Size-transformable antigen-presenting cell–mimicking nanovesicles potentiate effective cancer immunotherapy

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Artificial antigen-presenting cells (aAPCs) can stimulate CD8+ T cell activation. While nanosized aAPCs (naAPCs) have a better safety profile than microsized (maAPCs), they generally induce a weaker T cell response. Treatment with aAPCs alone is insufficient due to the lack of autologous antigen-specific CD8+ T cells. Here, we devised a nanovesicle for antigen-specific CD8+ T cell preactivation in vivo, followed by reactivation of CD8+ T cells via size-transformable naAPCs. naAPCs can be converted to maAPCs in tumor tissue when encountering preactivated CD8+ T cells with high surface redox potential. In vivo study revealed that naAPC’s combination with nanovesicle had an impressive antitumor efficacy. The methodology can also be applied to chemotherapy and photodynamic therapy. Our findings provide a generalizable approach for using size-transformable naAPCs in vivo for immunotherapy in combination with nanotechnologies that can activate CD8+ T cells.

INTRODUCTION

Artificial antigen-presenting cells (aAPCs) can be used in cancer immunotherapy for T cell expansion and activation (1–5). aAPCs provide three key signaling components: (i) major histocompatibility complex I/T cell receptor (MHC I/TCR) stimulatory signal, (ii) cluster of differentiation 80/cluster of differentiation 28 (CD80/CD28) costimulatory signal, and (iii) cytokine release [e.g., interleukin-2 (IL-2)] (2, 5). On the basis of size, aAPCs are characterized as nanosized aAPCs (naAPCs) or microsized aAPCs (maAPCs). naAPCs have good biocompatibility in vivo but have limited efficacy as monotherapeutics. maAPCs are more immunogenic given their larger surface area to form immunological synapses but are restricted to ex vivo settings due to safety concerns (2, 6–8). Adaptive aAPCs that can switch the size to leverage the merits of naAPCs and maAPCs have not been reported. A major application of aAPCs is ex vivo T cell expansion, which precedes adoptive T cell therapy (ACT) (4, 6). While promising for some malignancies, ACT is a resource-intensive process. There is some uncertainty about the bioactivity of the infused T cells. Therefore, a design based on aAPCs that can expand and activate T cells in vivo would be beneficial. We hypothesize that this can be achieved by preactivating antigen-specific CD8+ T cells before aAPCs treatment.

Because of their versatility, nanoformulations have been used to deliver vaccines (9–14), chemotherapeutics (15–19), and photosensitizers (20–23). Vaccines have gained increasing attention, largely because of the success of cancer immunotherapy (24–30). In general, a cancer nanovaccine is composed of antigen [e.g., tumor-associated antigen (TAA) and tumor-specific antigen (TSA)], immune adjuvant [e.g., resiquimod (R848)], and nanocarrier (31). In certain cases, nanocarriers that self-assemble from polymers can directly serve as adjuvants (10, 32, 33). Furthermore, some chemotherapeutics like doxorubicin (DOX) can induce immunogenic cell death (ICD) when administered at a low dose. As the cancer cells die, they secret TAAs that can be captured by immature dendritic cells (DCs) promoting maturation. DCs process the antigen and present them onto the surface to T cells to mount a response (34–37). With photodynamic therapy (PDT), cancer cells are eliminated via reactive oxygen species (ROS), which leads to ICD that is accompanied by an inflammatory response (38, 39). Because of the prominent antitumor effect, a plethora of work has been reported with PDT for cancer immunotherapy (38, 40, 41).

Here, we constructed three nanoplatforms on the basis of the copolymer poly(ethylene glycol)–poly(2-dimethylamino)ethyl methacrylate–poly(2-disopropylamino)ethyl methacrylate (PEG-PDMA-PDDA). The copolymer was used to encapsulate ovalbumin (OVA), DOX, and the photosensitizer 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH), to form nanoparticle (NP)–OVA, NP-DOX, and NP-HPPH, respectively (Fig. 1). We also developed size-transformable naAPCs using the redox-sensitive copolymer biotin–PEG–b–poly(N-2-hydroxypropyl methacrylamide-g-thiol)–b–poly[2-(dimethylamino)ethyl methacrylate] [biotin-PEG-PHPMA(-SH)-PDMA]. The surface of the naAPCs was modified with SIINFEKL (Ser-Ile-Asn-Phe-Glu-Lys-Leu) peptide-loaded MHC (pMHC) class I monomer and αCD28, while the watery core was loaded with IL-2. We demonstrate that using NP-OVA for nanovaccine, NP-DOX for chemotheraphy, or NP-HPPH for PDT can induce ICD in EG7-OVA tumor-bearing mice. This leads to antigen-specific CD8+ T cell preactivation. Notably, activated T cells undergo redox compartmentalization with increasing free thiols at the cell surface (42–44). We leverage this phenomenon using size-transformable naAPCs that convert from nanosize in circulation to microsize in tumor tissue once they encounter high redox potential on preactivated CD8+ T cell surface (43). In its microsize state, the aAPCs have longer residence time in tumor tissue, which helps to achieve a more potent CD8+ T cell response. The sequential administration of nanoformulation and naAPCs that can exert tumoricidal effects obviates the need to handle blood products and achieves the desired balance between naAPCs and maAPCs.
RESULTS
Construction of nanotechnology platforms and in vitro evaluation

We synthesized the pH-responsive copolymer PEG-PDMA-PDPA via reversible addition-fragmentation chain transfer (RAFT) polymerization (fig. S1A). The molecular weights of each component were 2.0, 4.0, and 1.8 kg/mol as confirmed by 1H nuclear magnetic resonance (NMR) spectrum (fig. S1C). Using similar methodology, we synthesized the copolymer 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid N-succinimidyl ester (CPAA)–PDMA–PDPA as a control (fig. S1, B and D). Dynamic light scattering (DLS) results indicated that NPs self-assembled from PEG-PDMA-PDPA had an average particle size of around 48 nm (fig. S2A and table S1). Meanwhile, control NPs (NPc) prepared by CPAA–PDMA–PDPA had a similar average particle size of about 50 nm (fig. S2C and table S1).

Transmission electron microscopy (TEM) was used to assess morphology of the NP (fig. S2B). NP-OVA appeared as spherical structures from TEM characterization and had an average particle size of around 48 nm (fig. S2A and table S1). Meanwhile, control NPs (NPc) prepared by CPAA–PDMA–PDPA had a similar average particle size of about 50 nm (fig. S2C and table S1).

Leveraging fluorescence detection, we used OVA-Cy5.5 in place of OVA to study in vitro protein loading and release. As shown in table S2, protein loading efficiency (PLE) of NP-OVA-Cy5.5 was close to 100% when protein loading content (PLC) was as low as approximately 2%. As shown in Fig. 2B, cumulative release of OVA-Cy5.5 from NP was around 60% in HOAc/NaOAc buffer (pH 5.0) within 48 hours. On the other hand, only 21% of OVA-Cy5.5 was released in PBS (pH 7.4) within 48 hours, suggesting the relatively high stability of the NP under physiological conditions (Fig. 2B).

