Supporting information

Immunogenic Cell Death Amplified by Co-localized Adjuvant Delivery for Cancer Immunotherapy

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Experimental Section

Synthesis of the maleimide-modified cationic lipid: Maleimide-modified N-(4-carboxybenzyl)-N,N-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOBAQ) was synthesized by EDC/NHS chemistry. In brief, DOBAQ (Avanti Polar Lipids), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, ThermoFisher Scientific), and N-Hydroxysuccinimide (NHS, Sigma-Aldrich) were dissolved in dichloromethane at a molar ratio of 1 : 1.5 : 1.5 and reacted for 0.5 h at room temperature, followed by addition of N-(2-Aminoethyl)maleimide hydrochloride (MAL, 1.2 molar fold to DOBAQ, TCI America) and adjustment of pH to 8-9 with triethylamine (Sigma-Aldrich). The reaction was kept for another 24 h and monitored by thin layer chromatography (TLC) using developing media composed of chloroform : methanol : acetonitrile : water = 70 : 21 : 5 : 4 (v/v/v/v) and colorization by iodine gas. Maleimide-modified DOBAQ (DOBAQ-MAL) was purified by multiple washes through 0.1 M hydrochloride solution, saturated sodium chloride solution, and acetonitrile. Reaction rate was quantified by high performance liquid chromatography (HPLC, SHIMADZU) using a diphenyl column (4.6 mm ID × 250 mm, GRACE) with elution phase composed of water : methanol : acetonitrile : trifluoroacetic acid = 34.98 : 32.48 : 32.48 : 0.05 (v/v/v/v), and detection wavelength at 220 nm. The molecular structure of DOBAQ-MAL was determined by ^{1}H-NMR (Varian, USA) using d^{6}-DMSO (Sigma-Aldrich) as the solvent.

Synthesis and characterization of lipid-polymer cross-linked nano-depots: Lipid-polymer cross-linked nanoparticles were synthesized using cationic lipids and thiolated hyaluronic acid (HA-SH). Briefly, 0.63 μmol of lipids including 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and DOBAQ-MAL (50:25:25, m/m/m) were dissolved in chloroform and dried under vacuum to form a lipid film, which was then hydrated with PBS and sonicated to produce unilamellar liposomes. One hundred μg of HA-SH and 25 μg of CpG were added to liposomes, followed by incubation for one hour at 37 ^{o}C with constant shaking to promote complexation and cross-linking between lipids and HA-SH. NPs were centrifuged (20817 ×g, 5 min) and washed three times with PBS, resuspended in PBS, dispersed by brief sonication, and stored at 4 ^{o}C until use. In some cases, portions of DOPC were replaced by fluorophore-labeled lipids, including TopFluor PC or Liss Rhod PE (both from Avanti Polar Lipids) to label NPs. Size distribution and zeta potential of NPs were measured by dynamic laser scattering (Zetasizer Nano ZSP, Malvern, UK). NPs were visualized by a transmission electron microscope (JEOL 1400 Plus, USA) with negative staining using uranyl acetate. The amount of CpG encapsulated in NPs was measured by absorbance at 260 nm using a plate reader (Synergy Neo, BioTek, USA). Reactive maleimide groups on the surfaces of NPs were measured by labeling maleimide with a fluorescent probe. In brief, Bodipy FL L-cystine (ThermoFisher Scientific) was reduce by 20 molar fold excess of tris(2-carboxyethyl)phosphine (TCEP, ThermoFisher Scientific) for two hours at 37 ^{o}C, then incubated with NPs for another 0.5 h at 37 ^{o}C, followed by removal of excess dye using a desalting column (MWCO 7 kD, ThermoFisher Scientific). The same amount of NPs were pre-incubated with an excess

amount of L-cysteine (Sigma-Aldrich) for 0.5 h at 37 ^{o}C to block reactive maleimide and served as the control. Fluorescence intensity at 488/520 nm was measured by a plate reader and converted to the number of reactive maleimide groups, while the number of NPs was calculated from particle concentration measured by nanoparticle tracking analysis (NTA, NanoSight, Malvern, USA).[1] The multilamellar structure of NPs was determined by a lamellarity assay,[2] using unilamellar liposomes as the control.

