Supporting Information

Cancer cell membrane-coated nanoparticles for anticancer vaccination and drug delivery

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Materials and Methods

Cancer cell membrane derivation. B16-F10 mouse melanoma cells (ATCC: CRL-6475) and MDA-MB-435 human melanoma cells (provided by Dr. Erkki Ruoslahti) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (Invitrogen). To harvest membrane, cells were grown in T-175 culture flasks to full confluency and detached with 2 mM ethylenediaminetetraacetic acid (EDTA, USB Corporation) in phosphate buffered saline (PBS, Invitrogen) and washed in PBS three times by centrifuging at 500 × *g*. The cells were suspended in a hypotonic lysing buffer consisting of 20 mM Tris-HCl pH = 7.5 (Mediatech), 10 mM KCl (Sigma Aldrich), 2 mM $MgCl_2$ (Sigma Aldrich), and 1 EDTA-free mini protease inhibitor tablet (Pierce) per 10 mL of solution and disrupted using a dounce homogenizer with a tight-fitting pestle. The entire solution was subjected to 20 passes before spinning down at 3,200 × *g* for 5 min. The supernatant was saved while the pellet was resuspended in hypotonic lysing buffer and subjected to another 20 passes and spun down again. The supernatants were pooled and centrifuged at 20,000 × *g* for 20 min, after which the pellet was discarded and the supernatant was centrifuged again at 100,000 × *g*. The pellet containing the plasma membrane material was then washed once in 10 mM Tris-HCl pH = 7.5 and 1 mM EDTA. The final pellet was collected and used as purified cancer cell membrane for subsequent experiments.

CCNP synthesis and characterization. To synthesize CCNPs, a previously reported extrusion approach was used[1]. Briefly, poly(lactic-*co*-glycolic acid) (PLGA, Lactel Absorbable Polymers) cores approximately 80-90 nm in size were prepared using a nanoprecipitation method. To prepare cancer cell membrane vesicles, membrane material derived as described above was physically extruded through a 400 nm polycarbonate membrane for 11 passes. The

resulting vesicles were then coated onto PLGA cores by co-extruding vesicles and cores through a 200 nm polycarbonate membrane. A ZEN 3600 Zetasizer (Malvern) was used to take dynamic light scattering (DLS) measurements for the characterization of particle size and zeta potential. Samples were suspended in water at 1 mg mL^{-1}. All measurements were done in triplicate at room temperature. Transmission electron microscopy (TEM) imaging was carried out by first glow discharging carbon-coated 400 square mesh copper grids (Electron Microscopy Sciences). Particles at 1 mg mL^{-1} were left on the grid for 1 min before being washed off with 10 drops of water. Grids were then negatively stained with 3 drops of 1% uranyl acetate (Sigma Aldrich). Excess solution was wicked away with absorbent paper and the samples were imaged using a Tecnai G2 Sphera (FEI) microscope at 200 kV.

Cancer cell membrane protein characterization. For protein characterization by SDS-PAGE, all samples were prepared at a final protein concentration of 1 mg mL^{-1} in lithium dodecyl sulfate (LDS) loading buffer (Invitrogen) as measured by a BCA assay (Pierce). CCNPs were purified by centrifugation at 18,000 × *g* to pellet the coated particles but not free vesicles or protein. Samples were heated to 70°C for 10 min and 20 μL of sample was loaded into each well of a NuPAGE Novex 4-12% Bis-Tris 10-well minigel (Invitrogen) in MOPS running buffer (Invitrogen) in an XCell SureLock Electrophoresis System (Invitrogen) based on the manufacturer's instructions. Protein staining was accomplished using SimplyBlue (Invitrogen) and destained in water overnight before imaging. For western blot analysis, protein was transferred to Protran nitrocellulose membranes (Whatman) using an XCell II Blot Module (Invitrogen) in NuPAGE transfer buffer (Invitrogen) per manufacturer's instructions. Membranes were probed using antibodies against pan-cadherin (C3678, Sigma Aldrich), Na^+/K^+-ATPase (A01483, GenScript), gp100 (EP4863(2), Abcam), histone H3 (Poly6019, Biolegend),

cytochrome c oxidase (A01396, GenScript), or glyceraldehyde 3-phosphate dehydrogenase (GT239, GeneTex) along with either horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Poly4053, Biolegend) or anti-rabbit IgG (Poly4064, Biolegend). Films were developed using ECL western blotting substrate (Pierce) and developed with the Mini-Medical/90 Developer (ImageWorks).