We studied the cellular internalization behavior of NP-OVA-Cy5.5 and free OVA-Cy5.5 in DC2.4 cells. As shown by flow cytometry (Fig. 2C) and confocal laser scanning microscope (CLSM) (Fig. 2D and fig. S3A) images, NP-OVA-Cy5.5 had more cellular uptake than free OVA-Cy5.5, probably because the negative charge of free OVA-Cy5.5 restricted its internalization. Following internalization, NP-OVA-Cy5.5 underwent endosomal escape in DC2.4 cells within 4 hours (fig. S3B).

After encapsulation with DOX (NP-DOX) or HPPH (NP-HPPH), the average particle size increased slightly (table S2). As shown in
Fig. 2. Construction and characterization of NP-based nanotechnology platform. (A) Mean diameter size and distribution of NP-OVA measured by DLS. Inset image shows the structure of NP-OVA characterized by TEM. (B) In vitro OVA-Cy5.5 release from NP-OVA-Cy5.5 within 48 hours in phosphate-buffered saline (PBS; pH 7.4) and HOAc/NaOAc (pH 5.0). Data are presented as means ± SD (n = 3). (C and D) Cellular internalization of OVA-Cy5.5 formulations in DC2.4 cells characterized via flow cytometry and CLSM, respectively. (E) Size and structure characterization of NP-HPPH by DLS and TEM, respectively. (F) In vitro DOX and HPPH release from NP-DOX and NP-HPPH, separately, both in PBS (pH 7.4) and HOAc/NaOAc (pH 5.0) within 24 hours. Data are shown as means ± SD (n = 3). (G) Cellular uptake of DOX and HPPH formulations in EG7-OVA cells after incubation for 24 hours. Red colors denote DOX and HPPH, respectively. (H) ROS generation by HPPH formulations in EG7-OVA cells with or without laser irradiation, using DCFH-DA as a probe. “−” represents no laser irradiation treatment. “+” represents laser irradiation (671 nm, 100 mW/cm², 1 min). Green color denotes DCF fluorescence. (I and J) Cell cytotoxicity of DOX and HPPH formulations to EG7-OVA cells after 48-hour treatment. HPPH-treated and NP-HPPH–treated cells received laser irradiation after 24-hour incubation. conc., concentration. Data are shown as means ± SD (n = 4). (K) ICD mediated by DOX and HPPH formulations as detected by flow cytometry. HPPH formulations received laser irradiation (671 nm, 100 mW/cm², 1 min). For the CLSM images, cell nuclei were stained with Hoechst 33342 (blue). Scale bars, 20 μm.
(1.5 μg/ml) formulation–treated cells, which also verified that PDT could induce ICD (Fig. 2K). Furthermore, NP alone did not bring about ICD as scarce fluorescence shift was observed from flow cytometry (Fig. 2K).

**In vitro and in vivo DC maturation and antigen cross-presentation**

As mentioned, nanocarrier self-assembled from polymers with positive charge can serve as immune adjuvant (32, 45). From Fig. 3 (A and B), we observed a fourfold increase in CD80+/CD11c+ DC2.4 cell population after NP treatment compared to the PBS group, illustrating the NP’s immune adjuvant function. This also corresponded to a twofold increase in CD80+/CD11c+ DC2.4 cells over NPc and commercial immune adjuvant Toll-like receptor (TLR) 7/8 agonist R848 treatment (Fig. 3, A and B). From Fig. 3 (C to E), higher levels of cytokines [IL-6, IL-12,(502,526),(531,621), and tumor necrosis factor–α (TNF-α)] were detected in serum from DC2.4 cells treated with NP at 24 hours, compared with NPc, R848, and PBS groups.

A key step for immune response induction is antigen cross-presentation by DCs to T cells via pMHC I complex/TCR signal. Because of OVA being used as the antigen herein, we used anti-SIINFEKL/H-2Kb to detect the specific H-2Kb–restricted peptide. Figure 3F results revealed that OVA alone did not effectively induce DC2.4 cells to present antigen (fig. S5A). OVA-loaded NP or NPc exhibited six- and threefold increase in SIINFEKL/H-2Kb+/CD11c+ DC2.4 cells, respectively, compared with the OVA group, indicating the adjuvant function of polymers. NP-OVA even displayed better efficacy in SIINFEKL/H-2Kb presentation than the combination of OVA plus R848 (Fig. 3F). We then explored whether ICD induced by NP-DOX and NP-HPPH will cause dying EG7-OVA cell to secrete TSA, which can be captured by DC2.4 cell, followed by SIINFEKL/H-2Kb presentation. Figure 3 (G and H) results revealed that EG7-OVA cell alone had high expression of SIINFEKL/H-2Kb in the mixed cells decreased, which is similar even after NP addition. When EG7-OVA cells were pretreated with free DOX, NP-DOX, free HPPH, and NP-HPPH with laser irradiation (671 nm, 100 mW/cm², 1 min), mixed with DC2.4 cell, separately, ratio of SIINFEKL/H-2Kb+/CD11c+ cells notably improved (1.4- to 1.7-fold) (Fig. 3, G and H, and fig. S5, B and C). Results from Fig. 3 (G and H) also indicated that chemotheraphy mediated by NP-DOX and PDT mediated by NP-HPPH were able to induce ICD with TSA.
secretion. This, in turn, facilitated antigen cross-presentation in DCs (fig. S5, B and C).

**In vitro characterization of size-transformable naAPCs**

To construct size-transformable naAPCs, we synthesized copolymer PEG5.0k-PHPMA14.2k-PDMA1.3k and biotin-PEG6.0k-PHPMA17.9k-PDMA1.7k via RAFT polymerization, respectively, molecular weights of which were characterized by $^1$H NMR spectra (fig. S6, A to D). Thiol-decorated graft copolymers were obtained via esterification reaction between PEG-PHPMA-PDMA, biotin-PEG-PHPMA-PDMA, and mercaptopropionic acid (MPA). Cross-linked nanovesicles (CNV) were self-assembled from the above copolymers, denoted as CNV and biotinylated CNV (BCNV). DLS results indicated that average particle sizes of CNV and BCNV were 104 and 110 nm, respectively (Fig. 4A and table S3). The larger size of CNV (104 nm) than the aforementioned NP (48 nm) probably lies in the different molecular weight, components of copolymers, and solubility differences in solvent (24). TEM results confirmed the hollow structure of BCNV (Fig. 4A). To simulate the redox potential of activated CD8$^+$ T cells, we incubated the BCNV in the presence of 1 mM glutathione (GSH) (43). The size of the BCNV changed from approximately 110 to 1500 nm within 24 hours (Fig. 4B). Moreover, BCNV revealed good stability in serum (fig. S6E).

