Supporting Information

Development of a whole cell vaccine to activate dendritic cells using polyvalent CpG

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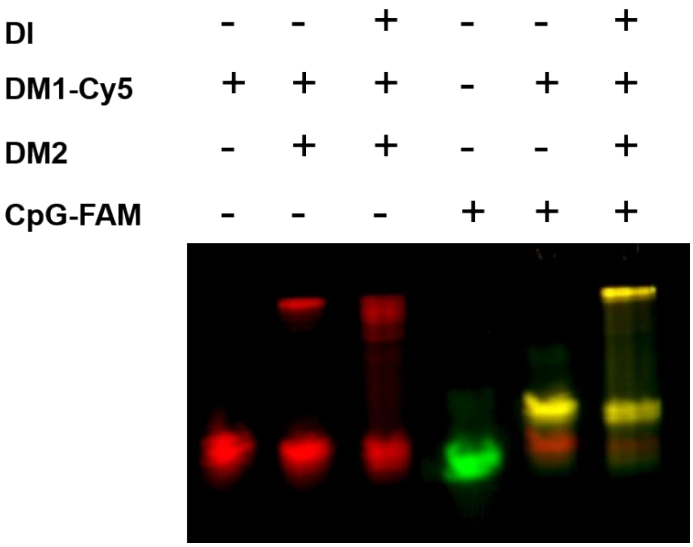

Fig. S1 Gel electrophoresis image showing the formation of DNA polymer with/without CpG loading.

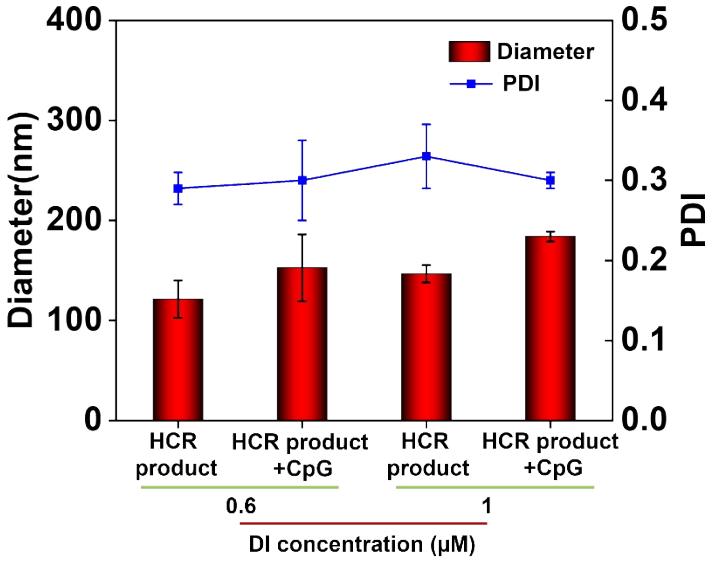

Fig. S2 Particle sizes and PDI of HCR products with/without addition of CpG.

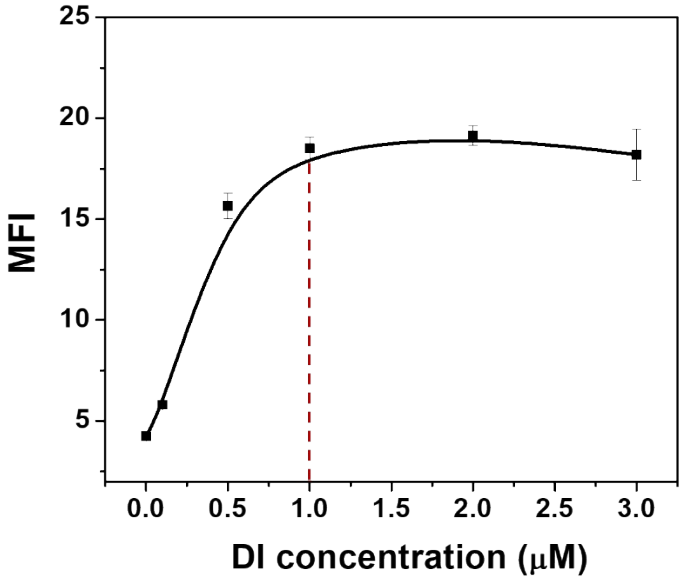

Fig. S3 Variation in the mean fluorescence intensity of 4T1 cells as a function of the DI concentration.

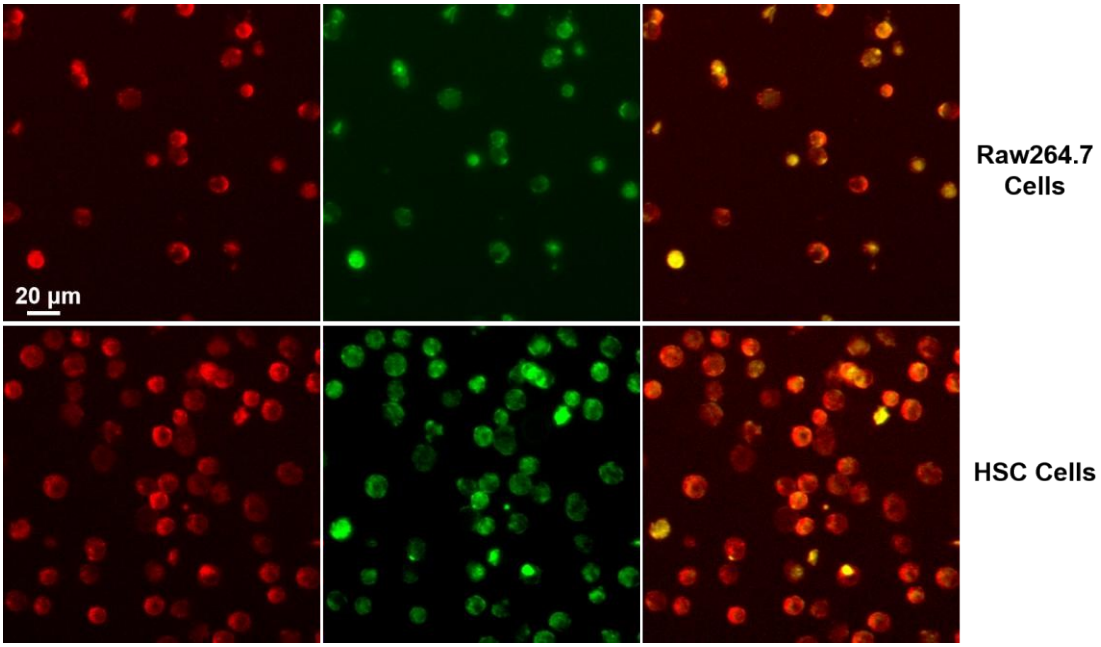

Fig. S4 Microphotographs of VPCpG-engineering Raw264.7 cells and HSC cells.