CCNP optimization and stability studies. To optimize the membrane ratio, CCNPs were synthesized at membrane-to-core weight ratios ranging from 0.125 to 4 mg of protein per 1 mg of PLGA. PLGA cores with no membrane were used as a control. Particle sizes were measured in triplicate by DLS right after synthesis, after adjusting the solution to 1X PBS using a 2X PBS stock, and over time for a period of 15 days in 1X PBS. To obtain dendritic cells for the fluorescent co-localization study, cells from the femur bone marrow of 6-week old female C56BL/6 mice (Charles River Laboratories) were derived and plated on non-treated 100 mm petri dishes at 1×10^6 cells per plate in Iscove's Modified Dulbecco's Media (IMDM, Mediatech) supplemented with 10% FBS, 2 mM L-Glutamine (Invitrogen), 50 μM β-mercaptoethanol (Invitrogen), penicillin-streptomycin, and 20 ng mL^{-1} recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF, Biolegend). On days 3, 6, and 8 after plating, half of the media was replaced with fresh media. On day 10 the cells were collected by scraping, washed in PBS, and plated at 50% confluency on 8-well Lab-Tek II CC2 chamber slides (Nunclon). CCNPs were synthesized using membrane labeled with NHS-fluorescein (Pierce). For the labeling, 10 μL of 10 mg mL^{-1} NHS-fluorescein in dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to 500 μg of cancer cell membrane vesicles. After 1 h, the membrane was coated onto PLGA cores loaded with 0.1 wt% 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo dicarbocyanine (DiD, Invitrogen) and the resulting CCNPs were purified using a Sepharose CL-

4B (Sigma Aldrich) size exclusion column. CCNPs were incubated at a final concentration of 100 μg mL^{-1} with the cells for 2 h. Afterwards, the cells were washed 3 times with PBS, fixed with 10% formalin (Millipore) and mounted with DAPI-containing Vectashield (Invitrogen). Imaging was done using a 100X oil immersion objective on an Applied Precision DeltaVision Deconvolution Scanning Fluorescence Microscope, and software deconvolution was done post-acquisition.

Dendritic cell maturation and T-cell activation studies. To test dendritic cell maturation, bone marrow-derived dendritic cells were cultured as described above and plated at 50% confluency in tissue culture-treated 6-well plates. CCNP samples were incubated in triplicate at a final concentration of 80 μg (polymer weight) per 1 mL media at a membrane-to-core ratio of 0.5 mg protein per 1 mg PLGA. Monophosphoryl lipid A (MPLA, Sigma) was incorporated with the CCNPs at 1 wt% of the polymer weight by gently agitating the sample in a Fisher Scientific FS30D bath sonicator. Samples were allowed to incubate for 48 h before the cells were collected by scraping and washed 3 times using PBS with 2% bovine serum albumin (BSA, Sigma Aldrich). The cells were then immunostained with FITC-conjugated anti-CD11c (N418, Biolegend) and Alexa-647-conjugated anti-CD40 (HM40-3, Biolegend), anti-CD80 (16-10A1, Biolegend), or anti-CD86 (GL-1, Biolegend) for 20 min on ice in the dark. Cells were washed twice more in PBS with 2% BSA before analysis. Flow cytometry data was collected using a Becton Dickinson FACSCanto II and analyzed using Flowjo software from Treestar.

To test for T-cell stimulation, bone marrow-derived dendritic cells were first plated at 75% confluency in 24-well plates. Dendritic cells were pulsed with CCNP samples in triplicate for 24 h at a concentration of 80 μg (polymer weight) per 1 mL media. Splenocytes were derived from a 10-week old female transgenic pmel-1 mouse (The Jackson Laboratory) and plated with

the pulsed dendritic cells at a density of 2×10^6 cells per well. Cells were co-cultured for 3 days. At each 24 h timepoint, a small sample of media was taken from the supernatant and replaced with fresh media. Interferon gamma (IFNγ) concentration was assayed using an IFNγ ELISA Ready-SET-Go! kit (eBioscience) per the manufacturer's instructions. At the conclusion of the study, the cells were gently washed with PBS and fixed in 10% formalin. Brightfield phase contrast imaging was performed using a Nikon TE300 inverted microscope.