To achieve the naAPCs, BCNV encapsulated with IL-2 were first prepared. After streptavidin incubation, biotinylated αCD28 and pMHC (molar ratio, 1:1) were added to form naAPCs. From DLS results, naAPCs increased in average size to approximately 164 nm compared to BCNV (Fig. 4C). TEM results characterized the spherical structure of naAPCs (Fig. 4C). To more easily evaluate the protein loading and in vitro release, we replaced IL-2 with OVA-Cy5.5 as before. From table S3, naAPCs almost completely encapsulated all available OVA-Cy5.5 at relatively low PLC. From Fig. 4D, 70% protein was released from naAPCs in the presence of 1 mM GSH within 48 hours. However, only 20% protein was released in PBS, which also indicated the physiological stability and redox responsiveness of naAPCs (Fig. 4D). According to the TEM images, naAPCs initially swelled to 200 nm at 4 hours, followed with aggregation at 8 hours (Fig. 4E). When incubation time increased to 24 hours, microsized particles formed, which illustrated the successful size transformation. We further investigated in vitro CD8$^+$ T cell proliferation.
proliferation through naAPC stimulation with or without any one of the pretreatments (e.g., nanovaccine, chemotherapy, or PDT) for T cell preactivation. According to Fig. 4F and fig. S7A, T cells were incubated with DC2.4 cells at a number ratio of 3:1. The pretreated DC2.4 cells with NP-OVA were able to preactivate T cells, leading to high CD8\(^+\) CD3\(^+\) expression. After the addition of the naAPCs, the numbers of activated CD8\(^+\) T cells increased as well (Fig. 4F). Nevertheless, naAPCs alone or with OVA scarcely activated CD8\(^+\) T cells.

Encouraged by the above results that naAPCs can expand preactivated CD8\(^+\) T cells by nanovaccine, we next investigated whether naAPCs could work with the chemotherapy or PDT approaches. As shown in Fig. 3 (F and G), ICD induced by DOX and HPPH formulations led to TSA secretion from EG7-OVA cells, which were subsequently captured by DC2.4 cells for antigen processing and presentation to T cells. According to Fig. 4G and fig. S7B, when T cells were incubated with DC2.4 cells premixed with dying EG7-OVA cells treated with either DOX or NP-DOX (ratio of 6:2:1 for cell type), we observed an increase in the population of CD8\(^+\)CD3\(^+\) T cells. When naAPCs were added to the above preactivated T cells, we observed additional proliferation of the activated T cells (Fig. 4G). With CD8\(^+\) T cells that were preactivated by PDT, naAPC treatment led to similar results (Fig. 4H and fig. S7C). While naAPCs can slightly stimulate CD8\(^+\) T cell activation, the effect can be significantly amplified when combined with different nanotechnologies.

**In vivo NIR imaging and in vivo CD8\(^+\) T cell proliferation**

Following the in vitro studies, we used in vivo near-infrared (NIR) fluorescence imaging to assess lymph node or tumor accumulation of the NPs and naAPCs. For the in vivo studies, we used C57BL/6 mice bearing EG7-OVA tumor xenograft. According to Fig. 5A, when NP-OVA-Cy5.5 was administered via subcutaneous injection in the right foot pad, it quickly accumulated in popliteal lymph node within 4 hours. At the 8-hour time point, accumulation in popliteal lymph node increased. After intravenous injection of NP-HPPH, visible HPPH fluorescence was observed in tumor tissue as early as 1 hour, probably because of its relatively small size (Fig. 5B). At 8 hours after injection, the highest tumor accumulation was achieved for NP-HPPH on the basis of fluorescence intensity observed. HPPH fluorescence intensity persisted throughout 48 hours, indicating the good tumor retention of NP-HPPH. This observation was also verified by ex vivo imaging at 48 hours (Fig. 5B). We tested BCNV to investigate tumor accumulation of our naAPC nanoplatform. From Fig. 5C, tumor accumulation of BCNV-OVA-Cy5.5 was the highest at 8 hours after injection. Even at 48 hours, fluorescence in the tumor region was still observable, showing their long retention time (Fig. 5C). This was also confirmed by ex vivo experiment (Fig. 5C). Probably because of the reticuloendothelial system uptake, strong fluorescence signals from BCNV-OVA-Cy5.5 were also observed in liver (46, 47). In addition, we speculate that NP-HPPH with smaller size may be more suitable for tumor penetration, illustrating longer retention time than BCNV.

We then evaluated the in vivo CD8\(^+\) T cell proliferation on the basis of naAPCs combined with different therapeutic nanoformulations (nanovaccine, chemotherapy, or PDT). From Fig. 5 (D to I) and fig. S8A, NP-OVA, NP-DOX, and NP-HPPH were able to preactivate CD8\(^+\) T cells to stimulate host immunity. The combination of naAPCs with each of the three therapeutic approaches led to higher increase in number of CD8\(^+\) T cells, than any nanof ormulation alone. Of the three, naAPCs combined with NP-OVA was the most potent for CD8\(^+\) T cell activation. Presumably, this is due to greater TSA secretion (OVA) when using nanovaccine, compared to TAA secretion generated by DOX or PDT. CRT exposure (red color) was observed in tumor tissues for mice treated with NP-DOX and NP-HPPH, compared with PBS and naAPCs groups, showing that both chemotherapy and PDT induce ICD in vivo (Fig. 5J and fig. S8B). According to Fig. 5 (K and L) and fig. S8C, the number of SIINFEKL/H-2K\(^\beta\) CD8\(^+\) DCs (2.0- to 2.9-fold) was higher in tumor tissue for mice treated with NP-DOX or NP-HPPH nanof ormulations compared with the PBS group. This indicated that ICD induced by DOX and PDT promoted DC recruitment, maturation, and antigen cross-presentation.

**In vivo antitumor efficacy of NP-drug/naAPCs**

We then investigated the in vivo antitumor efficacy of naAPCs in combination with NP-OVA, NP-DOX, or NP-HPPH in EG7-OVA tumor-bearing C57BL/6 mice. To explore the inhibition efficacy of more challenging large tumors, mice were treated at 8 days after tumor inoculation when average tumor volumes were 130 ± 11 mm\(^3\). At day 18, for mice treated with naAPCs/NP-OVA (415 ± 47 mm\(^3\)), tumor volume (Fig. 6A) was significantly smaller compared to NP-OVA (1023 ± 81 mm\(^3\)) and naAPCs (1431 ± 102 mm\(^3\)) alone (Fig. 6B). In mice just treated with PBS, average tumor volumes were as high as 2072 ± 186 mm\(^3\) at therapeutic ending point. NP-OVA as nanovaccine partially restricted tumor growth (Fig. 6B). Unexpectedly, the naAPCs was still able to inhibit tumor growth despite the absence of autologous antigen-specific CD8\(^+\) T cells (Fig. 6B). From Fig. 6C and fig. S9A, it was observed that tumors in mice treated with naAPCs/NP-OVA had the lowest weight and volume at euthanasia. The body weights of the mice remained fairly constant throughout the study, demonstrating the good biocompatibility of nanomaterials (fig. S9, B to D). Harvested tumors were lysed at study end point to determine the number of CD8\(^+\) T cells in the tumors. Figure 6 (D and E) and fig. S10A showed that NP-OVA resulted in partial CD8\(^+\) CD3\(^+\) T cells activation, which was better than naAPC treatment alone. When mice were treated with NP-OVA followed with naAPCs, more CD8\(^+\) CD3\(^+\) T cells were present.