Tumor cell culture and elicitation of immunogenic cell death by chemo-treatment: Murine melanoma B16F10, B16F10 expressing ovalbumin (B16F10OVA, both were provided by Dr. Darrell Irvine, MIT), and murine colon carcinoma CT26 (ATCC) were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 ^{o}C under 5% CO_2. Cells were tested free from mycoplasma. Tumor cells were treated by 10 μM mitoxantrone (Mit) for 12 h to induce immunogenic cell death. For determination of cell death and ICD, 10^5 tumor cells were seeded into a 12-well plate, followed by chemo-treatment described above, and measurements of cell death and cellular release of high mobility group box 1 (HMGB1) protein by Annexin V / PI staining using a flow cytometer (Cyan 5, Beckman Coulter, USA), and an ELISA kit (IBL International), respectively. Sulfhydryl groups expressed in cell-surface proteins were stained by Oregon Green-labeled maleimide (ThermoFisher Scientific). In brief, live or Mit-treated B16F10 cells were incubated with PBS or 1 mM TCEP for 0.5 h at room temperature with constant shaking, followed by washing and labeling with 1 μg/ml of Oregon Green-labeled maleimide for 15 min at room temperature. For maleimide blocking of the dye, 1 μg/ml of Oregon Green-maleimide was incubated with 100 molar

fold excess of L-cysteine for 1 h at room temperature, then used for staining. Cells were washed, resuspended in 2 μg/ml DAPI solution, and analyzed for fluorescence intensity of Oregon Green among the DAPI-negative population by flow cytometry.

Conjugation of CpG-loaded NPs (CpG-NPs) on the surfaces of dying tumor cells: Various amounts of CpG-NPs were incubated with 10^6 Mit- and TCEP-treated tumor cells suspended in 1 ml PBS for 12 h at 4 ^{o}C with constant shaking, followed by two washes with PBS using centrifuge (1500 ×g, 5 min), then resuspended in PBS and used freshly. The number of NPs associated per cell was measured using TopFluor PC-labeled NPs and calculated with particle concentration measured by NTA. Oregon Green-labeled dying tumor cells conjugated with Liss Rhod PE-labeled NPs on their surfaces were visualized by a confocal microscope (Nikon A1, Japan). The fraction of NPs on cell surfaces was quantified by a fluorescence quenching experiment using 0.25 mg/ml trypan blue, a membrane impermeable dye that can specifically quench cell surface-bound fluorescence, and calculated as $[1 – (F_3 – F_1) / (F_2 – F_1)] \times 100\%$, where F_1, F_2, and F_3 were geometric mean fluorescence intensity (MFI) of cells without particle conjugation, cells with particle conjugation but without quenching, and cells with particle conjugation and quenching, respectively.

Migration of dendritic cells to immunogenically dying tumor cells in vitro: Murine bone marrow-derived dendritic cells (BMDCs) were prepared as reported previously.[3] Migration of BMDCs was measured with the Transwell system (5 μm pore size, Costar 3421, Corning). Briefly, 2×10^5 live, Mit-treated B16F10 cells (Mit-B16F10), or Mit-

B16F10 conjugated with CpG-NPs (Mit-B16F10-CpG-NPs) were seeded into the lower chamber and cultured for 2 h for cell adherence, followed by seeding of 5×10^5 CFSE-labeled BMDCs into the upper chamber, and cell culture for another 12 h. Culture media without tumor cells in the lower chamber served as the negative control. DCs that migrated to the lower chamber were collected, and $CFSE^+DAPI^-$ DCs were counted by flow cytometry.

Uptake and cross-presentation of antigens by BMDCs: BMDCs were seeded into a 24-well plate at a density of 2×10^5 cells / well, followed by seeding of 6×10^5 Oregon Green-labeled, live or Mit-treated B16F10OVA cells with or without CpG, and co-culture for 24 h. DCs alone served as the negative control. Cells were collected, stained with a PE-labeled 25-D1.16 monoclonal antibody directed against SIINFEKL-H-$2K^b$ complexes (eBioscience 12-5743) and a PECy7-labeled anti-CD11c antibody (BD 558079), and analyzed for the percent of dye positive within the DC population, and MFI of PE within DCs engulfing tumor antigens by flow cytometry.

Activation of BMDCs: BMDCs were seeded into a 12-well plate at a density of 5×10^5 cells / well, followed by seeding of 10^5 live or Mit-treated B16F10OVA cells with or without CpG, and co-culture for 24 h. DCs alone cultured in media was the negative control. Co-culture supernatant was collected and analyzed for DC secretion of inflammatory cytokines, including IL-12p70, TNF-α, and IFN-β by ELISA kits (R&D Systems DY419, DY410, and Biolegend 79838, respectively). Cells were collected and

measured for expression of maturation markers, including CD86 (eBioscience 12-0862) and CD40 (eBioscience 12-0401) on DCs by flow cytometry.