Homotypic targeting studies. For flow cytometric analysis, MDA-MB-435 or HFF-1 (ATCC: SCRC-1041) human foreskin fibroblast cells were plated at 50% confluency in 6-well plates. RBCNPs were synthesized as previously reported[1]. All samples contained PLGA cores fluorescently labeled with 0.1 wt% DiD. CCNPs, RBCNPs, or bare PLGA cores were incubated with the cells in triplicate at a final concentration of 150 μg (polymer weight) per 1 mL media for 30 min. Afterwards, the wells were washed 3 times with PBS before incubating for 1 h in cell culture media. The cells were then detached with trypsin-EDTA solution (Invitrogen), washed twice with media, and data was collected using a Becton Dickinson FACSCanto II. Analysis was done using Flowjo software from Treestar.

For fluorescent imaging, MDA-MB-435 cells were plated at 75% confluency on 8-well Lab-Tek II CC2 chamber slides. DiD-labeled CCNPs, RBCNPs, or bare PLGA cores were incubated with the cells at a final concentration of 150 μg (polymer weight) per 1 mL media for 30 min. Afterwards, the wells were washed 3 times with PBS before incubating for 1 h in cell culture media. The wells were then washed 3 times with PBS, fixed with 10% formalin and mounted with DAPI-containing Vectashield. Imaging was done using a 20X objective on a Keyence BZ-9000 Fluorescence Microscope.

Supporting Figures

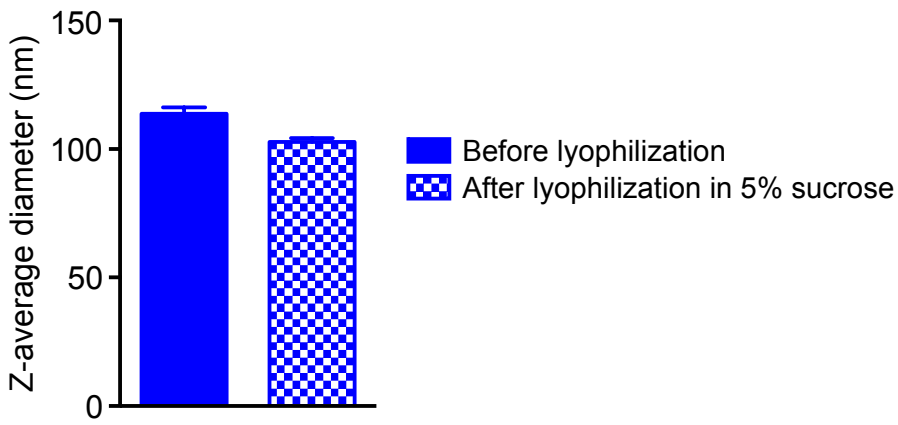

Figure S1. CCNP storage stability. CCNPs were synthesized at 1 mg mL^{-1}. The volume was then doubled using 10 wt% sucrose solution for a final particle concentration of 0.5 mg mL^{-1} in 5 wt% sucrose solution. Particles were measured by dynamic light scattering (DLS) before and after lyophilization. Bars represent means ± SD (n=3).