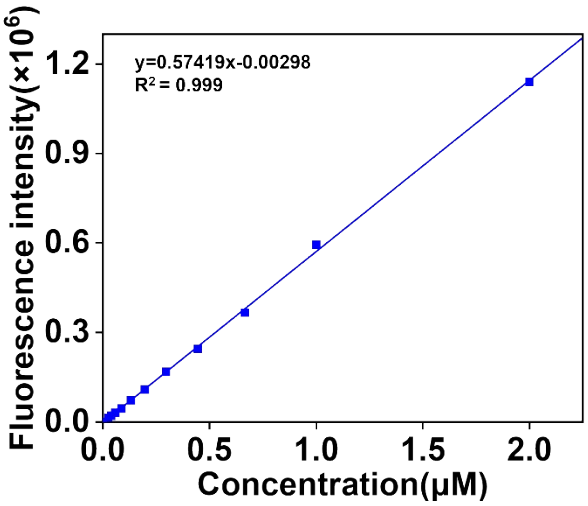

Fig. S5 Relationship between fluorescence intensity and CpG concentration (0-2 μM).

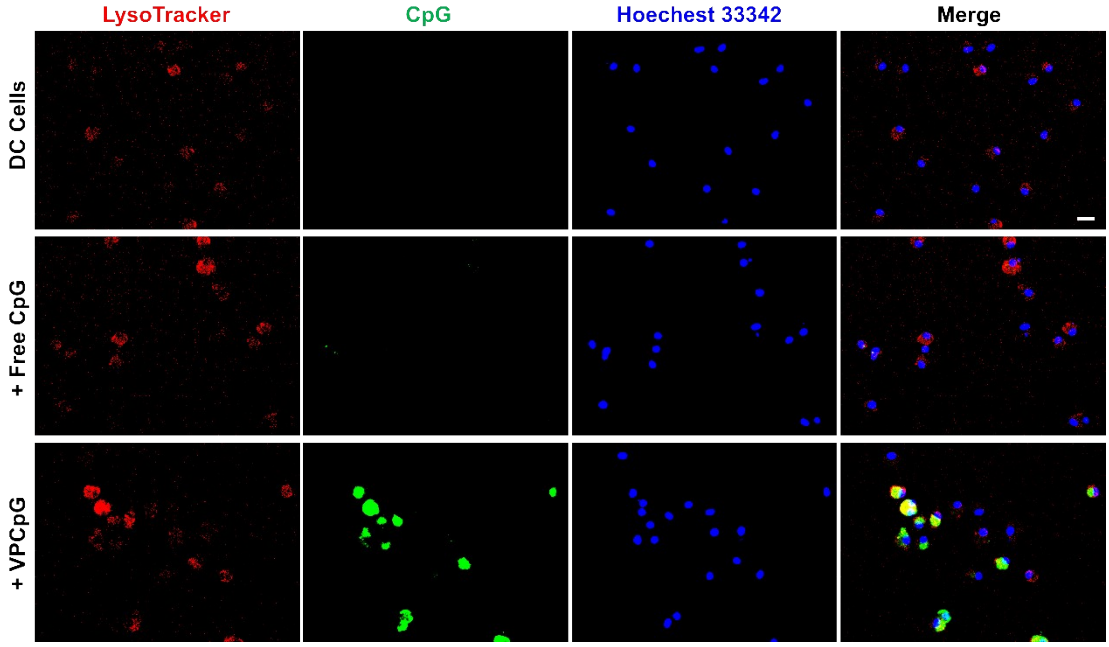

Fig. S6 Localization of green fluorescence CpG (both free CpG and VPCpG) in DC cells. Lysosomes and cell nuclei were stained with LysoTracker Red and Hoechst, respectively. The colocalization of CpG and lysosomes was visualized as yellow fluorescence. Scale bar, 20 μm.

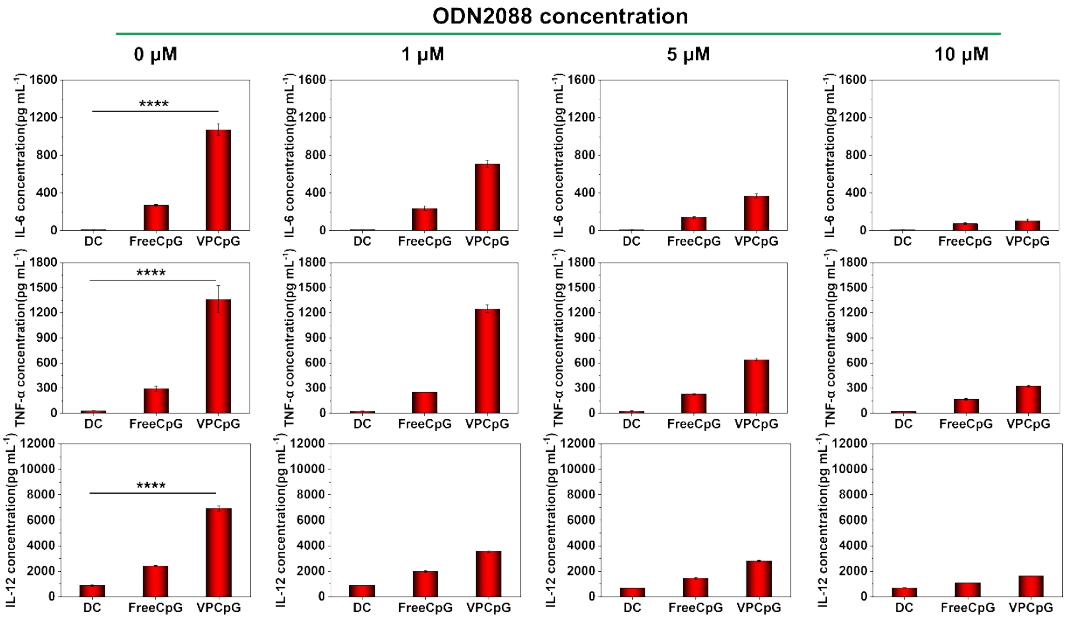

Fig. S7 Production of IL-6, IL-12 and TNF-α (detected by ELISA) in culture media in response to DCs stimulated by exposure to the various conditions before and after addition of ODN2088 at different concentrations. Value represents mean and s.d. (n = 3). Difference between groups were statistically significant. Data were analyzed using Student's t-test (****$p < 0.0001$ *versus* DC group).