In vivo immunization and cancer immunotherapeutic studies: All animal experiments were performed under approval from Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Female, 6 week-old C57BL/6 mice (Envigo) were subcutaneously (in two sides of the tail base) immunized with PBS, Mit-treated B16F10OVA cells with or without CpG on day 0, followed by assessment of SIINFEKL-specific $CD8\alpha^+$ T cells on day 7 by the tetramer staining assay. The single vaccine dose per mouse was composed of 4×10^6 Mit-treated tumor cells with or without 382 ng CpG. Mice were subcutaneously (in one side of flank) inoculated with 10^5 live B16F10OVA cells on day 8, and monitored for tumor initiation. In a separate experiment, ~$5x10^5$ splenocytes from immunized mice were collected on day 8 and co-cultured with 10^5 live B16F10OVA cells for 20 h in the presence of Brefeldin A, followed by staining with $CD8\alpha^+$ (BD 553035) and CD4 (BD 552775), fixation and permeabilization with 4% paraformaldehyde, and staining with IFN-γ (BD 562020). Percentages of IFN-γ^+ among $CD8\alpha^+$ and $CD4^+$ splenocytes were quantified by flow cytometry.

For therapeutic studies using the CT26 model, female, 6 week-old BALB/c mice (Envigo) were subcutaneously inoculated with 2×10^5 CT26 cells on day 0, followed by a single subcutaneous vaccine dose on day 4 with Mit-treated CT26 cells (Mit-CT26), Mit-CT26 conjugated with CpG-loaded NPs (Mit-CT26-CpG-NPs), Mit-CT26 admixed with CpG-loaded NPs, or Mit-CT26 admixed with soluble CpG. The single vaccine dose per mouse was composed of 10^6 Mit-CT26 with or without 117 ng CpG. For combination

therapy against CT26 tumors, mice were vaccinated with Mit-CT26-CpG-NPs as above, followed by intraperitoneal administration of an anti-PD1 IgG antibody (BioXcell BP0146, clone: RMP1-14; 100 μg / mouse) on days 5, 8, 11, 14, 17, 20, 23, and 26. Length and width of tumors were measured every two days starting day 6, and tumor volumes were calculated as [0.5 × length × width^2]. Mice were euthanized if the length of tumor > 1.5 cm, or tumors became ulcerated, according to requirements by IACUC. Mice cured by the combination therapy were re-challenged with 2×10^5 CT26 cells in the contralateral flank on day 70, and monitored for tumor initiation.

Statistical analysis: Data are presented as representative or compiled results obtained from two to three independent experiments. Animal experiments were performed after randomization. Data were analyzed by one- or two-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* tests for comparison of multiple groups or log-rank (Mantel–Cox) test using the GraphPad Prism 5.0 software. *P* values < 0.05 are considered statistically significant. All values are reported as means ± SEM with indicated sample size.

References

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[2] P. Girard, J. Pecreaux, G. Lenoir, P. Falson, J. L. Rigaud, P. Bassereau, *Biophys J* **2004**, 87, 419.
[3] M. B. Lutz, N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, G. Schuler, *J Immunol Methods* **1999**, 223, 77.