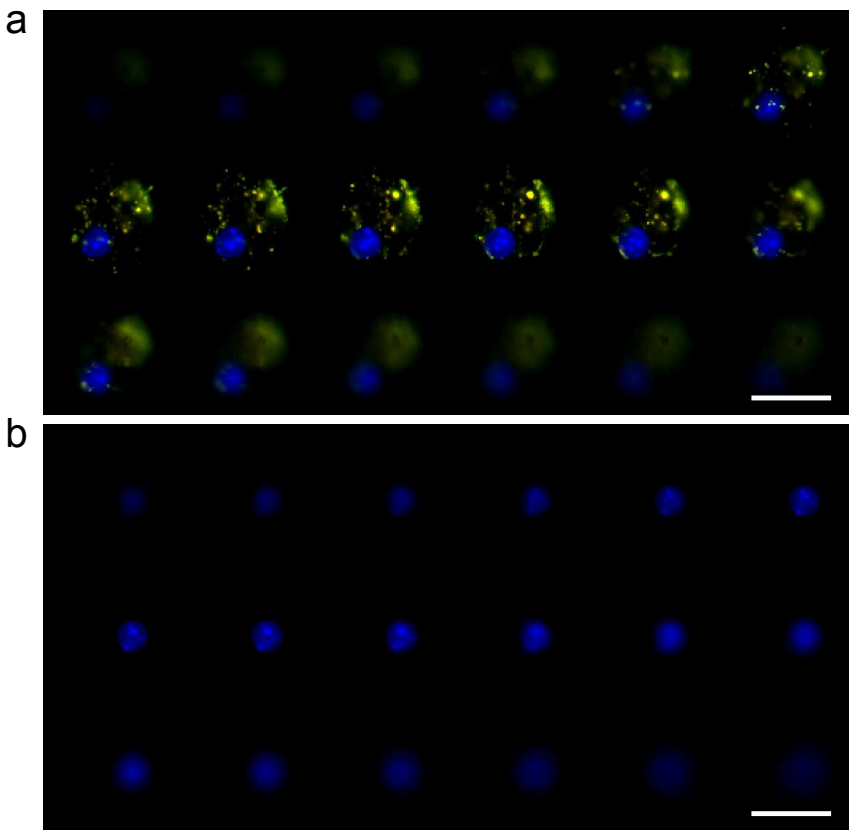

Figure S2. Intracellular uptake of CCNPs and cancer cell membrane vesicles. Samples were incubated with bone marrow-derived dendritic cells for 2 h at a concentration of 100 μg mL^{-1} polymer weight (or equivalent amount of vesicles) before imaging. Red channel = PLGA cores loaded with DiD; green channel = cancer cell membrane labeled with fluorescein; blue channel = nuclei stained with DAPI. **(a)** CCNPs show strong signal when nucleus is in focus (middle row), demonstrating cellular uptake. **(b)** Vesicles at the same exposure and gain on the green channel as **(a)** show an insignificant amount of uptake.

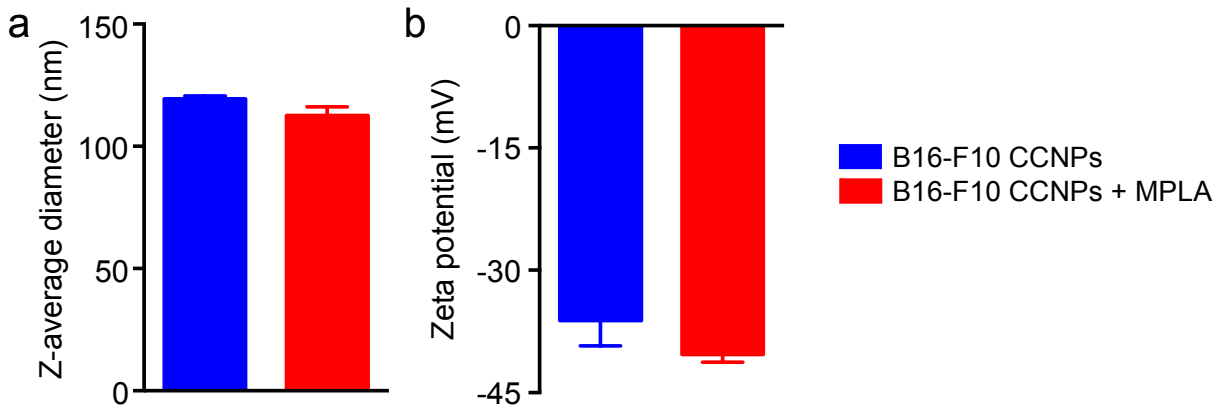

Figure S3. Physicochemical characterization of CCNPs with adjuvant. MPLA was incorporated with CCNPs at 1 wt% of polymer weight by gentle bath sonication. **(a)** Particle size and **(b)** surface zeta potential were measured by DLS and show little change after introduction of the adjuvant. Bars represent means ± SD (n=3).