With chemotherapy (Fig. 6F) or PDT (Fig. 6K), assisted naAPCs, NP-DOX/naAPCs (721 ± 45 mm\(^3\)), and NP-HPPH/naAPCs (497 ± 49 mm\(^3\)) showed improved tumor inhibition compared to NP-DOX (1072 ± 61 mm\(^3\)), NP-HPPH (878 ± 62 mm\(^3\)), or naAPCs (1431 ± 102 mm\(^3\)) alone (Fig. 6F). This observation was substantiated by tumor weight and volume measurements (Fig. 6, H and M, and fig. S9A). As before, we interrogated the T cell infiltration status in treated tumors. Figure 6 (I, J, N, and O) and fig. S10A indicated that treatments with NP-DOX and NP-HPPH alone were able to recruit CD8\(^+\) T cells to tumor site. When naAPCs were introduced after chemotherapy and PDT, more CD8\(^+\) CD3\(^+\) T cells were detected in tumor tissue, demonstrating synergy. Comparing the three nanof ormulations (NP-OVA, NP-DOX, and NP-HPPH) in combination with naAPCs, NP-OVA/naAPCs was the most effective at CD8\(^+\) T cell activation and tumor inhibition. We believe that this was due to the specific priming of T cells against the OVA antigen. On the other hand, ICD induced by chemotherapy and PDT generated a mixture of TSAs and TAs for the tumor model. It could be that DCs are better at processing the specific antigen or
Fig. 5. In vivo NIR fluorescence imaging, CD8^+ T cell proliferation, ICD, and antigen cross-presentation in EG7-OVA tumor-bearing C57BL/6 mice. (A) In vivo NIR lymph node imaging of NP-OVA-Cy5.5 via subcutaneous injection at right foot pad. (B and C) In vivo NIR tumor imaging of NP-HPPH and BCNV-OVA-Cy5.5 at different time points after tail vein injection. Mice were sacrificed at the last time point, and tumors and selected organs were extracted for the ex vivo imaging. (D to F) CD8^+ /CD3^+ T cell ratio for mice treated with naAPCs with or without nanoformula (NP-OVA, NP-DOX, and NP-HPPH) assistance. Data are presented as means ± SD (n = 3, one-way ANOVA with multiple comparisons). (G to I) CD8^+ CD3^+ T cell numbers in tumor tissue after treatment with naAPCs with or without nanoformula (NP-OVA, NP-DOX, and NP-HPPH) usage (n = 3, one-way ANOVA with multiple comparisons). (J) ICD induced by NP-DOX and NP-HPPH with laser irradiation (671 nm, 200 mW/cm^2, 10 min). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Exposed CRT was stained with anti-Alexa Fluor CRT-647. (K and L) DC maturation and antigen cross-presentation after NP-DOX and NP-HPPH (671 nm, 200 mW/cm^2, 10 min) treatment. Data are shown as means ± SD (n = 3, one-way ANOVA with multiple comparisons).
Fig. 6. In vivo antitumor efficacy of EG7-OVA tumor–bearing C57BL/6 mice. (A) Schematic illustration of naAPCs/NP-OVA study protocol. *s.c.*, subcutaneous; *i.v.*, intravenous. (B) Tumor volume monitoring for mice treated with PBS, naAPCs, NP-OVA, and naAPCs/NP-OVA. Data are presented as means ± SD (n = 5 per group). (C to E) Tumor weight (n = 5 per group), CD8+/CD3+ T cell ratio (n = 3 per group), and number of CD8+/CD3+ T cells (n = 3 per group) in tumor tissue at day 18 (D18) after treatment with PBS, naAPCs, NP-OVA, and naAPCs/NP-OVA. Data are shown as means ± SD. (F) Schematic diagram of naAPCs/NP-DOX study protocol. (G) Tumor volume monitoring for mice treated with PBS, naAPCs, NP-DOX, and naAPCs/NP-DOX. (H to J) Tumor weight (n = 5 per group), CD8+/CD3+ T cell ratio (n = 3 per group), and CD8+/CD3+ T cell numbers (n = 3 per group) in tumor tissue at end point in mice treated with PBS, naAPCs, NP-DOX, and naAPCs/NP-DOX. Data are displayed as means ± SD. (K) Schematic illustration of naAPCs/NP-HPPH PDT study protocol. (L) Tumor volume growth curve for mice treated with PBS, naAPCs, NP-HPPH, and naAPCs/NP-HPPH. (M to O) Tumor weight (n = 5 per group), CD8+/CD3+ T cell ratio (n = 3 per group), and CD8+/CD3+ T cell numbers (n = 3 per group) in tumor tissue at D18 in each group for mice treated with PBS, naAPCs, NP-HPPH, and naAPCs/NP-HPPH. Data are displayed as means ± SD. (P to R) CD8+/CD3+ T cell distribution [(P) scale bars, 20 µm], OVA-specific CD8+ T cell activation and proliferation ([Q] scale bars, 200 µm; [R] scale bars, 100 µm) of tumor tissue for mice treated with PBS, naAPCs, NP-DOX/naAPCs, NP-HPPH/naAPCs, and NP-OVA/naAPCs. Red triangle represents apoptosis, and green triangle means necrosis of tumor cell. The NP dose used in antitumor efficacy was 5 mg/kg. One-way ANOVA was used to evaluate the statistic comparisons among groups.
that T cells may be more reactive toward this antigen. Future investigation is required to test these hypotheses.

To visualize the CD8+ T cells in tumor tissue, we performed immunofluorescence staining and acquired images by CLSM. For mice treated with nanoformulation alone (Fig. 6P and fig. S10B), only a portion of CD8+ T cells were activated with some red fluorescence in tumor tissue. A small population of CD8+ T cells was observed in tumors of mice treated with naAPCs alone (Fig. 6P and fig. S10B), probably because of the absence of antigen-specific CD8+ T cells and the lack of immunogenicity of naAPCs in its smaller state. We observed an increase of CD8+ T cells (red fluorescence) in tumor tissue for mice that were treated with nanotechnology and naAPCs (Fig. 6P and fig. S10B), which again indicated that naAPCs could target to the preactivated CD8+ T cells and help with their recruitment. Then, we examined whether naAPCs assisted by nanotechnology could induce OVA-tetramer CD8+ T cells proliferation in mice after treatment. As shown in Fig. 6Q, mice that underwent naAPCs alone rarely induced OVA-specific CD8+ T cell activation and proliferation. Mice after NP-OVA, NP-DOX, or NP-HPPH treatment elicited slightly higher activation of OVA-tetramer CD8+ T cells. When naAPCs were combined with nanotherapeutics (Fig. 6Q and fig. S10C), the numbers of OVA-tetramer CD8+ T cells increased significantly.