Supporting figures and tables

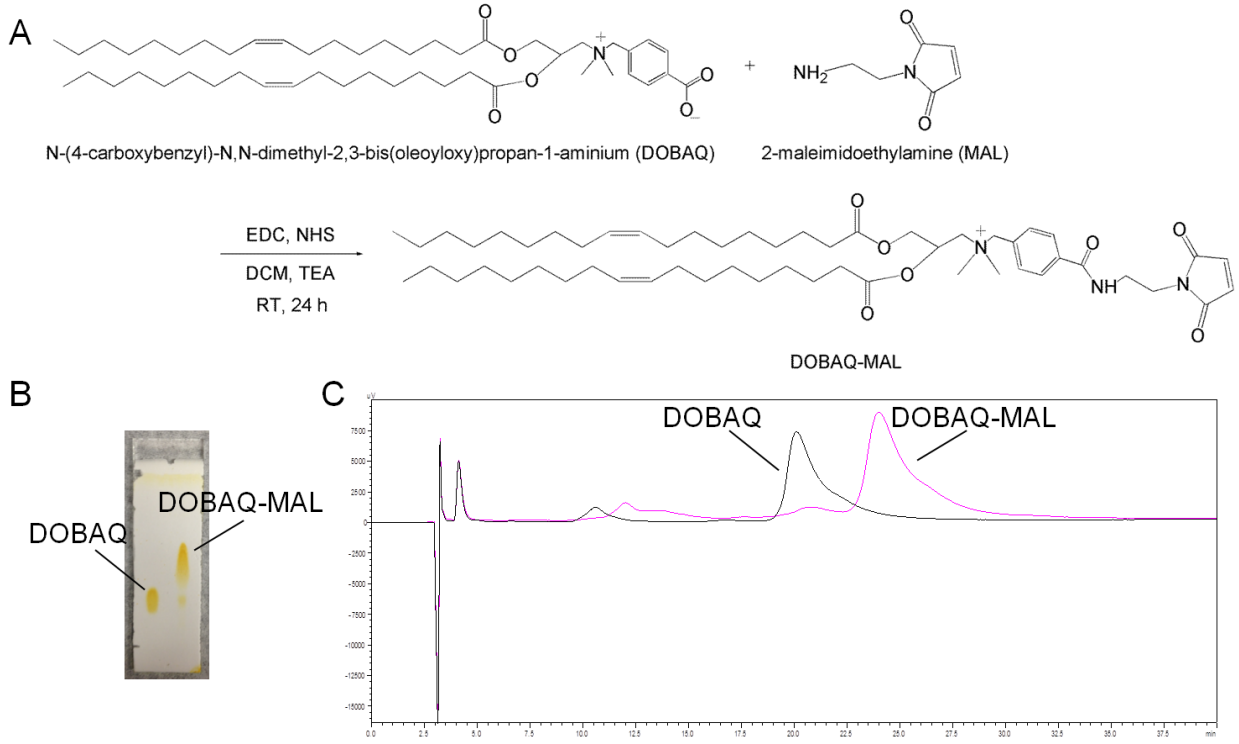


Figure S1. Synthesis of DOBAQ-MAL. (A) Synthesis scheme. DOBAQ was modified with maleimide via EDC/NHS chemistry. (B) Thin layer chromatography (TLC) and (C) high performance liquid chromatography (HPLC) show successful modification of maleimide to DOBAQ, with reaction efficiency = 95.0 ± 1 % (mean ± SEM, *n* = 3) calculated by peak areas from HPLC results.