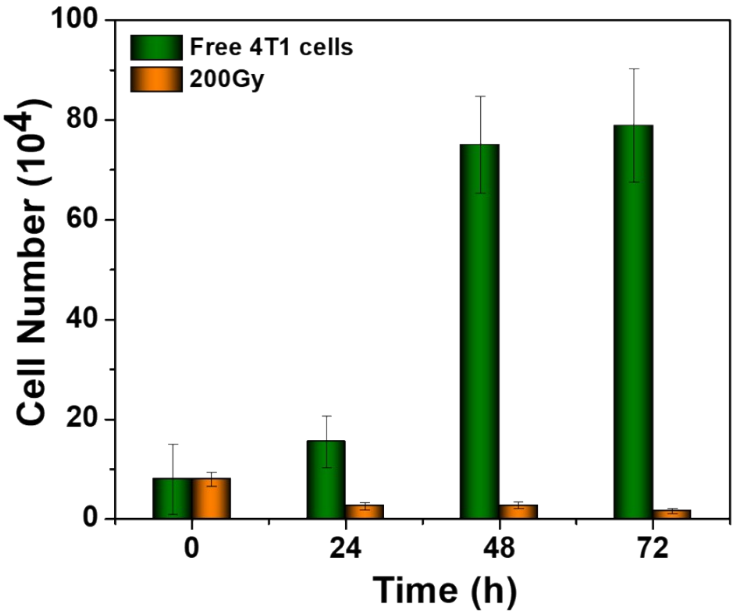

Fig. S8 *In vitro* proliferation assay of 4T1 cells and irradiated 4T1 cells (200 Gy) as a function of time.

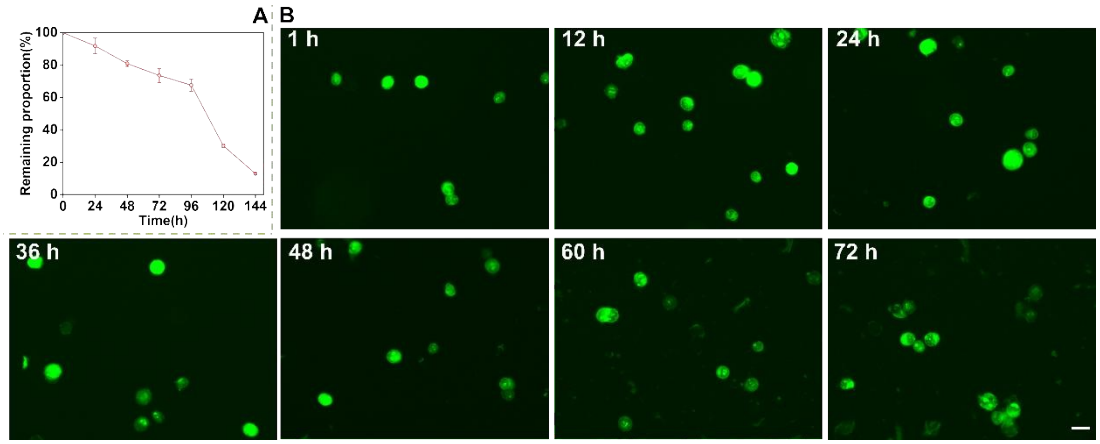

Fig. S9 A) The curve of the fluorescence intensity on the surface of vaccine over time; B) The fluorescence intensity of CpG on the surface of vaccine changes over time. Scale bar, 20 μm.

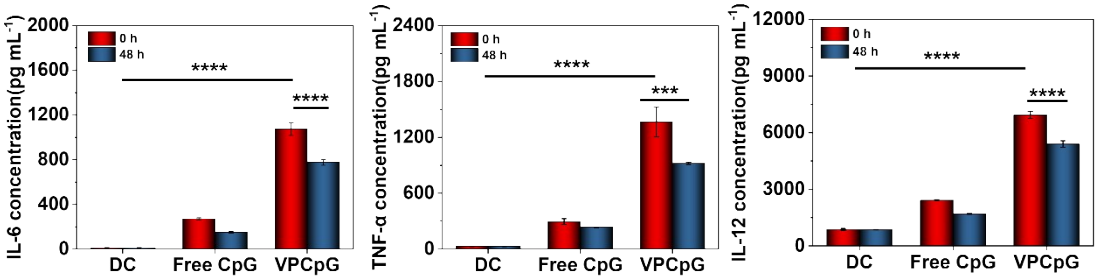

Fig. S10 Production of IL-6, TNF-α and IL-12 (detected by ELISA) in culture media in response to DCs stimulated by exposure to the various conditions. The Free CpG and VPCpG was stored in DMEM medium containing 10% FBS at room temperature for 2 days. Value represents mean and s.d. (n = 3). Difference between groups were statistically significant. Data were analyzed using Student's t-test (***$p < 0.001$ and ****$p < 0.0001$).