We performed hematoxylin and eosin (H&E) staining in tumors and selected organs to determine tissue damage. Significant tumor cell death was observed where some cells merely had red endochyloma (red triangle) or wrinkled cell nuclei (green triangle) for mice treated with NP-OVA/naAPCs, NP-DOX/naAPCs, or NP-HPPH/naAPCs (Fig. 6R and fig. S11), while less tumor cell death was observed for the nanoformula alone–treated group. naAPCs alone induced slight tumor damage, illustrating the necessity of nanof ormulation (Fig. 6R and fig. S11). Negligible damage to normal organs was observed for all the mice, demonstrating the good biocompatibility of the nanomaterials (fig. S11).

DISCUSSION

Although therapeutic vaccine has achieved some antitumor efficacy and elicited clinical response, sipuleucel-T was the exclusive one, which acquired U.S. Food and Drug Administration approval (48). A number of vaccines were in clinical trial stages or underwent failure due to the tumor heterogeneity (48, 49). Here, we constructed a multifunctional nanoplatform, which could be widely used for nanovaccine (NP-OVA), chemotheraphy (NP-DOX), and PDT (NP-HPPH) to form cancer vaccination in vivo via ICD, to activate CD8+ T cells. We here tried to build a general cancer nanovaccination platform as a tool to preactivate antigen-specific CD8+ T cell in vivo, which could be better combination with other nanotechnology for cancer treatment. The biomaterials we used were easily synthesized, providing the possibility for clinical translation. Concomitantly, nanocarrier alone had immune adjuvant function even better than commercial TLR7/8 agonist R848 at a proper concentration, which avoided extra adjuvant addition and simplified vaccine components. For further study, the smart nanoplatform can also be widely applied into photothermal therapy and radiotherapy to be tumor vaccine in vivo via ICD. The limitation of the cancer nanovaccination platform lied in the insufficient antigiterimmunity, probably because of the initial large tumors (130 ± 11 mm3) when treatment started, as well as the single injection.

Subsequent naAPCs could specifically target to the preactivated antigen-specific CD8+ T cell, which were generated by the above-mentioned approaches stimulation. Then, naAPCs were able to transform to maAPCs when they encounter the high redox potential on activated CD8+ T cell surface. The larger size of transformed maAPCs enhanced immunogenicity by allowing for greater immunological synapse contact and also prolonged retention time in tumor tissue. In addition, the transformed maAPCs ensured the paracrine delivery of low dose IL-2, which had better efficiency and safety than the direct IL-2 administration as reported (4, 50, 51). This transformation about naAPCs in blood to maAPCs in tumor site leveraged their individual advantages including good safety of naAPCs and impressive immunity provocation of maAPCs. As reported, a lipid-decorated silica microrod APCs (SMR-APCs) led to a massive CD8+ T cell expansion in vitro, even better than commercial magnetic bead (4). Probably in consideration of potential safety issues, the authors exploited SMR-APCs to proliferate CD8+ T cell, followed by further ACT. Our promising approach that subtly resolved the safety issues of maAPCs can potentially be an alternative to adoptive cell therapy, pending further investigations. We harnessed the local redox potential differences between naive and primed CD8+ T cell surface to make the size transformation and cytokine paracrine delivery. Theoretically, any other nanoplatform that is redox sensitive can accomplish APC size transformation. Moreover, the naAPC platform still can be improved if the fluid lipid structure was used. Despite the relatively complicated synthesis of the redox-sensitive copolymer, the polymer has good biocompatibility, when multiple HPMA prodrugs have been applied into clinical trials (52).

The creative combination between cancer nanovaccination and naAPCs for sequential treatment methodology successfully accomplished size transformation to maAPCs for antigen-specific CD8+ T cell reactivation, significantly suppressing tumor progression. In contrast, naAPCs alone exhibited poor antitumor efficacy with minimal CD8+ T cell activation probably because of the absence of autologous antigen-specific T cell and small size of naAPCs. While we investigated three nanoformulations for cancer nanovaccination, it is conceivable that other treatment modalities may be feasible as long as they can preactivate antigen-specific CD8+ T cells. This research demonstrates that aAPCs can be combined with nanotechnology to achieve enhanced immuno therapy. We simultaneously expect that this work can open a new avenue for people to use aAPCs to treat cancers.

MATERIALS AND METHODS

Synthesis and characterization of pH-responsive copolymer PEG-PDMA-PDPA

The macromolecular RAFT reagent PEG-CPAA was obtained by amidation between PEG-NH2 (number-average molecular weight = 2.0 kg/mol, Biochempep) and CPAA (Sigma-Aldrich) according to the previously published methods (45). Copolymer PEG-PDMA-PDPA was synthesized via RAFT polymerization. Briefly, PEG-CPPA (200 mg, 0.1 mmol), monomer 2-(dimethylamino)ethyl methacrylate (DMA; 3.18 mmol, Sigma-Aldrich, 98%), and initiator azobisisobutyronitrile (AIBN; 0.015 mmol, Sigma-Aldrich) were dissolved in tetrahydrofuran (THF) and added to a Schlenk flask at nitrogen atmosphere. After 30 min of nitrogen protection and continuous stirring, the reaction was placed into a 70°C oil bath for 24 hours. Then, at nitrogen environment, the second monomer
2-(diisopropylamino)ethyl methacrylate (DPA; 0.94 mmol, Sigma-Aldrich, 97%) was added with AIBN (0.01 mmol) for an additional 24 hours. DMA and DPA were passed through an aluminum oxide (neutral) column before use. The final reaction product was obtained via precipitation in anhydrous diethyl ether, centrifugation, and vacuum drying. The molecular weights of PEG-PDMA-PDPA were 2.0, 4.0, and 1.8 kg/mol, which were characterized by $^1$H NMR (Bruker Avance III-300) spectrum. The control copolymer CPAA-PDMA-PDPA was also synthesized via RAFT polymerization with the similar method. The $^1$H NMR spectrum result showed that the molecular weights of each segment were 4.7 and 2.3 kg/mol.

### Preparation and characterization of pH-responsive NP

NPs self-assembled from PEG-PDMA-PDPA were achieved via solvent-exchange method. In short, 50 μl of polymer (THF, 10 mg/ml) was added dropwise into 950 μl of PBS (pH 7.4) until uniform diffusion. After volatilization and dialysis to remove THF, NP was obtained and characterized by DLS (Horiba SZ-100, HORIBA Ltd., Kyoto, Japan) and TEM (Tecnai TF30, FEI, Hillsboro, OR). Npc self-assembled from CPAA-PDMA-PDPA was prepared similarly.

As for the pH-responsiveness study, NP (1 ml, 0.2 mg/ml) was added separately to PBS (pH 7.4) and HOAc/NaOAc (pH 5.0) and placed on an orbital shaker (37°C, 200 rpm). Size change of NP in low pH was monitored via DLS measurement at 0, 4, 8, and 24 hours, respectively. NP in PBS was tested at 0 and 24 hours, as a control.