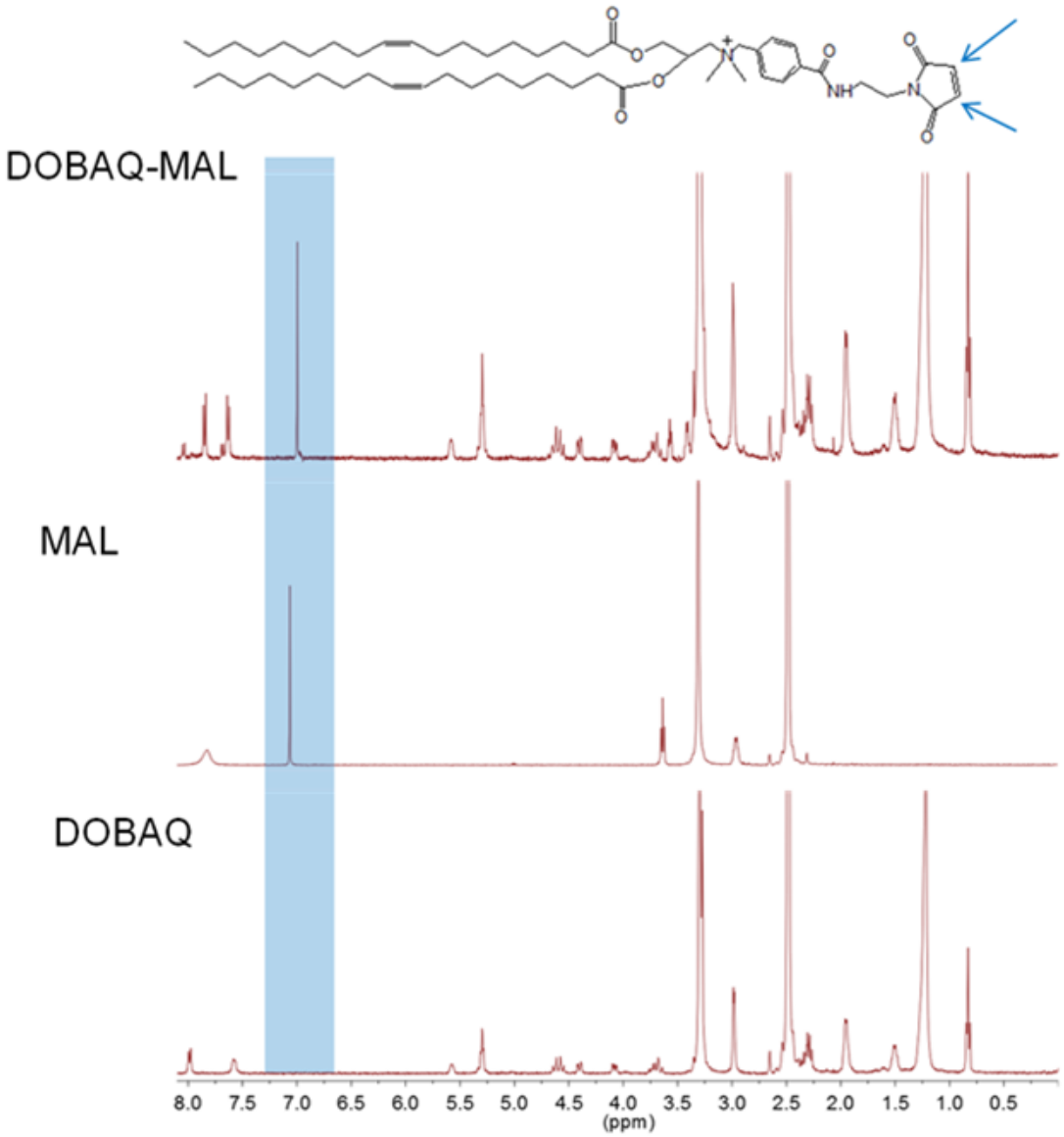


Figure S2. ^{1}H-NMR identification of DOBAQ-MAL, MAL, and DOBAQ. The shaded area indicates the characteristic peak of hydrogen atoms in the maleimide group.

Table S1. Characterization of CpG-loaded NPs. The data show mean ± SEM, *n* = 3.

Particles	Z-average (nm)	PDI	Zeta potential (mV)	% Encapsulation efficiency of CpG	Reactive maleimide on NP surfaces (ave. # x10^3 molecules / particle)
Unilamellar liposomes	108 ± 4	0.234 ± 0.02	20.3 ± 0.6	N.A.	N.A.
Blank NPs without CpG	254 ± 10	0.222 ± 0.03	-16.4 ± 0.4	N.A.	2.19 ± 0.3
CpG-loaded NPs	290 ± 10	0.177 ± 0.02	-17.7 ± 0.5	81.2 ± 2	2.34 ± 0.4

Table S2. Lamellarity of liposomes and CpG-loaded NPs. The data show mean ± SEM, *n* = 3.

Particles	Unilamellar liposomes	CpG-loaded NPs
Lamellarity	0.45 ± 0.04	0.28 ± 0.02