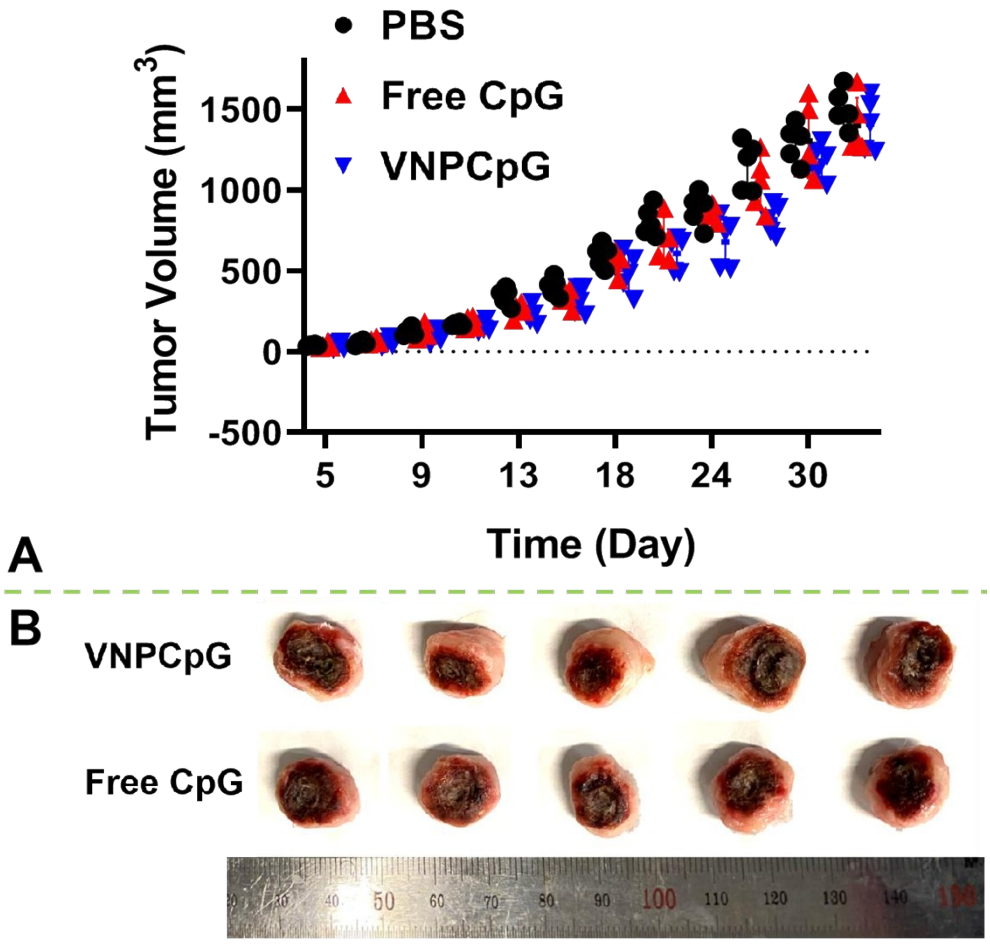

Fig. S11 A) Tumor volume of the mice treated with PBS, VNPCpG and Free CpG. (n = 5); B) Images of tumors after different treatments. The CpG concentration was fixed at 1 μM.

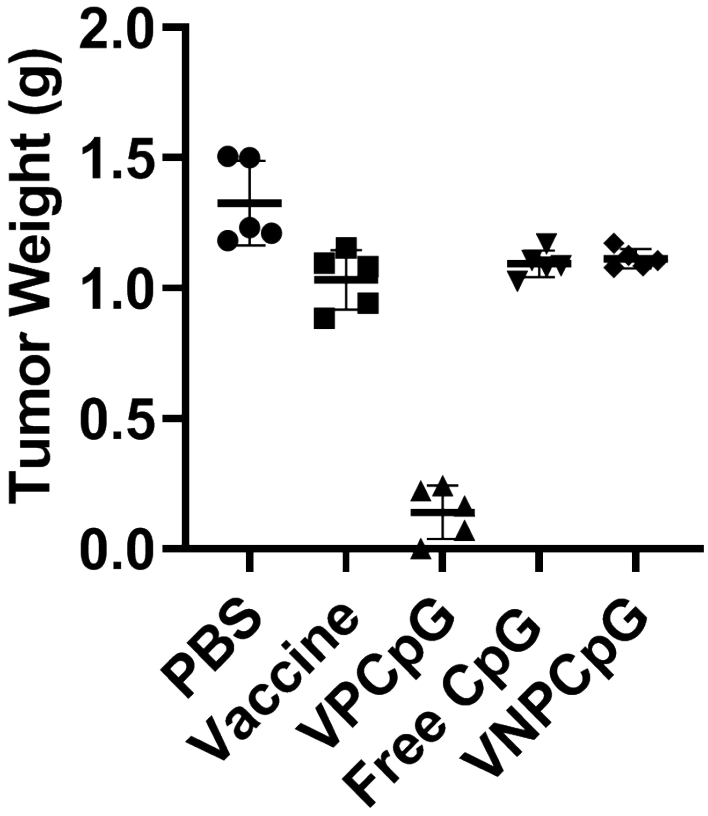

Fig. S12 Weight of tumors (n = 5).

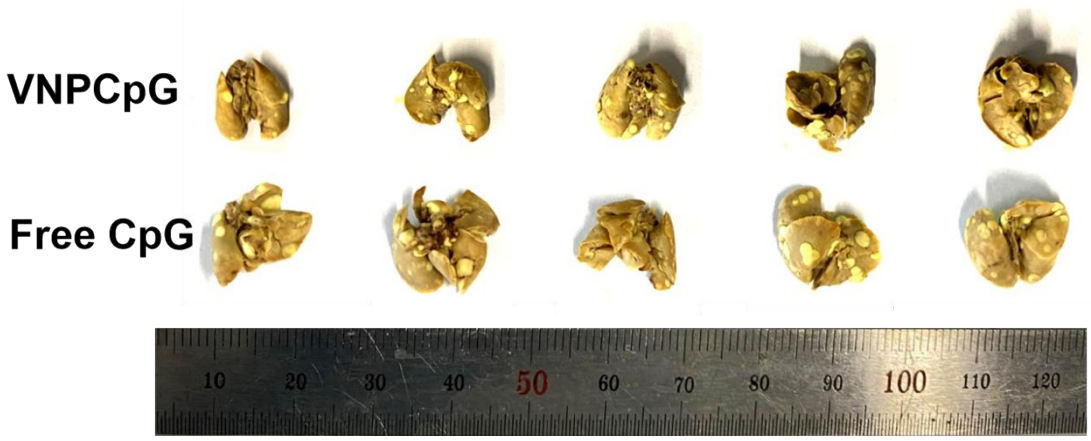

Fig. S13 Images of tumor nodules on the lung surface.