### Preparation of NP-OVA (nanovaccine) and in vitro OVA release

NP-OVA was prepared in a similar way to NP, just OVA (10 μg, Sigma-Aldrich) before dissolving in PBS (950 μl, pH 7.4) needed. To calculate PLC, PLE, protein release, and cellular uptake, we used OVA-Cy5.5 (Nanocs) in place of OVA. PLC and PLE were measured using a fluorescence spectrophotometer (Hitachi F-7000) according to the following formulas

\[
\text{PLC} = \frac{\text{mass of actual protein encapsulation}}{\text{mass of (actual protein encapsulation + polymer)}} \times 100\%
\]

\[
\text{PLE} = \frac{\text{mass of actual protein encapsulation}}{\text{mass of theoretical protein encapsulation}} \times 100\%
\]

For in vitro OVA-Cy5.5 release behavior, we tracked the accumulative release by high-speed centrifugation measurement. In brief, 1 ml of NP-OVA-Cy5.5 solution (pH 7.4 or pH 5.0) was placed in a 2-ml Eppendorf tube (n = 3). The sample was then placed on an orbital shaker (37°C, 200 rpm). At fixed time points, samples were centrifuged (15,000 rpm, 5 min), and 200 μl of supernatant was removed and tested by fluorescence spectrophotometer. After each removal, 200 μl of fresh medium was added. The drug-release profiles were studied over a period of 48 hours.

### Cellular internalization of NP-OVA-Cy5.5 in DC2.4 cells

The cellular internalization of NP-OVA-Cy5.5 was characterized using a BD Beckman Coulter flow cytometer (Brea, CA) and a Zeiss 780 microscope (CLSM). For flow cytometry, DC2.4 cells were seeded in a six-well plate at 5 × 10⁵ cells per well. After incubation overnight, OVA-Cy5.5 and NP-OVA-Cy5.5 (OVA-Cy5.5 concentration, 10 μg/ml) were added at preset time point, separately. Cells were trypsinized, washed, centrifuged, and suspended in 0.5 ml of PBS followed by flow cytometry measurement.

For CLSM characterization, DC2.4 cells (2 × 10⁶ per well) were seeded in an eight-well plate. At 70% cell confluence, OVA-Cy5.5 and NP-OVA-Cy5.5 (OVA-Cy5.5 concentration, 10 μg/ml) were added at specific time points. After PBS washing, cell nuclei were stained with Hoechst 33342 (2 μg/ml, Thermo Fisher Scientific). Following another PBS wash, mounting medium (Vector Laboratories) was added onto the cells. The cells were covered by coverslip and sealed with nail polish. The images were acquired by CLSM. The endosomal escape was performed similarly. Seeded cells were washed by PBS and stained with LysoTracker green DND-26 (100 nM, Thermo Fisher Scientific) and Hoechst 33342 (2 μg/ml, respectively).

### Preparation and characterization of NP-DOX and NP-HPPH

NP-DOX was self-assembled in a similar manner to NP, just the in-advance mixture between 5 μl of DOX (5 mg/ml) and 50 μl of polymer (10 mg/ml) needed. The drug loading content (DLC) and drug loading efficiency (DLE) were tested by ultraviolet-visible (UV-vis) (UV3100PC, VWR, Radnor, PA), calculated by the similar equations to PLC and PLE. Preparation of NP-HPPH (HPPH concentration, 15 μg/ml) was almost similar to NP-DOX, just replacing DOX with HPPH; DLC and DLE were measured by UV-vis. The structures of NP-DOX and NP-HPPH were confirmed via TEM.

### In vitro DOX and HPPH release

In vitro DOX (Sigma-Aldrich) and HPPH (MedKoo Biosciences) release behaviors were studied per published procedures (45). Briefly, both 0.5 ml of NP-DOX (DOX concentration, 25 μg/ml) and NP-HPPH (HPPH concentration, 15 μg/ml) were added inside filter bags (molecular weight cutoff of 10 kDa). The filter bags were placed in a solution of 25 ml of PBS (pH 7.4) or HOAc/NaOAc (pH 5.0). The solutions were placed on an orbital shaker (37°C, 200 rpm). At selected time points, 5 ml of solutions was removed and subsequently replaced with 5 ml of fresh buffer. The accumulative release of the two drugs was measured by fluorophotometer.

### Cellular uptake and ROS generation

The cellular uptake of NP-DOX and NP-HPPH in EG7-OVA cells was investigated by CLSM and flow cytometry in a similar manner to cellular internalization studies of NP-OVA in DC2.4 cells. For ROS generation in EG7-OVA cell by HPPH formulations, we used CLSM for characterization. In short, EG7-OVA cells (2 × 10⁴ per well) were seeded in Nunc Glass Bottom Dishes (Thermo Fisher Scientific) and allowed to grow for 24 hours. Then, HPPH (n = 2) and NP-HPPH (n = 2) (final HPPH concentration, 1.5 μg/ml) were added, respectively. One dish of each group received laser irradiation (671 nm, 100 mW/cm², 1 min) for 4-hour incubation. Afterward, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (final, 30 μM; Crescent Chemical Company) was added for 30 min. Cells were washed by
PBS and stained with Hoechst 33342 for 10 min. Following another PBS wash, cells were analyzed by CLSM.

**MTT assays**
The cytotoxicity of NP-DOX and NP-HPPH in EG7-OVA cell was investigated by MTT assays. Shortly, EG7-OVA cells (5 × 10^4 per well) were seeded in a 96-well plate for growth overnight. DOX formulations of different concentrations (0.001, 0.01, 0.1, 0.5, 1, 2, and 5 µg/ml) and HPPH formulations of different concentrations (0.001, 0.01, 0.1, 0.2, 0.5, 1, and 1.5 µg/ml) were added, respectively. After 24-hour incubation, cells were washed with PBS, centrifuged, and stained with Alexa Fluor 488 anti-CRT antibody (Abcam). After 40-min staining, cells were washed with PBS, centrifuged, and resuspended in 0.5 ml of PBS. Cells were assayed by flow cytometer. For HPPH formulation-mediated ICD, EG7-OVA cells (5 × 10^5 per well) were seeded in a six-well plate for 24 hours, followed by addition of NP, DOX, and NP-DOX (final DOX concentration, 5 µg/ml), respectively. After 24-hour incubation, cells were washed by PBS, centrifuged, and stained with Alexa Fluor 488 anti-CRT antibody (Abcam). After 40-min staining, cells were washed with PBS, centrifuged, and resuspended in 0.5 ml of PBS. Cells were assayed by flow cytometer. For HPPH formulation–mediated ICD, EG7-OVA cells (5 × 10^5 per well) were seeded in a six-well plate for 24 hours, followed by addition of NP, HPPH, and NP-HPPH (final HPPH concentration, 1.5 µg/ml). After 24-hour incubation, laser irradiation (671 nm, 100 mW/cm², 1 min) was carried out. After 4 hours, cells were washed by PBS, centrifuged, and stained by Alexa Fluor 488 anti-CRT antibody (Abcam) for 40 min. Cells were washed with PBS, centrifuged, resuspended in 0.5 ml of PBS, and assayed on a flow cytometer.

