Supporting Information

"Modular Vaccine Design Using Carrier-Free Capsules Assembled from Polyionic Immune Signals"

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Supporting Methods

Materials. SIINFEKL (SIIN) and SIINFEKL-R_9 (SIIN*) were synthesized by Genscript with >98% purity, with or without a FITC label on the N-terminus. Polyinosinic-polycytidylic acid, low molecular weight (polyIC) was purchased from Invivogen. Non-immunostimulatory control oligonucleotide (ODN, TCCTGAGCTTGAAGT) was synthesized with a phosphorothioate backbone by IDT. Label-IT nucleic acid labeling kits (Cy5) were purchased from Mirus Bio LLC. PolyIC was labeled according to the manufacturer's protocol.

iPEM capsule synthesis. iPEM capsules were synthesized by coating sacrificial colloidal supports with PEMs consisting of SIIN* and polyIC, followed by removal of the core. To form the sacrificial templates from $CaCO_3$, spherical particles were precipitated by adding equal volumes of 0.33 M $CaCl_2$ (Sigma) into 0.33 M $Na_2CO_3.2H_2O$ (Sigma) while mixing at 800 rpm on a stir plate for 5 min. SIIN* and polyIC were prepared in PBS with 0.5 M NaCl and adjusted to the indicated pH values using 0.1 M NaOH. Wash buffer consisted of pH 8, 0.05 M NaCl in PBS. 300 μL of $CaCO_3$ containing 3.69 mg of particles was initially washed twice with wash buffer by incubating particles for 30 sec, then centrifuging particles for 5 sec using a Quickspin Micro 1207 Microcentrifuge (VWR). This sequence was repeated for the second wash. The templates were then suspended in 300 μL of SIIN* solution (1.0 mg/mL) for 1 min and washed three times as above. The washed particles were then collected and suspended for 1 min in 300 μL of either polyIC (1.0 mg/mL) or ODN (1.0 mg/mL). Particles

were washed three times as above and the sequence was repeated for up to 3 cycles to form $CaCO_3$ particles coated with $(SIIN^*/polyIC)_3$ or $(SIIN^*/ODN)_3$. The sacrificial templates were removed by collecting the particles with centrifugation (1 min, 1000 g), followed by resuspension in 300 μL of 0.1 M EDTA at the indicated pH values for 30 min. Particles were then washed twice to remove EDTA and finally resuspended in PBS.

iPEM characterization. iPEM build up on silicon and quartz chips was measured by a LSE stokes ellipsometer (Gaertner Scientific Corporation) and Evolution 60 UV-visible spectrophotometer (Thermo Scientific) to assess iPEM thickness and relative cargo loading, respectively. UV-visible spectrophotometry was used to assess relative cargo loading on quartz chips by measuring absorbance values from 200 nm to 700 nm at 1 nm intervals using a solid state sample holder. Wavelengths of 260 nm and 508 nm indicated loading of nucleic acid and peptide, respectively. At least five regions throughout each chip were measured after every 2 bilayers. A Leica SP5X confocal microscope was used to visualize co-localization of both fluorescently-tagged SIIN* (FITC) and polyIC (Cy5) in iPEM capsules. Loading of antigen and adjuvant on sacrificial cores was assessed by measuring the absorbance of the nucleic acid (260 nm) and peptide (FITC, 495 nm) dipping solutions and wash buffer by UV-visible spectrophotometery. Loading of immune signals in iPEM capsules was assessed by incubating capsules in 300 μL of trypsin (0.05%) at 37°C for 1 hr, then measuring the peptide (FITC; Ex: 495, Em: 520) and polyIC (Cy5; Ex: 650, Em: 670) signals by fluorimetry using a Gemini XPS fluorescence microplate reader (Molecular Devices). Capsule sizes were measured using ImageJ to analyze diameters of at least 50 particles. For stability studies, capsules were incubated in PBS, incomplete media (RPMI), or complete DC media (with 10% FBS; detailed below) at 37 °C and the size was measured at the indicated times.