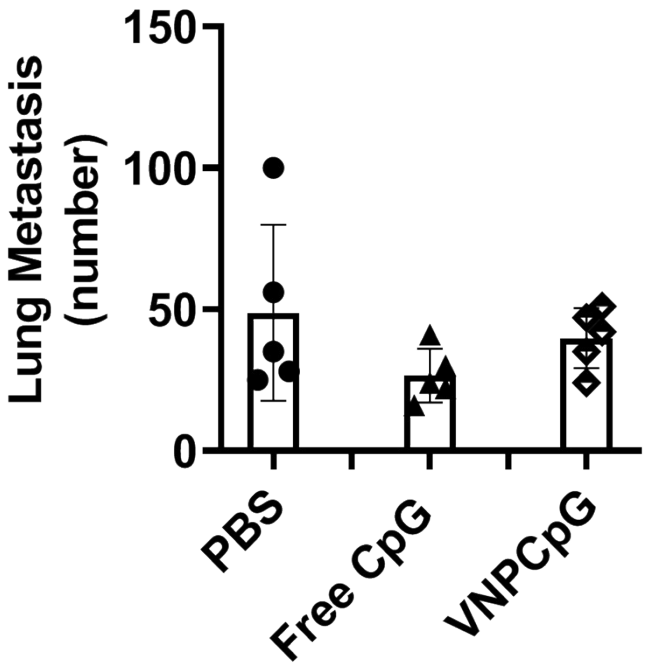

Fig. S14 Macroscopic tumor nodules on the lung surface (n = 5).

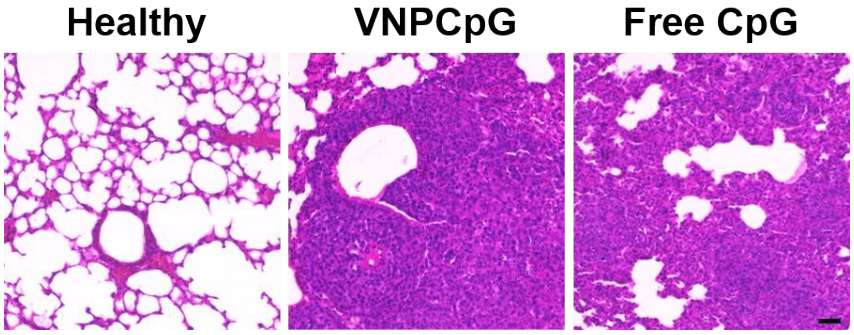

Fig. S15 Representative H&E sections of lung for the different groups on day 32 of tumor inoculation, bar represents 50 μm.

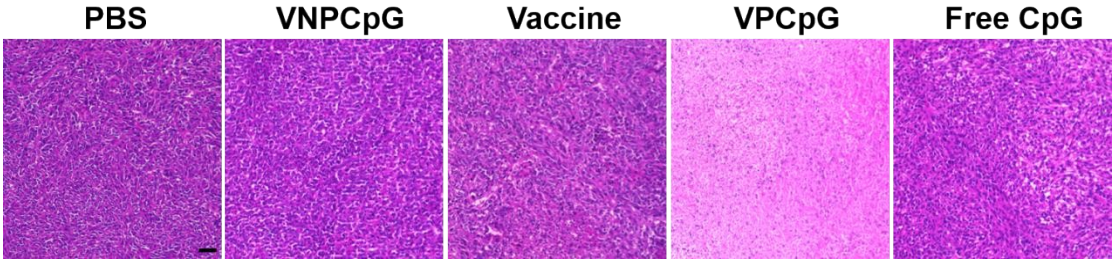

Fig. S16 Representative H&E sections of tumor for the different groups on day 32 of tumor inoculation, bar represents 50 μm.

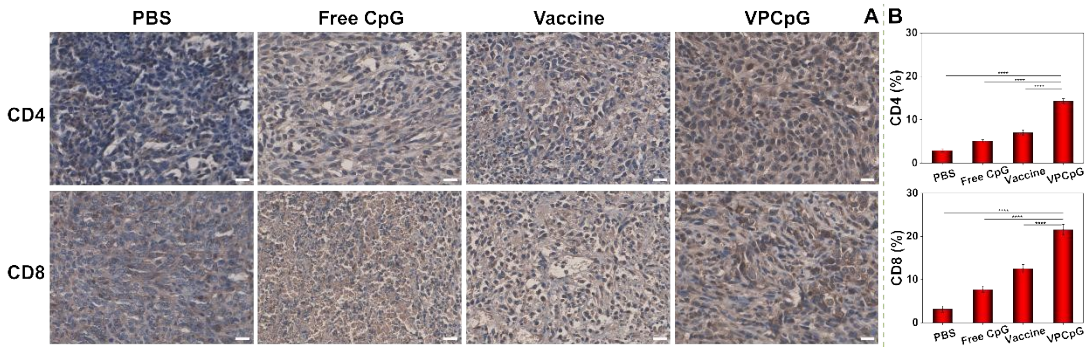

Fig. S17 A) Immunofluorescence staining of CD4 and CD8 in tumors from the PBS, Free CpG, vaccine and VPCpG treatment groups. Scale: 100 μm; B) Quantification analysis of CD4 and CD8 in tumors from the PBS, Free CpG, vaccine and VPCpG treatment groups was performed using Image J software (n = 3). Scale bar, 20 μm.

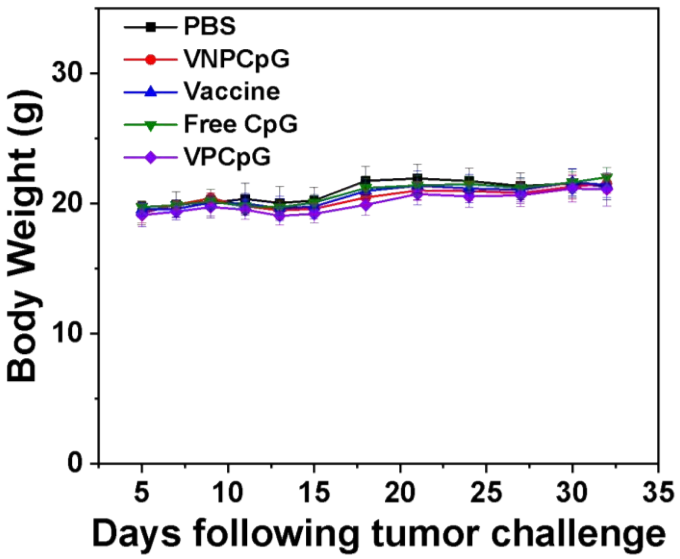

Fig. S18 Analysis of mice body weight during 32 days with different treatments.

