation**"

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1. Materials and methods

Methoxyl poly (ethylene glycol) (Sigma, M_n= 2000 g/moL) was dried at 40 ^oC under vacuum for 24 h. Stannous octanoate, copper (I) bromide (CuBr), 2,2′-bipyridine (Bpy), and butanone were purchased from sigma and used as received. ε-Caprolactone (CL, Sigma) was dried over calcium hydride for 48 h at room

temperature and distilled under reduced pressure. γ-(2-Bromo-2-methylpropionate)-ε-caprolactone (BMPCL) was synthesized according to a previously reported procedure (J. Mater. Chem., 2010, 20, 6935-6941). S-ethylisothiourea hydrobromide and ovalbumin (OVA, grade V) were purchased from Sigma. *N*-(*tert*-butoxycarbonyl) amino ethyl methacrylate (tBAM) was synthesized as previously described (J. Am. Chem. Soc., 2005, 127, 4128-4129). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Japan). Hamster anti-mouse CD11c-PE, Rat anti-mouse CCR7-PE, CD86-PE, CD40-PE and Rat IgG2a K Isotype Control PE were purchased from eBioscience (San Diego, CA). Fluorescein isothiocyanate labeled ovalbumin (OVA-FITC) was from Beijing Solarbio Science & Technology Co.. Rhodamine B thioisocyanate labeled ovalbumin (OVA-RBTIC) was from Beijing Biosynthesis Biotechnology Co., Ltd. Recombinant mouse GM-CSF, IL-4 and IL-2 were purchased from Peprotech (Rocky Hill, USA). Amantadine hydrochloride, amiloride hydrochloride, genistein and chlorquine diphosphate salt were purchased from Sigma. Alexa Flour® 594 Phalloidin was provided by Thermo Fisher Scientific.

Animals: BALB/C mice, C57 BL/6 and transgenic OT-I mice aged 6-8 weeks were used in this study. All experiments were performed in accordance with the guidelines of and approved by the Animal Care and Use Committee of Chinese Academy of Medical Sciences and Peking Union Medical College.

1.1 Synthesis of the macro-initiator mPEG-*b*-PCL-Br

The macro-initiator, methoxyl poly(ethylene glycol)-*block*-poly(*ε*-caprolactone-*co*-BMPCL) (mPEG-*b*-PCL-Br), was synthesized by the ring-opening polymerization (ROP) of CL and BMPCL using mPEG as an initiator and stannous octoate as a catalyst. Briefly, mPEG (M_n = 2000 g/moL) (1.0 g, 5.0 mmoL) was dissolved in the Schlenk tube with 6 mL toluene, and the toluene was distilled off completely to remove the residual water. Then, *ε*-caprolactone (18.24 g, 160 mmoL), BMPCL (4.87 g, 17.5mmol) and stannous octoate (58 μL, 0.18 mmoL) were added to the mixture under dry nitrogen and the tube was sealed. The reaction was continued at 130 ^{o}C for 12 h under stirring. After cooling the reaction system to room temperature, methylene chloride was added to dissolve the crude products and then precipitated by slowly adding the solution into cold diethyl ether. The precipitate was collected and the residual solvent was removed under vacuum. The final yield was over 90%.

1.2 Synthesis of mPEG-*b*-PCL-*g*-PtBAM

A typical atom transfer radical polymerization (ATRP) procedure was employed to prepare methoxyl poly(ethylene glycol)-*block*-poly(*ε*-caprolactone)-*graft*-poly(*N*-(*tert*-butoxycarbonyl) amino ethyl methacrylate) (mPEG-*b*-PCL-*g*-PtBAM). Briefly, mPEG-*b*-PCL-Br (626 mg, 0.1 mmoL), tBAM (1.52 g, 6.6 mmoL) and Bpy (36.5 mg) was dissolved in 3 mL butanone in a Schlenk tube. The mixture was freezed in liquid nitrogen and degassed by three vacuum/nitrogen cycles. Then, CuBr (14.4 mg) was added and the mixture was further degassed. Polymerization was carried out at 60 ^{o}C for 12 h. The product was recovered by dialysis in ultra-pure water, and lyophilized to

obtain the powder of mPEG-*b*-PCL-*g*-PtBAM.

1.3 Deprotection of Boc groups on mPEG-*b*-PCL-*g*-PtBAM

Deprotection of Boc groups on mPEG-*b*-PCL-*g*-PtBAM was achieved by the treatment of trifluoroacetic acid (TFA). Briefly, mPEG-*b*-PCL-*g*-PtBAM (1.0 g) was dissolved in 5 mL TFA, and the mixture was stirred for 2 h at room temperature. TFA was removed by rotary evaporation and the residue was dissolved in DMF. The resultant solution was then precipitated in diethyl ether and the precipitate was collected by filtration, washed twice by diethyl ether, and dried overnight under vacuum.