***In vitro* dendritic cell studies.** All animal and cell experiments were approved by the institutional animal care and use committee (IACUC) at University of Maryland, College Park. Splenic dendritic cells (DCs) were isolated from C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME) with CD11c positive magnetic isolation kit (Miltenyi) following the manufacturer's instructions. Briefly, after euthanizing mice, spleens were harvested and minced by forceps. Minced spleens were incubated with 4 mL of spleen dissociation medium (Stemcell) for 30 min at 37°C followed by homogenizing with a 16 G needle and 3 mL syringe. To the homogenized medium, 80 µL of 0.5 M EDTA was added at a final concentration of 10 mM and incubated for 5 min at room temperature, then passed through a 70 µm cell strainer (BD Biosciences) and centrifuged at 300 g for 10 min at 4°C to acquire splenocytes. Cells were then resuspended with 400 µL of MACS buffer (1% BSA + 2 mM EDTA in PBS) per 10^8 cells and mixed with 100 µL of CD11c microbeads per 10^8 cells and incubated for 15 min at 4°C. After incubation, cells were washed with 49 mL buffer and centrifuged at 200 g for 10 min at 4°C. Washed cells were then re-suspended in 500 µL buffer per 10^8 cells and passed through a pre-wetted LS column (Miltenyi) in a magnet followed by washing three times with 3 mL MACS buffer. After the last wash, the LS column was removed from the magnet and flushed with 5 mL buffer. The flushed cell suspension was then centrifuged at 300 g for 10 min at 4°C to collect pelleted cells. The cell pellet was then resuspended with 1 mL DC medium (RPMI1640, 10% FBS, 0.5% Penicillin Streptomycin, 50 µM 2- mercaptoethanol (2-ME)) prior to use.

For cytokine studies, capsules were serially diluted and added to DCs ($1x10^5$ cells/well) to reach final capsule concentrations of 42, 21, 10, 5, 3, or 1 µg/mL. Controls included untreated DCs, soluble peptide SIIN (5 µg/mL), LPS (1 µg/mL), polyIC (10 µg/mL), LPS (1 µg/mL) + SIIN (5 µg/mL), and polyIC (10 µg/mL) + SIIN (5 µg/mL). After 24 hrs, supernatants were collected and analyzed by ELISA.

TLR3 signaling assay. The ability of iPEMs to activate TLR3 signaling was investigated using HEK-Blue mTLR3 reporter cells (Invivogen). Cells were seeded in 96 well plates with $5x10^5$ cells/well in 200 µL of HEK-Blue™ detection medium. Cells were treated with TLR2a: Pam3CSK4 (200 ng/mL; Invivogen), TLR3a: polyIC (10 µg/mL; Invivogen), TLR4a: LPS (1 µg/mL; Sigma), non-immunogenic control ssDNA: ODN (10 µg/mL), iPEM capsules $(SIIN*/polyIC)_3$, or non-immunogenic iPEM capsules $(SIIN*/ODN)_3$. After 24 hrs, TLR3 signaling was measured by absorbance at 625 nm using a spectrophotometer.

***In vitro* CD8⁺ T cell expansion.** OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) were purchased from The Jackson Laboratory (Bar Harbor, ME).[1] Three days after immunization, CD11c-enriched DCs from naïve, soluble vaccine-immunized, and iPEM capsule-immunized C57BL/6 mice were isolated. T cells were isolated from the spleens of OT-I mice using a negative selection CD8 isolation kit (Stemcell). Briefly, splenocytes were resuspended at $1x10^8$ cells/mL (up to 8 mL total volume) followed by adding 50 µL of normal rat serum (Stemcell) per 1 mL of cells. After mixing, 50 µL of mouse $CD8^+$ T Cell Isolation Cocktail (Stemcell) per 1 mL of cells was added and incubated at room temperature for 10 min. Streptavidin RapidSpheres (Stemcell) were then added to the cell suspension at 125 µL/mL of cells and incubated at room temperature for 5 min. After incubation, the cell suspension volume was brought to a total volume of 5 mL (for $<4x10^8$ cells) with recommended medium (0.5% BSA and 0.2 mM EDTA in PBS). The cell suspension was placed in a 14 mL tube without a cap in the magnet for 2.5 min and carefully the desired $CD8^+$ T cells were poured into a new tube. Resulting cells were washed twice to remove any serum and labeled with 5 µM of proliferation dye eFluor 670 (eBioscience) for 10 min at 37°C in the dark. After 10 min, the dye was neutralized with 5 times volume of T cell medium (RPMI1640, 10% FBS, 1x non-essential amino acid, 10 mM HEPES, 2 mM L-glutamine, 0.5% Penicillin