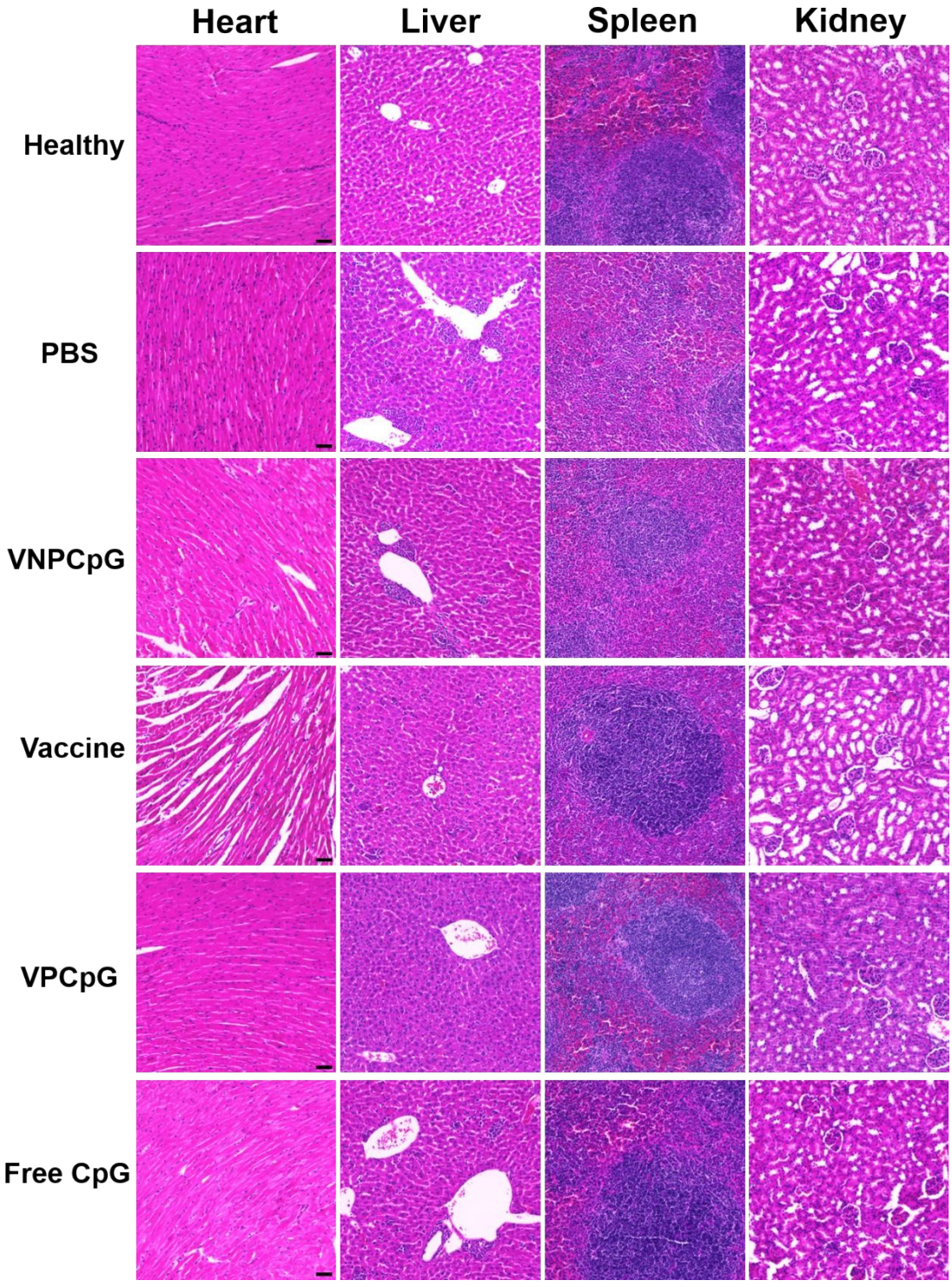

Fig. S19 HE staining of major organs collected on day 32 of tumor inoculation, bar represents 50 μm.

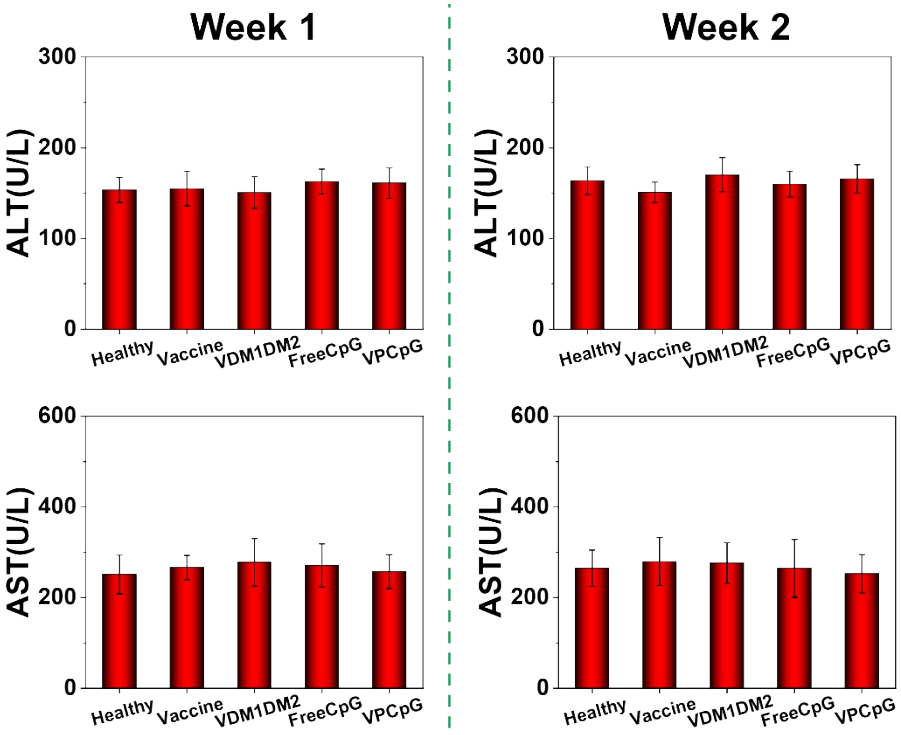

Fig. S20 Measurement of serum AST and ALT in healthy mice and the mice treated with vaccine, VDM1DM2, Free CpG and VPCpG nanosystem. (n = 3)

Table S1. DNA sequences.