1.4 Guanidinylation of primary amines to obtain mPEG-*b*-PCL-*g*-PGEM

Finally, the primary amines on mPEG-*b*-PCL-*g*-PAEM were guanidinylated in 0.1 M $NaHCO_3$ solution. After addition of 2 equivalent moles of *S*-ethylisothiourea hydrobromide, the mixture was stirred for two days at room temperature. Then, the mixture was sealed in a dialysis bag with the MWCO of 3.5 kDa and dialyzed against ultra-pure water for 3 days. The final methoxyl poly(ethylene glycol)- *block*-poly(*ε*-caprolactone)-*graft*-poly(2-(guanidyl) ethyl methacrylate) (mPEG-*b*-PCL-*g*-PGEM, PECG) polymer was recovered by lyophilization. The synthesis route of PECG copolymers was shown in Fig. S1.

1.5 Polymer characterization

The chemical structures and molecular weights of all polymers including the macro-initiator, intermediate and final products were determined by ^{1}H NMR (Varian Unity Inova 500 MHz, Fig. S2). The molecular weight distribution of PECG was determined by the gel permeation chromatography (GPC) system, which was equipped with a CoMetre 6000 LDI pump, a Schambeck SFD GmbH RI2000 refractive index detector and a ShodexOHpak SB-802.5 column. The mobile phase was HPLC grade dimethylformamide containing 1.0 M acetic acid. The flow rate is 0.5 mL/min and the detection temperature was 40 ^{o}C. All samples were filtered through a 0.22 μm filter before analysis. The physicochemical properties of PECG copolymers are summarized in Table S1.

1.6 Preparation of blank or antigen-formulated PEC or PECG nanoparticles

Blank PEC or PECG nanoparticles (NPs) were prepared by the self-assembly of PECG copolymers in PBS buffer (pH=7.2, 0.01M). Briefly, 20 mg PECG was dissolved in 1 mL of trifluoroethanol and then added the solution dropwise into 10 mL PBS buffer at an injection speed of 0.1 mL/min. The solution was continuously stirred for 24 h to evaporate trifluoroethanol and then dialyzed in water for two days. The concentration of PECG NPs solution was adjusted to 2 mg/mL. The size and zeta potential were characterized by DLS (Malvern Zetasizer Nano ZS). The morphology of nanoparticles was observed by TEM (JEOL JEM100CXII). To prepare antigen/adjuvant vaccines, certain amount of OVA was added into the solution of PEC or PECG NPs at 4 ^{o}C, which was gently mixed for 30 min. To verify the

absorption of antigens to NPs, the antigen loading capacity was determined by ultra-centrifuging the mixture at 100,000 g for 30 min. The amount of unbound OVA in supernatant was determined using Pierce™ BCA Protein Assay Kit. The loading amount (w/w) and encapsulation efficiency (%) were expressed as the amount of loaded OVA/the amount of PECG and the amount of loaded OVA/the amount of feeding OVA, respectively. Three parallel experiments were implemented and the results were expressed as mean ± standard deviations (SDs).

1.7 Generation of murine bone marrow-derived dendritic cells (BMDCs) and in vitro cytotoxicity

Immature BMDCs were prepared following the previous methods (Nanoscale, 2011, 3, 2307-2314; Acta Biomater., 2011, 7, 2857-2864). Cell viability of BMDCs in the presence of PECG NPs was evaluated by CCK-8 assay. Briefly, 5×10^4 cells were seeded in 96-well plates, incubated overnight and then treated with different concentrations of NPs for 48 h. Cells were washed and the CCK-8 reagent was added in a dose of 10 μL/well and incubated for 3 h. Fluorescence measurements were performed at 450 nm using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Cell culture medium was used as the negative control. Three parallel experiments were implemented and the results were expressed as mean ± standard deviations (SDs).

For plasma cell membrane staining, BMDC cells were cultured in 2 mL of RPMI-1640 media containing 10% FBS, 20 ng/mL IL-4, 20 ng/mL GM-CSF on 18-mm

glass-base dishes at 2×10^5 cells/well. After 24 h, cells were washed with DPBS and cultured with 15 μg NPs or medium only for 12 h. The nucleus and plasma membranes were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue fluorescence) and Alexa Fluor® 488 Phalloidin (ThermoFisher Scientific, green fluoresence). Cells were observed using confocal laser scanning microscopy (CLSM, Zeiss LSM 710 Meta). The Lactate dehydrogenase (LDH) assay was also performed to quantify the cell membrane breakage following the manufacturer's protocol (Takara Biochemicals, Osaka, Japan). Cells were seeded in 96-well plastic plates at a density of 5×10^4 cells/well. 24 h later, cells were treated with NPs, and the LDH leaked into the culture medium was measured after 12 h. The LDH activity was determined at 450 nm using a microplate reader (Thermo Scientific Varioskan Flash).