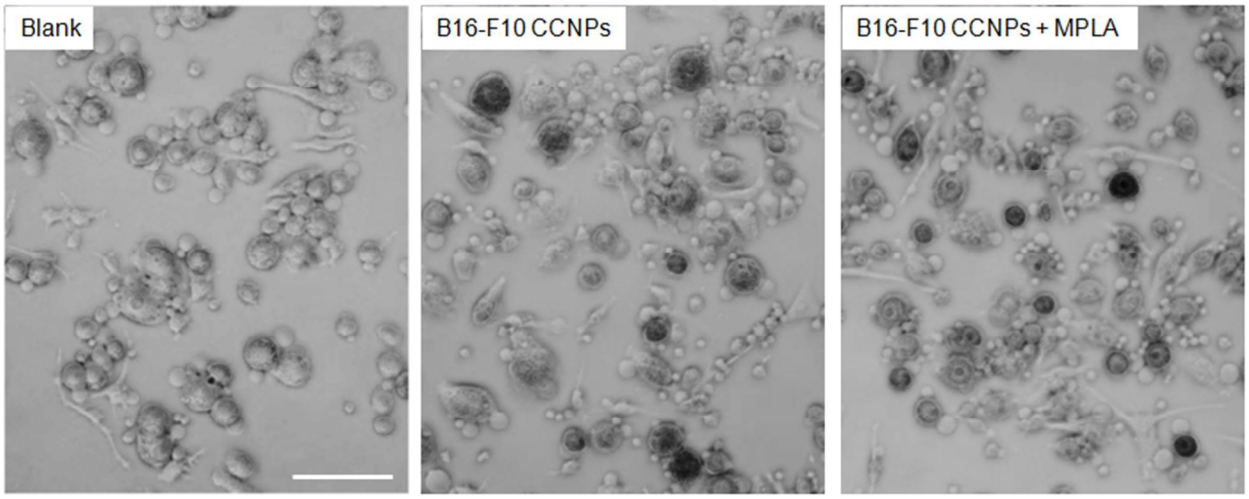

Figure S4. Phase contrast microscopy images of CCNP-pulsed dendritic cells co-incubated with pmel-1 splenocytes. Samples were pulsed into dendritic cells for 24 h before co-incubation with splenocytes for 72 h. Loosely adherent cells were gently washed away with PBS and the remaining cells were fixed in 10% formalin. Nanoparticle uptake can be visualized by darkening of cells. Stimulated T-lymphocytes can be found interacting with dendritic cells.

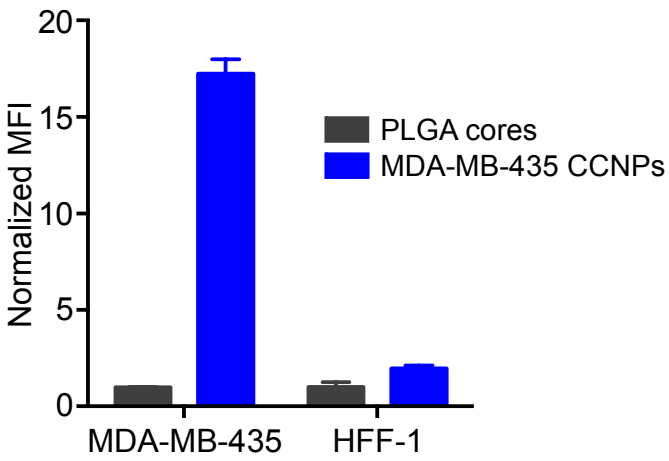

Figure S5. Comparison of preferential uptake of CCNPs by the source cell line versus a heterotypic normal cell line. PLGA cores or MDA-MB-435 CCNPs were incubated with either MDA-MB-435 cells or HFF-1 cells for 30 min before washing away particles and incubating for another 1 h in fresh media. Cells were detached with trypsin-EDTA and analyzed by flow cytometry. Mean fluorescence intensity (MFI) was independently normalized to the signal of the PLGA cores sample within each cell line.

Reference

(1) Hu, C. M.; Zhang, L.; Aryal, S.; Cheung, C.; Fang, R. H.; Zhang, L. *Proc. Natl. Acad. Sci. U.S.A.* **2011,** *108*, 10980-10985.