**In vitro ICD**
CRF expression was measured by flow cytometry. For DOX-mediated ICD, EG7-OVA cells (5 × 10^5 per well) were seeded in a six-well plate for 24 hours, followed by addition of NP, DOX, and NP-DOX (final DOX concentration, 5 µg/ml), respectively. After 24-hour incubation, cells were washed by PBS, centrifuged, and stained with Alexa Fluor 647 anti-CRT antibody (Abcam). After 40-min staining, cells were washed with PBS, centrifuged, and resuspended in 0.5 ml of PBS. Cells were assayed by flow cytometer. For HPPH formulation–mediated ICD, EG7-OVA cells (5 × 10^5 per well) were seeded in a six-well plate for 24 hours, followed by addition of NP, HPPH, and NP-HPPH (final HPPH concentration, 1.5 µg/ml). After 24-hour incubation, laser irradiation (671 nm, 100 mW/cm², 1 min) was performed on HPPH formulation–treated cells. After further 24 hours, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 hours. After centrifugation, the supernatant was aspirated and replaced with 150 µl of dimethyl sulfoxide. Absorbance at 570 nm was measured using the BioTek Synergy H4 microplate reader.

**Synthesis and characterization of redox-sensitive copolymer biotin-PEG-PHPMA(-SH)-PDMA**
The redox-sensitive copolymer PEG-PHPMA-PDMA was synthesized via RAFT polymerization according to previously published research (53). In brief, PEG-CPAA (100 mg, 0.02 mmol), HPMA (320 mg, 2.23 mmol), and AIBN (0.49 mg, 0.003 mmol) were dissolved in THF and added to a Schlenk flask at nitrogen atmosphere. After sealing, the flask was placed into 70°C oil bath. After 24 hours, in the presence of nitrogen, the second monomer DMA (40 mg, 0.25 mmol) in dimethyl formamide (DMF) was added with AIBN (0.33 mg, 0.002 mmol) for another 24 hours. The final product was obtained after precipitation in anhydrous diethyl ether, centrifugation, and vacuum desiccation. The molecular weights of the copolymer were 5.0, 14.2, and 1.3 kg/mol, which were characterized by ^1H NMR spectrum.

The graft copolymer PEG-PHPMA(-SH)-PDMA was acquired by esterification reaction between PEG-PHPMA-PDMA and 3-MPA (Sigma-Aldrich, 99%). Briefly, PEG-PHPMA-PDMA (200 mg, 0.0098 mmol), MPA (62.3 mg, 0.59 mmol), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (Sigma-Aldrich, 98%) (226.2 mg, 1.18 mmol), and 4-dimethylaminopyridine (DMAP) (72.08 mg, 0.59 mmol) were dissolved in DMF and reacted at room temperature (r.t.) for 24 hours. The desired product was obtained after dialysis in deionized water and lyophilization. The biotin-PEG-PHPMA-PDMA was also synthesized by RAFT polymerization. After ^1H NMR characterization, the molecular weights of biotin-PEG-PHPMA-PDMA were 6.0, 17.9, and 1.7 kg/mol. Biotin-PEG-PHPMA(-SH)-PDMA was also obtained via esterification reaction similar to the above PEG-PHPMA(-SH)-PDMA synthesis method.

**Preparation and redox responsiveness of CNVs**
CNV self-assembled from PEG-PHPMA(-SH)-PDMA was also acquired via solvent-exchange method same as the above NP preparation. In short, 50 µl of polymer (10 mg/ml) in DMF was added dropwise into 950 µl of PBS (pH 7.4). After uniform dispersion, CNVs were obtained after dialysis, and size and size distribution of which were measured by DLS. BCNV was prepared in a similar pathway to CNV, for polymer, just replacement with 5% biotin-PEG-PHPMA(-SH)-PDMA and 95% PEG-PHPMA(-SH)-PDMA mixture (molar ratio). The hollow structure of BCNV was confirmed by TEM characterization. The redox responsiveness of BCNV was examined in PBS buffer.
with or without 1 mM GSH, and size of which was monitored by DLS at preset time point.

**Preparation of naAPCs and in vitro protein release**

To get naAPCs, we first prepared BCNV-IL2 with the similar method to the above BCNV, just pre-dissolution of IL-2 in PBS needed. Then, streptavidin at around 30% (molar ratio) of biotin was added and reacted with BCNV for approximately 1 hour at r.t. to obtain streptavidin-BCNV. After addition of biotin-labeled anti-CD28 (Thermo Fisher Scientific) and biotin-labeled MHC SIINFEKL-H-2Kb monomer (product code: TB-5001-M, MBL International Corporation) (molar ratio, 1:1), reaction was allowed to proceed for 1 hour at r.t. After dialysis, naAPCs were obtained, and size and structure of which were separately characterized by DLS and TEM.

For in vitro protein release, we used OVA-Cy5.5 as a surrogate for IL-2. In brief, 1 ml of naAPCs-OVA-Cy5.5 (n = 3) dispersed in PBS with or without 1 mM GSH was put into a 2-ml Eppendorf tube onto an orbital shaker (37°C, 200 rpm). At selected time points, high-speed centrifugation (15,000 rpm, 5 min) was performed, and 200 µl supernatant was extracted to test the protein released by fluorophotometer. Each time an aliquot was removed, 200 µl of fresh buffer was added.

Moreover, the size change of naPACs was monitored at reductive microenvironment via TEM characterization. In short, naPACs in PBS with 1 mM GSH was placed in a shaking bed (37°C, 200 rpm). At selected time point, one drop solution was acquired to lay in TEM copper grid for air drying at r.t. The particle’s images were obtained by TEM.

**In vitro CD8⁺ T cell expansion via naAPCs with NP-drug preactivation**

DC2.4 cells (4 x 10⁵ per well) were seeded in a six-well plate. After 24-hour growth, PBS, OVA, and NP-OVA were added, respectively. After 48-hour incubation, each group received naïve T cells (1.2 x 10⁶ per well). After 48-hour incubation, naAPCs were added to half of wells in each group. After an additional 48 hours, cells were washed by PBS and stained with APC anti-CD8α and PE anti-CD3e. After PBS washing and centrifugation, cells were firstly suspended in 0.5 ml of PBS and tested via flow cytometer. For NP-DOX-mediated CD8⁺ T cell preactivation, EG7-OVA cells (2 x 10⁶ per well) were seeded in a six-well plate for 24 hours. Afterward, PBS, DOX, and NP-DOX were added, respectively, for 24-hour incubation. DC2.4 cells (4 x 10⁵ per well) were mixed with above-treated EG7-OVA cells. After 48-hour coinoculation, naïve T cells (1.2 x 10⁶ per well) were added for preactivation. After 48-hour stimulation, half of the wells in each group were treated with naAPCs for the further CD8⁺ T cells amplification. Forty-eight hours later, all cells in each group were processed at a similar way to the above NP-OVA part and detected via flow cytometer.

For NP-HPPH–mediated CD8⁺ T cell preactivation, the method was similar to the above NP-DOX, but laser irradiation (671 nm, 200 mW/cm², 1 min) was needed after 24-hour incubation of the HPPH formulations.