1.8 Cellular uptake and localization of PECG nanoparticles

A novel Cy7 derivative (Cy7 bearing carboxyl, information of this dye is shown in Fig. S4) was conjugated to PEC or PECG copolymer to label corresponding NPs. Boc-protected PEG was used to prepare PEG-PCL copolymer and after TFA treatment, dye was conjugated to copolymer via amidation. Then, following procedures described in section 1.3-1.4 were implemented to prepare dye-labeled PECG copolymers. BMDCs (2×10^5/well) were seeded in a 12-well plate and treated with PEC or PECG NPs at 37 °C for 0.5 h. The uptake of dye-labeled NPs was analyzed by flow cytometry. For the nanoparticle localization study, BMDCs (1×10^6 cells/mL) were cultured with dye-encapsulated NPs at 37 °C for 1 h. Then, cells were

stained with 50 nM Lyso-Traker Green (Invitrogen, CA, USA) for 60 mins to label lysosomes. Intracellular localization of NPs was examined by confocal laser scanning microscope (CLSM, TCS SP8, Leica). DAPI was used to label the nuclear.

The uptake mechanism of NPs was measured as below. Briefly, iBMDCs were seeded in 96-well flat bottom plate (1.5×10^5/well, 150 μL) and treated with one of the following pharmacological inhibitors: amantadine hydrochloride (1 mM), chloroquine diphosphate salt (50 mM), genistein (20 mM), or amiloride hydrochloride (2 mM) for 0.5 h. NPs were then added into the medium with a final concentration of 20 μg/mL and co-incubated for 18 h. Cells untreated with inhibitors were used as normal controls. To check the energy dependency of NP internalization, BMDCs were treated at 4 °C for 0.5 h and then co-cultured with NPs for another 4 h. Flow cytometry was used to analyze the internalization of NPs. Three parallel experiments were implemented and the mean fluorescence intensity was normalized to that of untreated cells. Results were expressed as mean ± SD (n=3).

1.9 *In vitro* antigen cross-presentation and intracellular antigen localization

A co-culture assay was used to characterize antigen delivery into the MHC class I processing pathway. B3Z cell, a SIINFEKL-specific $CD8^+$ T cell hybridoma line, could be activated by SIINFEKL, which was one peptide fragment of OVA antigens cross-presented by DCs, the production of β-galactosidase upon binding ovalbumin class I antigenic epitope SINFEKL complexed with MHC-I H-$2K^b$ present on DCs, can be used as a reporter cell. iBMDC cells were cultured overnight (6×10^4

cells/well) in 96-well U-bottom cell culture plates. One day later, OVA-containing nanoparticles or free OVA (50 μg) were added into the plate and incubated with BMDC cells for further 5 h at 37 °C in a 5% CO_2 incubator. Medium was used as the negative control. Cells were then carefully rinsed three times with DPBS, and 5×10^5 B3Z cells were added to each well and co-cultured for 24 h in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 55 μM β-mercaptoethanol, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were then pelleted via centrifugation (7 min, ~500 rcf), media were carefully aspirated, and 150 μL of CPRG/lysis buffer (0.15 M chlorophenol red-β-d-galactopyranoside (Calbiochem), 0.1% Triton X-100, 9 mM MgCl, 100 μM mercaptoethanol) was added. Plates were incubated at 37 °C in the dark for 20 h, then 100 μL samples was transferred to 96-well clear flat-bottom plates and the absorbance of released chlorophenol red was measured at 570 nm.

For intracellular antigen localization study, BMDCs (5×10^5 cells/mL) were treated with free Rhodamine B-labeled OVA or OVA/NPs complex formulations at 37 °C for given times (2, 4, 8 h). The dose of OVA was 25 μg. Then, cells were washed, collected and stained with 50 nM Lyso-Traker™ Green (Invitrogen, CA, USA) for 60 mins to label lysosomes. The intracellular localization of OVA was examined by the confocal laser scanning microscope (CLSM, LSM710, Zeiss).

1.10 Lymph node draining and the stimulation of immune response *in vivo*

BALB/C mice were intradermally immunized with nanoparticles (100 μL) at the

posterior end. The presence of NPs in the injection site and draining lymph nodes was visualized by the IVIS spectrum preclinical in vivo imaging system (PerkinElmer) with the excitation and emission wavelength of 630 nm and 767 nm, respectively. The fluorescence intensity at the injection site and draining lymph nodes was recorded. The duration kinetics of NPs was expressed as the fluorescence decay in the injection site or draining lymph nodes by comparing the fluorescence intensity at different scheduled time points to the maximum recorded value. Besides, inguinal reflux lymph nodes were isolated at 12 h after immunization, infiltrated by opti-mum cutting temperature compound (OCT) at room temperature for 4 h, and cut to 5 μm thick by Cryostat Microtome (Lecia CM 1950). Sections were stored on glass slides, mounted by Dapi-Fluoromount-G and covered by glass coverslips. The distribution of nanoparticles in lymph nodes was observed using fluorescence microscope.