DNA name	Sequence (5-->3)
Cholesterol-TEG-DI	CCTCATCCCACTCCTACCTAAACCAAAAAAAAAA/3CholTEG/
DM1	GGTTTAGGTAGGAGTGGGATGAGGCCAAATCCTCATCCCACT CCTACCCCAAATTTCCCTTATATTCTCTCTC
DM1-Cy5	GGTTTAGGTAGGAGTGGGATGAGGCCAAATCCTCATCCCACT CCTACCCCAAATTTCCCTTATATTCTCTCTC/3Cy5Sp/
DM2	AAAAACCTCATCCCACTCCTACCTAAACCGGTAGGAGTGGGA TGAGGATTTGG
CpG'	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*G*G*CCAAAGA GAGAGAATATAAGGGAAA
DM2	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*G*G*CCAAAGA GAGAGAATATAAGGGAAA/36-FAM/

Materials and methods

Materials and instrumentation. Oligonucleotides (Supplementary **Table S1**) were purchased form integrated DNA Technologies (Coralville, IA). Vybrant DiO/DiD celllabeling solution and Fluorescein isothiocyanate (FITC)-conjugated MHCII (11-532281) were supplied by Invitrogen (Carlsbad, CA). phycoerythrin (PE)-conjugated CD86
(MABF726); (FITC)-conjugated CD11(MABF530) were obtained from Sigma. RPMI (Roswell Park Memorial Institute) 1640, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS) were purchased from Thermo Fisher Scientific; Enzyme-linked immunosorbent assay (ELISA) kits were obtained from PeproTech (Rocky Hill, NJ). The murine mammary carcinoma cell line 4T1 were supplied by ATCC (Manassas, VA).

The gel electrophoresis was run for 50 min at 80 mV and a CRI Maestro EX System (Woburn, MA) was used to record the images of gels. Flow cytometry analysis was performed using a guava easyCyteTM flow cytometer (Millipore). Cell images were recorded using Olympus IX73 inverted microscope.

General cell culture conditions. The murine mammary carcinoma cell line (4T1, CRL-2539) was incubated in RPMI-1640 supplemented with 10% FBS in a humidified incubator with 5% CO_2 at 37 ^{o}C.

Quantitation of DI on the cell surface. To test the effect of DI concentration on the display of DI on the cell surface, 5×10^5 of cells were incubated with Cholesterol-TEGDI (0.1-3 μM) in DPBS for 30 min. After that, DI-modified cells were collected,

washed and subsequently stained with DM1-Cy5 (1 μM). The mean fluorescence intensity (MFI) of cells in each group was measured using a guava easyCyteTM flow cytometer.

Synthesis of polyvalent CpG (PCpG) in aqueous solution. Prior to use, all DNA monomers and CpG at desired concentration in DPBS were heated at 95 ^{o}C for 5 min. Then they were cooled at room temperature for 1 h to allow sequences to form energetically favorable secondary structures. Firstly, the DNA polymer scaffolds were prepared by mixing DI-Chol with DM1-Cy5 and DM2 at room temperature for 3 h. Next, the CpG-FAM solution was added and incubated at room temperature for another

45 min to allow the hybridization of CpG with the toeholds of each DM1. Synthesis of PCpG was characterized by gel electrophoresis. The gel electrophoresis was run at 80 mV for 50 min and a CRI Maestro EX System (Woburn, MA) was used to record the images of gels. The hydrodynamic size and polydispersity index (PDI) of DNA polymer scaffolds with/without CpG loading were determined using Dynamic Light Scattering (DLS) (Malvern Instrument, Nano-ZSZEN3600) at 25 ^{o}C. The data were presented as mean ± standard deviation (SD) based on three independent measurements.

Polyvalent engineering of 4T1 cells and the synthesis of polyvalent CpG modified vaccine (VPCpG). To immobilize DNA initiators on the cell surface, 1×10^6 of 4T1 cells were incubated with DI-Chol for 30 min in 400 μL of reaction buffer solution (1 μM). DI-functionalized cells were collected, washed and subsequently mixed with 400 μL of DNA monomers (each at 1 μM, reaction buffer) for 3 h to form the DNA polymer scaffolds. To hybridized the CpG with DNA scaffolds to form PCpG, the scaffoldsfunctionalized cells were incubated in a CpG solution (1 μM) for 0.5 h. Finally, the cells modified with PCpG were collected by centrifugation and washed three times with reaction, then detected with a fluorescence microscope and a flow cytometer. As a control, DI-functionalized cells were incubated in sequential solutions of DM1 and CpG to generate monovalent CpG (MCpG) modified cells.

To generate the PCpG modified vaccine (VPCpG) and the MCpG modified vaccine (VMCpG), the 4T1 cells were irradiated by X-Ray (200 Gy). The whole cell vaccine was synthesized by irradiating the pure 4T1 cells with X-Ray (200 Gy).

***In vitro* proliferation assay.** The 4T1 cells were irradiated at 200 Gy using an X-Ray irradiator to form the whole cell vaccine. The 4T1 Cells or whole cell vaccine were then plated on a 96-well plate with a density of 7×10^4 and incubated for 24 h, 48 h or 72 h at 37°C in a humidified incubator with 5% CO_2. At each time point, the cells were collected and the number was accounted using the Olympus IX73 inverted microscope. Data were expressed as mean ± standard deviation (SD) based on three independent measurements.