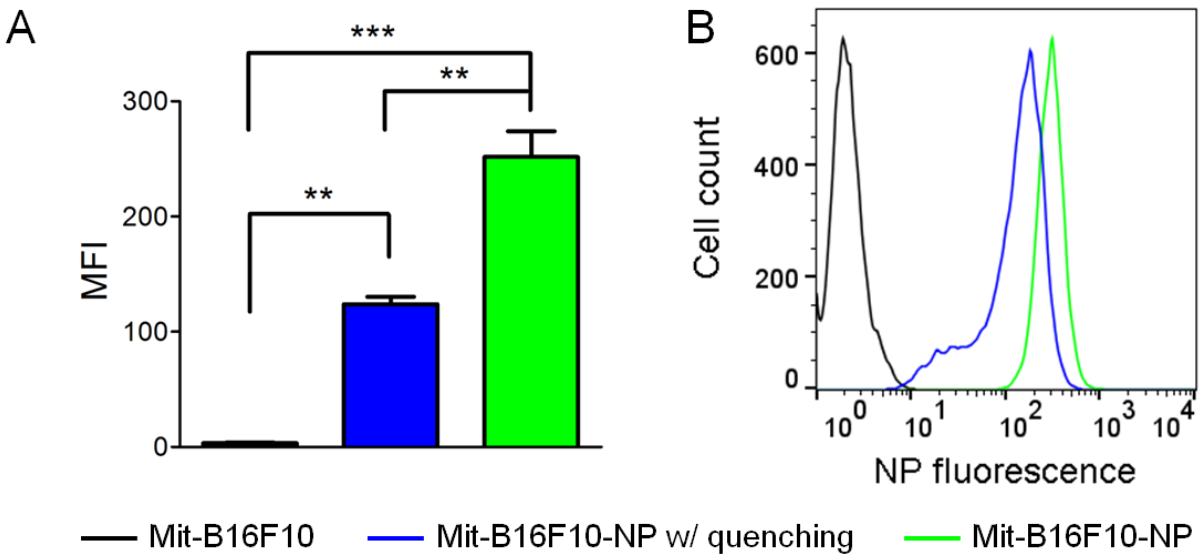


Figure S3. Incubation of chemo-treated tumor cells with NPs at 37 ^{o}C results in significant cellular uptake of NPs. Fluorophore-labeled, CpG-loaded NPs were incubated with Mit-treated B16F10 cells at 37 ^{o}C for 1 h with constant shaking, followed by washing and quantification of cell-surface bound NPs as in Figure 2F. Cell-surface fraction of NPs = 50.3 ± 5 %, determined by MFI. The data show mean ± SEM, n = 3. ** P < 0.01, *** P < 0.001, analyzed by one-way ANOVA with Bonferroni multiple comparison post-test.

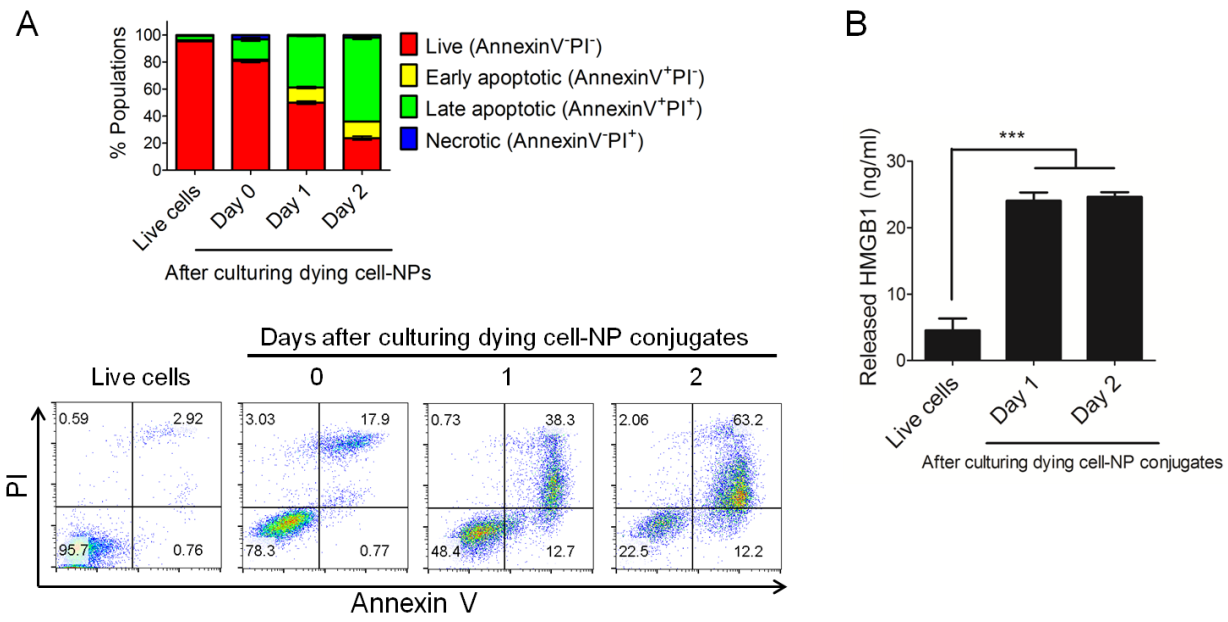


Figure S4. Dying tumor cell-NP conjugates undergo immunogenic cell death. B16F10OVA cells were treated with 10 μM mitoxantrone for 12 h, followed by conjugation with NPs on their surfaces and cell culture for two days. (A) Cell death and (B) cellular release of HMGB1 were measured by Annexin V / PI staining and ELISA, respectively. The data show mean ± SEM, $n = 3$. *** $P < 0.001$, analyzed by one-way ANOVA with Bonferroni multiple comparison post-test.