For animal immunization, BALB/C mice (n=5) were immunized with OVA/nanoparticle formulations (antigen dose, 50 μg) by intradermal injection with pre-determined immunization dose 3 times at 1 week interval. 7 days after last immunization, splenocytes or lymphocytes (5×10^6 cells/mL) were isolated from spleen and draining lymph nodes, respectively, and re-stimulated with soluble OVA (50 μg/ml) in a 24-well plate at 37 °C for 96 h. The production of IFN-γ, IL-4, IL-10 and TNF-α was measured by corresponding ELISA kits acquired from Dakewe Biotech Company (ShenZhen, China).

1.11 Antigen cross-presentation in vivo

To evaluate the antigen presentation *in vivo*, C57 BL/6 mice were intradermal vaccinated with NPs-formulated OVA or free OVA at the dose of 50 μg. Two days after vaccination, OVA-specific $CD8^+$ T cells were isolated from the lymph nodes and spleen of OT-I mice (C57BL/6-Tg (Tcra, Tcrb) 1100Mjb/J, The Jackson Laboratory) by using the mouse $CD8^+$ T cell isolation Kit (YOUZRE BIOTECH CO. LTD., lot#21-00800-11). OVA-specific OT-I $CD8^+$ T (5×10^5 cells / mice) were injected to C57 BL/6 mice by tail intravenous injection. 4 days later, mice were sacrificed, splenocytes were harvest and stained with fluorescence-labeled antibodies for CD8β (eBioscience, lot#12-0083-82). The OT-I congenic marker CD90.1 (Thy1.1, eBiosience, lot#17-0900-82) was analyzed by flow cytometry. T cell expansion is expressed as the number of transferred (OT-I) T cells as a percentage of total $CD8^+$ T cells.

1.12 Statistical analysis

Data are expressed as mean ± standard deviation (SD). The statistical differences among groups were determined using one-way ANOVA analysis or student's *t*-test by Graphpad prism 6.01.

2. Figures

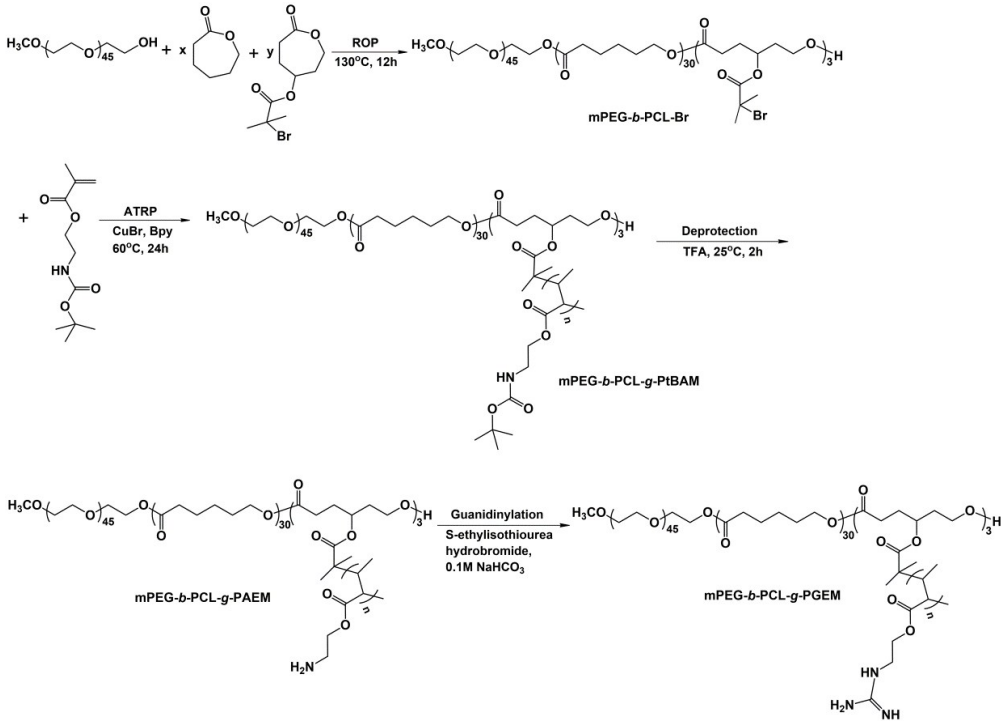
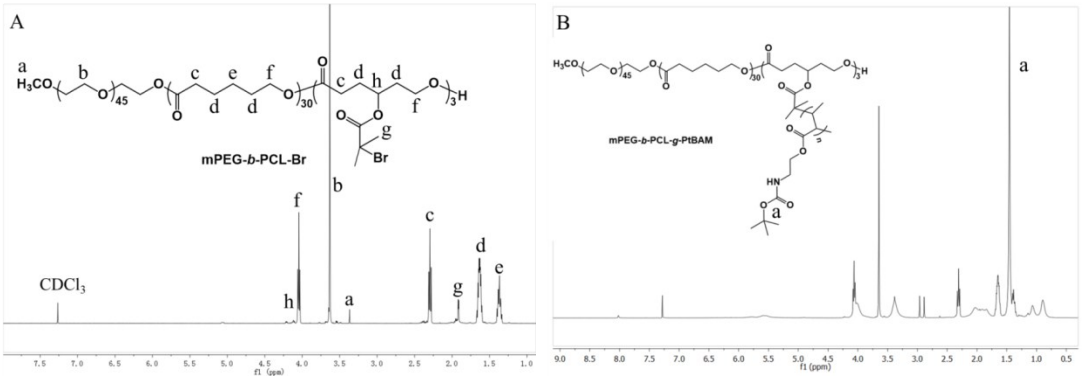