Naïve T cells were extracted from spleens of C57BL/6 mice. The spleens were cut into pieces and digested by collagenase and deoxyribonuclease (DNase) for 30 min at 37°C. Then, the tissues were lysed by red blood cell (RBC) lysis buffer with centrifugation to remove RBCs. After filtration through a 70-µm filter, lymphocytes were isolated via ficoll-Paque Plus (VWR) according to the manufacturer’s protocol.

**In vivo NIR imaging**

Female C57BL/6 mouse (6 to 8 weeks, 20 g) was inoculated 0.5 x 10⁶ EG7-OVA cells at right flank. Two weeks later, NP-OVA-Cy5.5 was administered via subcutaneous injection at right foot pad. Mice were anesthetized and imaged using the PerkinElmer’s in vivo imaging system (IVIS spectrum) at 1, 4, 8, 24, and 48 hours. NP-HPPH was injected intravenously via tail vein in EG7-OVA tumor–bearing C57BL/6 mouse. The biodistribution of NP-HPPH was monitored using IVIS spectrum at 1, 4, 8, 24, and 48 hours. At the last time point, mice were sacrificed. Tumor and normal organs including heart, liver, spleen, lung, and kidney were extracted for ex vivo imaging. To explore whether naAPCs could accumulate in tumor tissue, we took BCNV-OVA-Cy5.5 as an example for the NIR imaging. Similarly, BCNV-OVA-Cy5.5 was carried out intravenous injection via tail vein, and the in vivo distribution of which was observed through IVIS spectrum at 1, 4, 8, 24, and 48 hours. At the last time point, mice were euthanized and processed for ex vivo imaging.

**In vivo CD8⁺ T cell activation via NP-drug/naAPCs**

To study the in vivo CD8⁺ T cell activation, 0.5 x 10⁶ EG7-OVA cells per mouse were inoculated at the right flank of C57BL/6 mice. When tumor reached 200 to 300 mm³, mice were randomly divided into eight groups: PBS, naAPCs, NP-OVA, NP-OVA/naAPCs, NP-DOX, NP-DOX/naAPCs, NP-HPPH, and NP-HPPH/naAPCs (n = 3). Furthermore, NP-OVA and NP-OVA/naAPCs were administered via subcutaneous injection at right foot pad. Simultaneously, mice treated with PBS, naAPCs, NP-DOX, NP-DOX/naAPCs, NP-HPPH, and NP-HPPH/naAPCs were performed intravenous injection via tail vein. Mice treated with NP-HPPH received laser irradiation (671 nm, 200 mW/cm², 10 min), 24 hours after the injection. For mice treated with naAPC’s combination with nanotechnology, naAPCs were administered via intravenous injection after NP-OVA, NP-DOX, and NP-HPPH treatment for 48 hours. After 48 hours, all mice were euthanized with tumors extracted. The tumors were cut into pieces and digested by collagenase and DNase for 2 hours at 37°C. After filtration through 70-µm filter, the tissues were centrifuged and stained with APC anti-CD8α and PE anti-CD3e. After PBS washing and centrifugation, cells were suspended in 0.5 ml of PBS and measured by flow cytometer. CD8⁺ CD3⁻ T cell numbers in tumor tissues were calculated and compared among different groups.

**In vivo DC maturation and in vivo ICD detection**

Tumor tissues from mice treated with NP-DOX and NP-HPPH formulations were cut into pieces, digested by collagenase and DNase, filtered by 70-µm filter, and stained with PE anti-SIINFEKL H-2Kb and PerCP-Cy5.5 anti-CD80. Eventually, cells were suspended in 0.5 ml of PBS and detected by flow cytometer.

To investigate the in vivo ICD, tumor tissues from mice with NP-DOX and NP-HPPH treatment were processed by immunofluorescence staining. In detail, tumor tissues were placed in Tissue-Tek O.C.T. Compound (Sakura) at −80°C for 48 hours. Then, tumors were processed as tissues sections via a freezing microtome (Thermo Fisher Scientific). Each tissue section was put on individual microslides and fixed with Z-Fix solution. Mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) was applied, and tissues
were also stained with anti-Alexa Fluor CRT-647 (Abcam) to identify CRT exposure. Tissue sections were imaged via CLSM.

**In vivo antitumor efficacy of NP-drug/naAPCs**

All animal experiments were performed under an NIH Animal Care and Use Committee–approved protocol. Female C57BL/6 mice (6 to 8 weeks, 18 to 20 g) were inoculated EG7-OVA cells (0.5 × 10⁶ per mouse) at right flank. When tumor grew to around 130 mm³ at day 8 after tumor inoculation, mice were randomly divided into eight groups: PBS, naAPCs, NP-OVA (OVA dose, 20 μg per mouse), NP-OVA/naAPCs (OVA, 20 μg per mouse; IL-2, 50 ng per mouse), NP-DOX (DOX, 0.3 mg/kg), NP-DOX/naAPCs (DOX, 0.3 mg/kg; IL-2, 50 ng per mouse), NP-HPPH (HPPH, 0.5 mg/kg), and NP-HPPH/naAPCs (HPPH, 0.5 mg/kg; IL-2, 50 ng per mouse) (n = 5). Moreover, NP-OVA and NP-OVA/naAPCs were administered via subcutaneous injection at right foot pads of mice, while the other groups were given as intravenous injection via tail vein. Thereafter, tumor volume and body weight of all the mice were measured every 2 days from days 8 to 18 after tumor inoculation. The tumor volume was calculated according to the formula

\[ V = \frac{1}{2}(L \times W^2) \]

At day 18, mice were sacrificed. Tumors and normal organs were harvested for H&E staining. Images were acquired using a digital microscope (Olympus BX41). Moreover, tumors from mice in each group were weighed. Afterward, tumor tissues were digested, filtered, centrifuged, and stained with 0.5 ml of PBS, the procedures of which are similar to the above in vivo CD8⁺ T cell activation section. CD8⁺ ratio in CD3⁺ T cells and CD8⁺ CD3⁺ T cells numbers in tumor tissues were evaluated according to flow cytometry results. To investigate the OVA-specific CD8⁺ T cell proliferation, partial tumor cells were stained with APC anti-CD8a and PE-conjugated SIINFEKL H-2K⁺ tetramer (MBL International Corporation). After PBS washing, centrifugation, and suspension in 0.5 ml of PBS, the cells were also tested by flow cytometry.

To acquire the immunofluorescence staining images, some tumors were embedded with Tissue-Tek O.C.T. Compound at ~80°C for at least 48 hours. The tissues were processed and placed onto microslides. Then, the tissue sections were fixed by Z-fix solution, stained with PE anti-CD3e and APC anti-CD8a, infiltrated with mounting media with DAPI, covered by coverslip, and sealed with nail polish. The images of tumor tissues were acquired by CLSM.

**Statistical analysis**

All data analysis was executed using GraphPad Prism 7.0 software where one-way analysis of variance (ANOVA) was used. A value of *P* < 0.05 was regarded as statistically significant. **P** < 0.01 and ***P*** < 0.001.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/50/eabd1631/DC1

View/request a protocol for this paper from Bio-protocol.
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Size-transformable antigen-presenting cell–mimicking nanovesicles potentiate effective cancer immunotherapy

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