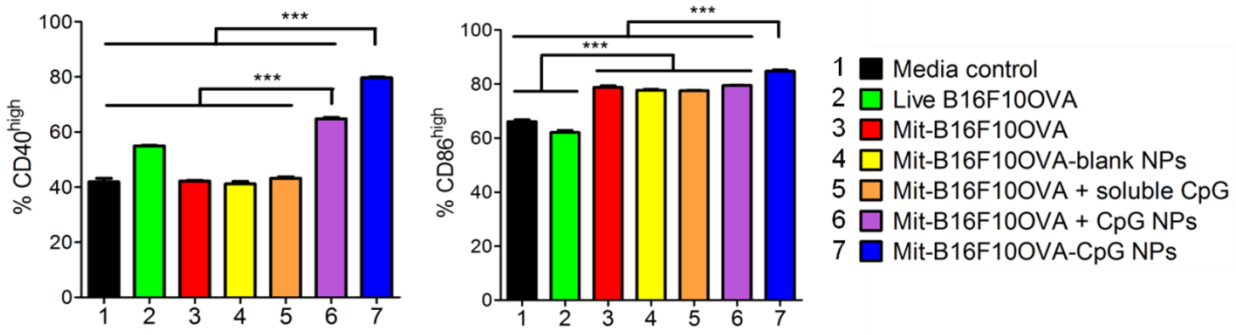


Figure S5. Percents of CD40^{high} or CD86^{high} BMDCs shown in Figure 3D and 3E. The data show mean ± SEM, from a representative experiment (*n* = 3) from 2-3 independent experiments. *** $P < 0.001$, analyzed by one-way ANOVA with Bonferroni multiple comparison post-test.

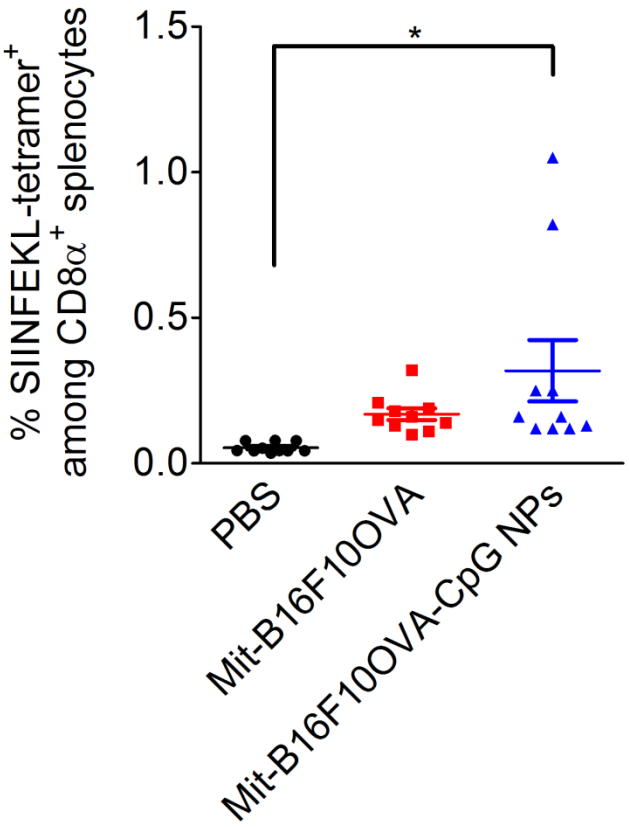


Figure S6. Immunogenically dying tumor cells membrane-decorated with CpG-NPs elicit tumor-specific CD8$^+$ T cells in spleen. Following the same immunization scheme shown in **Figure 4**, tetramer staining was performed using splenocytes on day 8. The data show mean ± SEM, n = 10, analyzed by one-way ANOVA with Bonferroni multiple comparison post-test * $P < 0.05$.