Fig. S1 The synthesis route of mPEG-*b*-PCL-*g*-PGEM (PECG) copolymers.



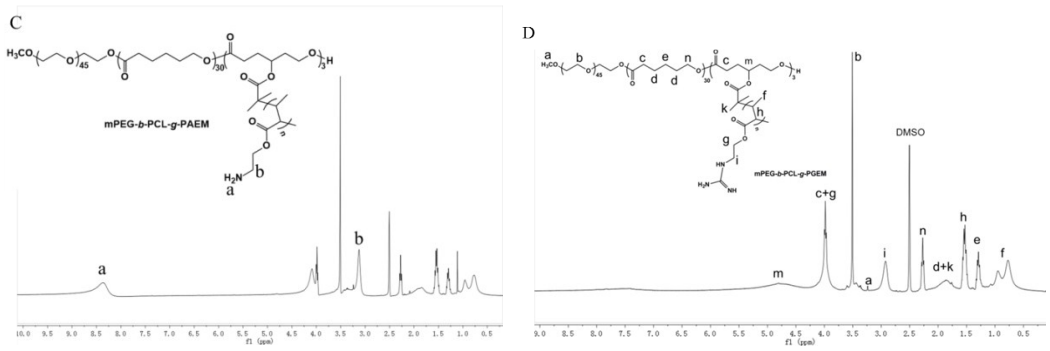


Fig. S2 ^{1}H NMR spectrum of mPEG-*b*-PCL-Br (A; solvent, $CDCl_3$), mPEG-*b*-PCL-*g*-PtBMA (B; solvent, $CDCl_3$), mPEG-*b*-PCL-*g*-PAEM (C; solvent, DMSO-d_6) and mPEG-*b*-PCL-*g*-PGEM (PECG; solvent, DMSO-d_6).

Table S1 Characterization of PEC or PECG copolymers and nanoparticles

Samples	DP [a]		M_n [a] (g/moL)	PDI [b]	Size [c] (nm)	PDI [c]	ξ [c] (mV)
	CL	GEM					
PEC	33	0	6254	1.19	51.66±1.22	0.24±0.002	0.34±0.18
PECG-1	33	60	17426	1.21	48.01±0.56	0.24±0.007	12.89±3.80
PECG-2	33	105	25805	1.22	52.01±0.23	0.28±0.033	32.98±7.61

[a] Determined by ^{1}H NMR. DP and M_n indicate the degree of polymerization and number-average molecular weight, respectively. [b] Polydispersity index (PDI) of the molecular weight of PECG copolymers determined by GPC. [c] Size and zeta potential (ξ) of PEC or PECG nanoparticles at a concentration of 2 mg/mL.

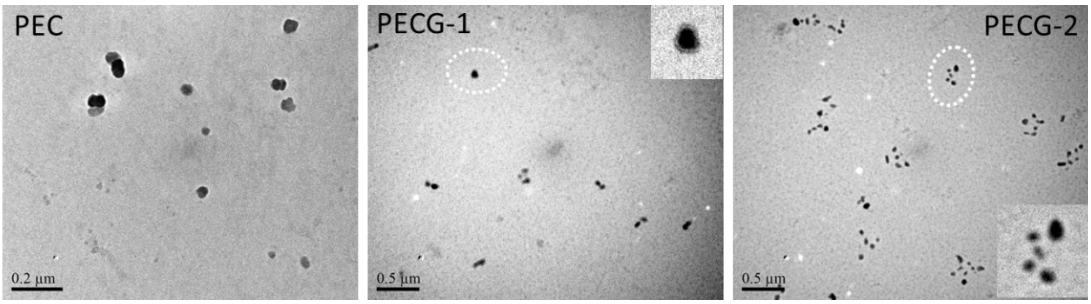


Fig. S3 Representative TEM images of nanoparticles and the inserted photos are magnified nanoparticles in the circle with white dotted line.