Generation of BMDCs. Bone marrow-derived DCs (BMDCs) were generated from hindlimb bones isolated from murine. The hindlimb bones were placed in 70% ethanol for 2 min and then washed in DPBS solution. Then the both distal bone ends were excised and the marrow was flushed with DPBS using a Syringe with a 27 G needle. The vigorous pipetting was used to disintegrate the Clusters within the marrow suspension. The red blood cells were lysed with ACK Lysing Buffer (gibco). The remaining cells were centrifuged for 10 min at 300 g. One million of cells in 10 mL of complete culture medium containing 20 ng/mL granulocyte-macrophage colonystimulating factor (GM-CSF) were seeded in a 60 mm sterile petri dish. At day 3, 6 and 8, half of the culture medium from the plate was harvested and replaced by half of fresh medium containing GM-CSF (20 ng mL^{-1}). After 7 days, nonadherent and loosely adherent cells were collected and used as the source of immature DCs. At day 10, the adherent cells were used as DCs in the subsequent experiments.

***In vitro* internalization of polyvalent CpG modified vaccine (VPCpG) by DCs.** 4T1 cells were irradiated with X-Ray irradiator (200Gy), then the irradiated 4T1 cells were modified with PCPG and MCpG. After that, the vaccines were labeled with a green

fluorescent dye (DiO) and the mature DCs were collected and labeled with red fluorescent dye (DiD) according to the manufacturer's instruction. Then the dye-labeled DCs (5×10^5 cells) were cultured with the equal numbers of different dye-labeled vaccine at 37 ^oC for 24 h. After incubation, the cells were washed 3 times with 500 μL of DPBS. The internalization of green fluorescent vaccine by fluorescent DCs was evaluated by the Olympus IX73 inverted microscope or a flow cytometer.

To verify the targeting of CpG to TLR9, the vaccines were labeled with a green fluorescent dye (DiO) according to the manufacturer's instruction and co-cultured with equal numbers of mature DCs (5×10^5 cells) for 24 h at 37 ^oC. Following incubation, the cells were washed three times with 500 μL of PBS. Next, LysoTracker Red (1 μM) in DMEM was added to the dishes and incubated for 20 minutes. The medium was then removed and the cells were washed for 3 times. Subsequently, Hoechst 33342 (5 μg/mL) in DMEM was added to the dishes and cultured for an additional 15 minutes. After removing the cluture medium and washing the cells three times with PBS, subcellular imaging was performed using an inverted fluorescence microscope (Invitrogen, EVOS
M5000).

In vitro **DCs activation assays.** To evaluate the activation of DCs exposed to polyvalent CpG modified vaccine (VPCpG), monovalent CpG modified vaccine (VMCpG) and vaccine. The DCs were co-cultured with VPCpG, VMCpG and vaccine separately in complete culture medium (RPMI 1640 medium supplemented with 10% FBS) for 2 days in a 6-well plate for a 1:1 (DCs *versus* vaccine) ratio. Free CpG and DCs cultured in the absence of vaccine were used as a control. The concentration of CpG was fixed at 1 μM. The maturation of DCs was evaluated by flow cytometry using the following antibody conjugated to fluorescent markers (2.5 μg/mL) for surface staining. To further investigate the role of CpG in TLR9 activation, 1-10 μM of ODN2088 (a TLR9 inhibitor) was added to DCs 1 h prior to Free CpG or VPCpG treatment, and the DCs were co-incubated with Free CpG or VPCpG for 2 days.

Supernatants were subsequently collected and the levels of IL-6, IL-12 and TNF-α were then analyzed with ELISA (PeproTech) according to the manufacturer's instructions.

Characterization of the *in vitro* stability of polyvalent CpG modified vaccine (VPCpG). To evaluate the structural stability of VPCpG, FAM-labeled CpG was introduced into the DNA polymer scaffold and stored in DMEM containg 10% FBS. At specified time intervals, VPCpG samples were collected via centrifugation, and the fluorescence intensity of the pellet was measured using a microplate reader (Synergy H1, BioTek). Furthermore, the fluorescence signal intensity on the surface of vaccine was visualized using an inverted fluorescence microscope (Invitrogen, EVOS M5000). To further assess the biological activity of CpG after storage for 2 days, the stored VPCpG was co-cultured with DCs for an additional 2 days in a 6-well plate. The supernatants were then collected, and the concentrations of cytokines (IL-6, IL-12 and TNF-α) were quantified using ELISA according to the manufacturer's instructions.

***In vivo* dendritic cells recruitment and T cells activation.** All animal experiment protocols were approved by the Animal Ethical and Welfare Committee. DPBS, whole cell cancer vaccine and polyvalent CpG modified vaccine (1×10^6) were injected subcutaneously into the left flank every 7 days, two times. At 14 day, the BALB/c mice were inoculated subcutaneously with 1×10^5 of 4T1 cells in the right flank. At 21 days of tumor challenging, mice were sacrificed and inguinal lymph nodes (LN) were isolated. Then a single cell suspension was collected from the dissected LN in ice-cold PBS, smashed through 70 μm cell strainers, washed with PBS and stained with the following primary labeled monoclonal antibodies: FITC-conjugated CD11c, FITCconjugated CD8a (CD8 T cells), FITC-conjugated CD3. The labelled cells were analyzed using flow cytometry. Immunohistochemical analysis was performed on tumor tissue sections to analyze the phenotype of T cells ($CD4^+$, $CD8^+$) in the tumor tissues.

***In vivo* antitumor effect.** The BALB/c mice were randomly assigned to 5 groups. PBS, whole cell cancer vaccine (vaccine), polyvalent CpG modified vaccine (VPCpG), CpG unloaded vaccine (VNPCpG) (1×10^6) and Free CpG were injected subcutaneously into the left flank every 7 days, two times. The concentration of CpG was fixed at 1 μM. At 14 day, the BALB/c mice were inoculated subcutaneously with 2×10^5 of 4T1 cells in the right flank. Untreated BALB/c mice served as controls. After 5 days, the tumor size was monitored every day or every two days. The tumor volume was defined as $V = W^2L/2$, where the W and L refered to the shortest and longest diameters respectively. During the entire process, mice body weight were recorded. All mice were euthanized at day 32, the organs were collected and fixed by 4% formalin or Bouin's solution. After that, the tissues were paraffin and sectioned for histological examinations by hematoxylin and eosin (H&E) staining. The stained slices were imaged with inverted microscope (Invitrogen，EVOS M5000) at 10 magnifications.

Statistical analysis. All data were expressed as the mean ± standard deviation (S.D.) (n = 3~5). Student's t-test was used to evaluated the statistical significance between different groups. $p<0.05$ is statistically significant, which was indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.