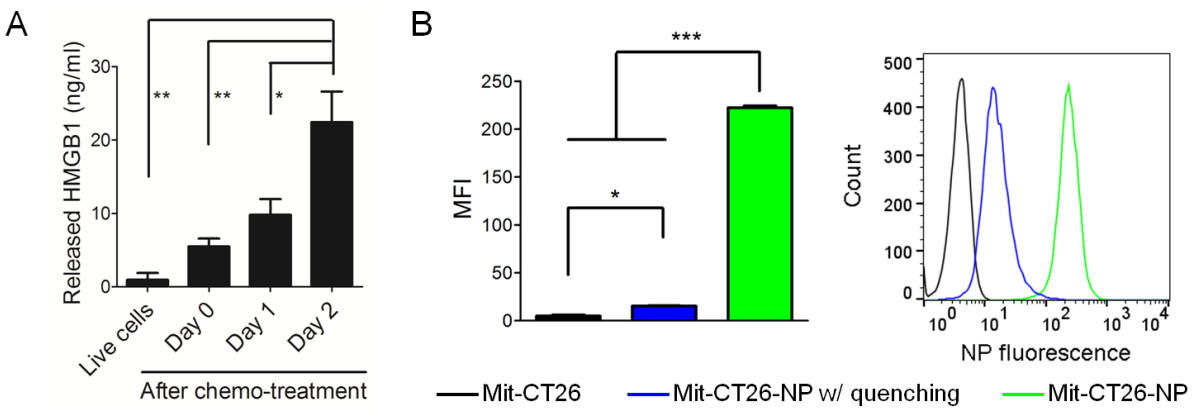


Figure S7. ICD and cell-surface conjugation of NPs on CT26 cells. (A) HMGB1 release from mitoxantrone-treated CT26 cells. CT26 cells were treated by 10 μM mitoxantrone for 12 h, followed by media change and cell culture for two days. Cellular release of HMGB1 was measured by ELISA. (B) NPs were mainly located on the surfaces of dying CT26 cells. Cell-surface fraction of NPs = 94.6 ± 0.2 %, determined as in Figure 2F. The data show mean ± SEM, n = 3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, analyzed by one-way ANOVA with Bonferroni multiple comparison post-test.

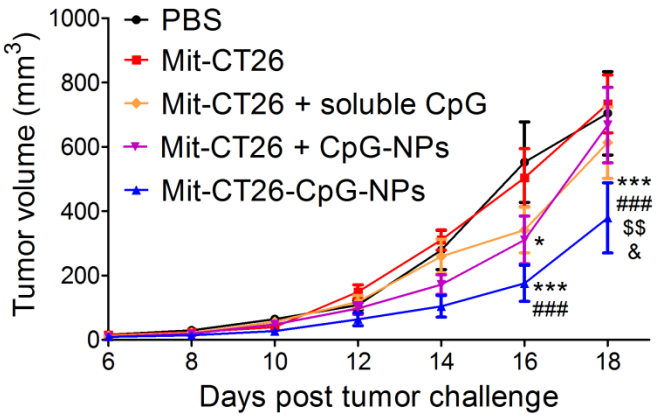
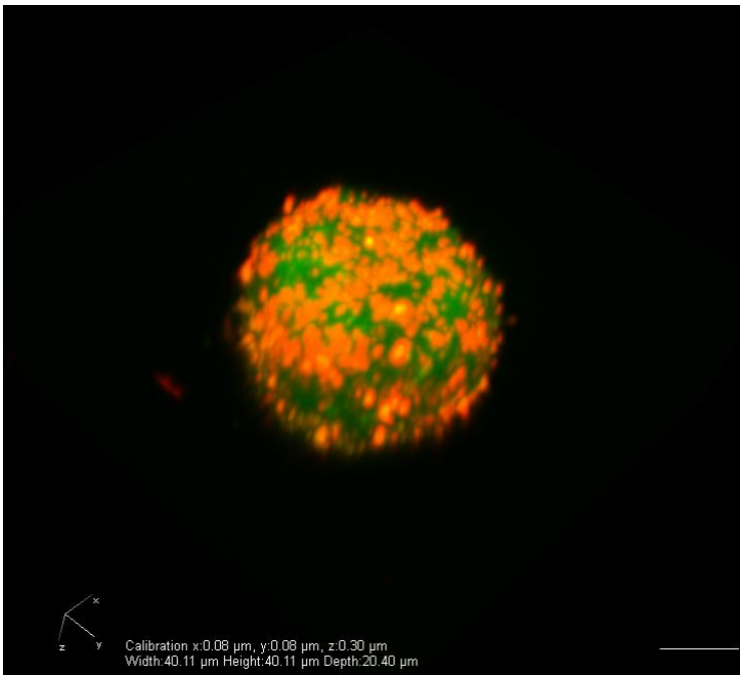


Figure S8. Anti-tumor efficacy of dying tumor cells decorated with CpG-loaded NPs. BALB/c mice were inoculated with $2x10^5$ CT26 cells subcutaneously on day 0, and on day 4 when tumors were palpable, animals were treated with a single dose of whole-cell vaccines along with various CpG formulations (10^6 Mit-CT26 cells plus 117 ng CpG / mouse). The average tumor growth volumes are shown until day 18 when mice bearing large or ulcerated tumors had to be euthanized. The data show mean ± SEM, from a representative experiment (n = 8) from two independent experiments, analyzed by two-way ANOVA with Bonferroni multiple comparison post-test. * $P < 0.05$, *** $P < 0.001$ vs. the PBS control; $^{\#\#\#}$ $P < 0.001$ vs. Mit-CT26; $^{\$\$}$ $P < 0.01$ vs. Mit-CT26 + CpG NPs; $^{\&}$ $P < 0.05$ vs. Mit-CT26 + soluble CpG.



Movie S1. Representative confocal 3D reconstruction of a dying tumor cell conjugated with NPs. Green, a Mit-treated tumor cell labeled with Oregon Green; red, Rhodamine-labeled NPs; scale bar, 5 µm.