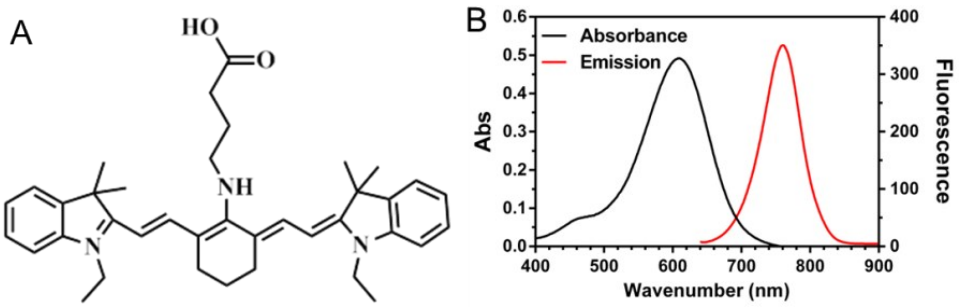


Fig. S4 The chemical structure (A) of the Cy7 derivative and its absorbance (black line) and emission (red line) curves (B).

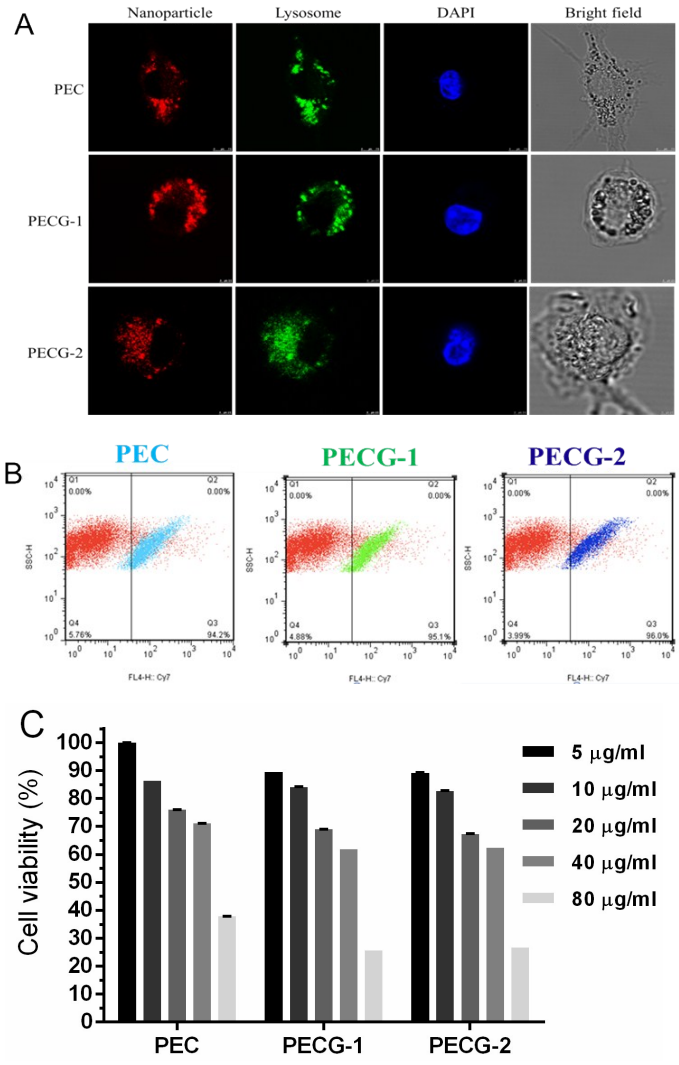


Fig. S5 (A) Representative CLSM images of the cellular uptake of NPs by DCs. NPs and

lysosomes were labeled by the Cy7 derivative and Lyso-Tracker-Green, respectively. (B) Representative flow cytometry profiles of the internalization of NPs. (C) *In vitro* cytotoxicity of NPs against BMDCs. Data are expressed as mean ± SDs (n=3).

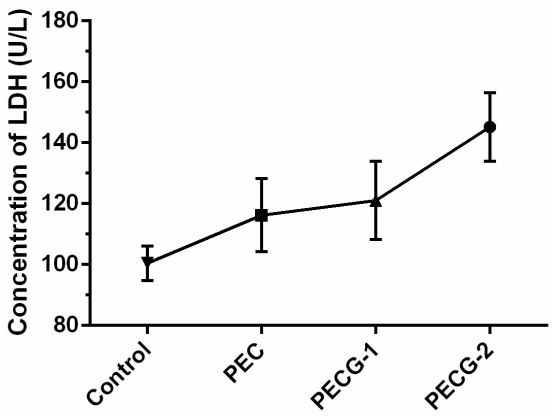
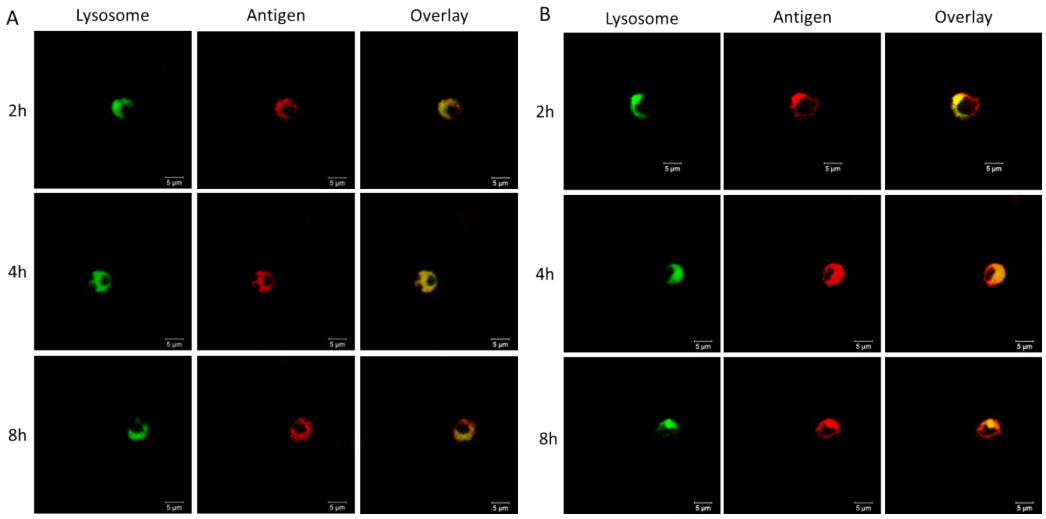


Fig. S6 The result of lactate dehydrogenase (LDH) assay. The LDH released into the culture medium of BMDCs treated with NPs was measured and the data were shown as mean ± SDs (n=3).



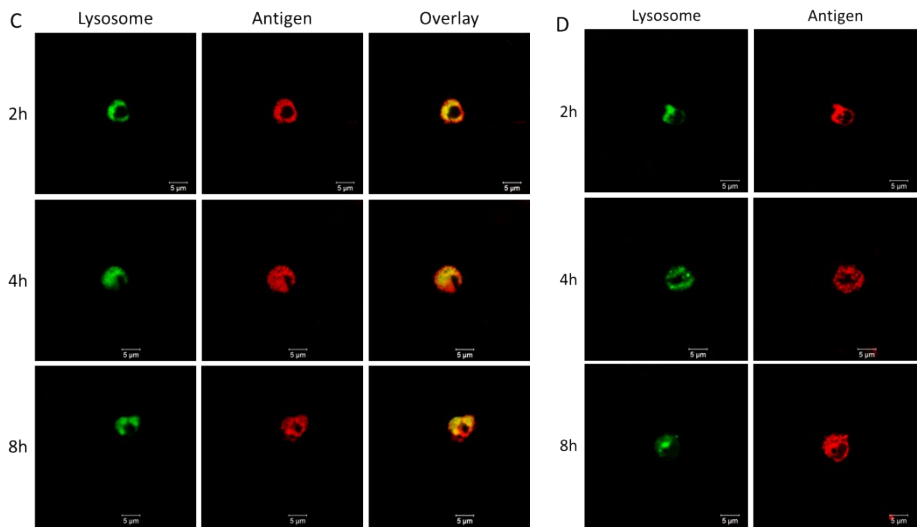


Fig. S7 Representative CLSM images of the intracellular antigen location at different time points (A, free OVA; B, OVA/PEC NPs; C, OVA/PECG-1 NPs; D, OVA/PECG-2 NPs). BMDCs were treated with various OVA formulations for given times and then observed by CLSM. Lysosomes were stained by the LysoTracker™ Green probe (green color) and antigen was labeled by Rhodamine B (red color).

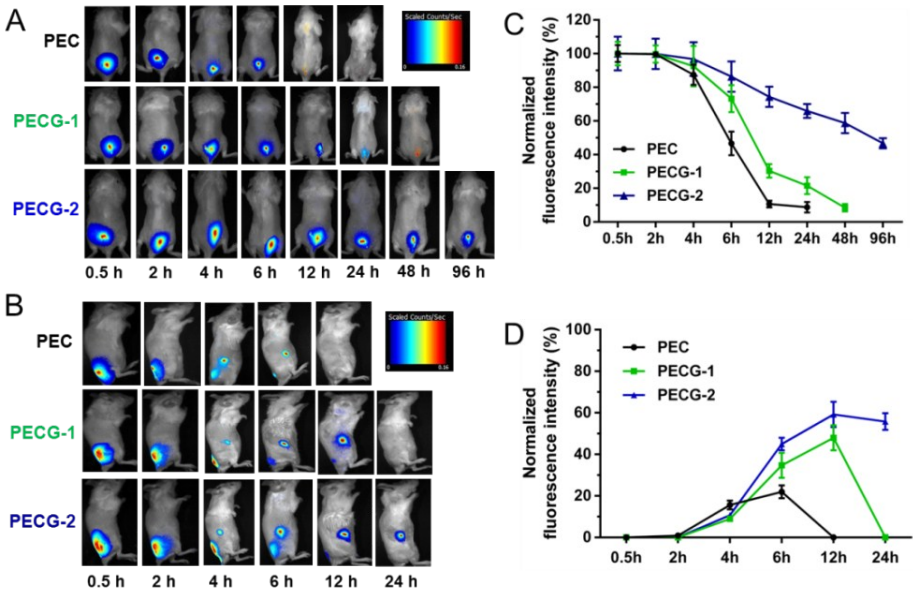


Fig. S8 In vivo duration of NPs at the injection site and draining lymph nodes. (A) The representative fluorescence images of NPs at the injection site and (B) the migration of NPs to the

draining lymph node. The normalized fluorescence intensity decay at the injection site (C) and at the draining lymph node (D).

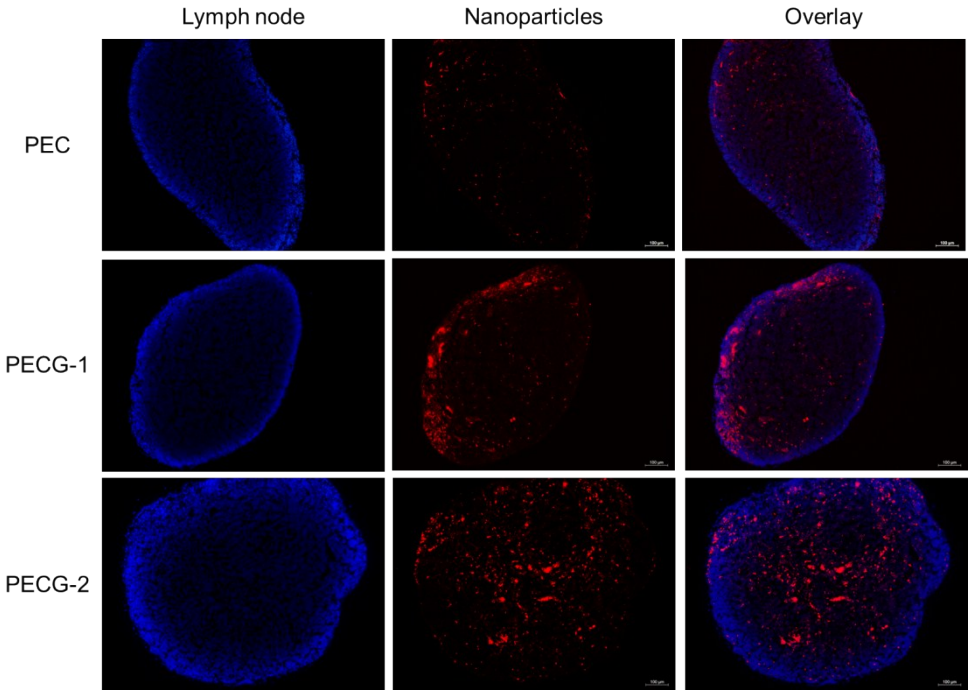


Fig. S9 Representative frozen sections of the separated lymph nodes 12 h post the nanoparticle injection. Lymph nodes were stained by DAPI (blue) and nanoparticles were labeled by the Cy7 derivative (red).

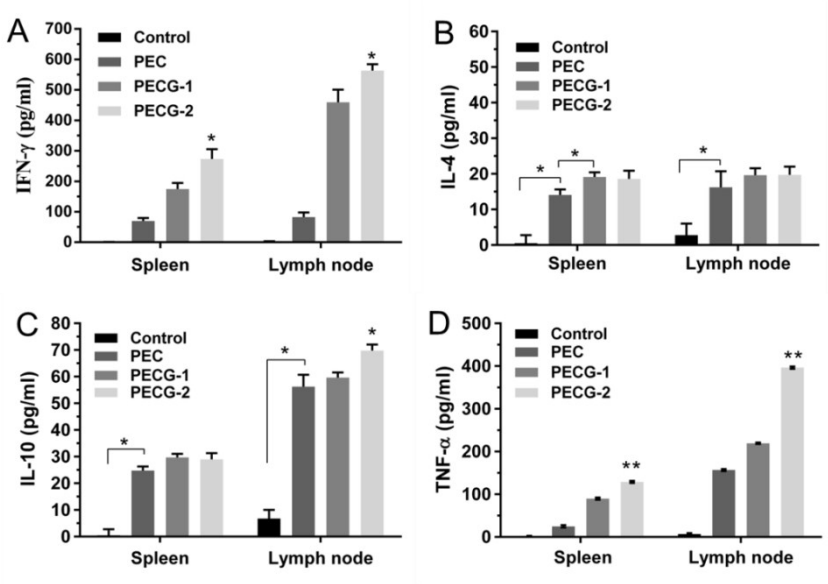


Fig. S10 The production of cytokines in the spleen and draining lymph nodes after the immunization with diverse OVA/NPs mixture formulations in mice. Data are shown as mean ± SDs (n=5).