Streptomycin, 50 μM 2-ME) followed by washing three times with T cell medium. Resulting cells ($3x10^5$ cells/50 μL) were added into wells containing DCs/capsules and incubated for 48 hrs for the T cell co-culture assay. After 48 hrs, the resulting cell population was divided into two portions for proliferation analysis and intracellular cytokine staining (ICCS).

To assess T cell proliferation, cells were blocked with anti-CD16/32 as described previously and stained with anti-CD3e (PE-Cy7) and anti-CD8a (APC) for $CD8^+$ T cells. For ICCS staining, culture medium was replaced with T cell medium supplemented with 1/1000 dilution of brefeldin A (BFA, eBioscience) and incubated for 4 hrs at 37 °C. Cells were then washed with ice cold FACS buffer, and blocked with anti-CD16/32 for 10 min, followed by staining for CD3e (PE-Cy7) and CD8a (APC) for 20 min at 4°C. After surface staining, cells were washed twice with ice cold FACS buffer and fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences). Briefly, 100 μL of fixation solution was added to each well and incubated at 4°C for 20 min followed by washing twice with 200 μL of permeabilization washing buffer. Anti-IFN-γ (PE) antibody was diluted in permeabilization washing buffer and cells were stained for 30 min at 4°C. After staining, cells were washed twice with 200 μL of permeabilization washing buffer and resuspended in 100 μL FACS buffer prior to flow cytometry analysis.

Immunization. Six to eight week old C57BL/6 female mice from The Jackson Laboratory, in groups of 8 were used in immunization studies, along with untreated control groups (N=4). Mice were immunized by intradermal injection (25 μL) on each flank with either capsules, free SIINFEKL and polyIC, or left untreated. Capsule vaccines and soluble vaccines formulated in simple mixtures were prepared and administered using matching doses of peptide (60 μg) and polyIC (240 μg). Mice were injected at day 0 and in some studies boosted at day 15 and day 28 as indicated in the main text.

Tumor studies. Six to eight week old C57BL/6 female mice from The Jackson Laboratory were randomized in groups of 6. Mice were then immunized at day 0 and boosted at day 15 and day 28 with the formulations indicated in the main text. At day 36, mice were inoculated subcutaneously in the flank using an aggressive dose of 1 x 10^6 B16 tumor cells expressing OVA. Tumor cells were a generous gift from Dr. Kenneth Rock. Body weight was monitored, and tumor burdens were calculated daily as the product of two orthogonal diameters. Mice were euthanized according to IACUC-approved humane endpoints when the aggregate burden reached 150 mm^2.

***In vivo* analysis of antigen-specific CD8$^+$ T cell expansion.** Following immunization, blood samples were collected by submandibular bleeding at days 7, 14, 22, 29, and 41. Blood was treated with 1 mL ACK lysing buffer (Life Technologies) for 3 min and centrifuged at 500 g for 5 min. This process was repeated and cells were then washed once in PBS. To assess the frequency of antigen-specific CD8$^+$ T cells, samples were blocked with anti-CD16/32 for 10 min, followed by staining with MHC-I SIINFEKL tetramer (PE-conjugated, MBL International Corp.) for 30 min. Cells were then stained with anti-CD8a (APC) for 20 min, washed twice with FACS buffer, and resuspended in 100 μL FACS buffer containing DAPI for measurement by flow cytometry.

ELISA assay. All ELISA assays were conducted using mouse IL-1β, IL-6, and IFN-γ OptEIA reagents according to the manufacturer's instructions (BD Biosciences). Supernatants were collected and analyzed without purification using 4-10x dilutions.

Mechanistic *in vivo* studies. Six to eight week old C57BL/6 female mice from The Jackson Laboratory were immunized by intradermal injection with capsules, soluble SIIN and polyIC, or left untreated as above. Three days after immunization, DCs from draining lymph nodes and spleens were isolated by positive CD11c selection as described above. Cells were then

stained with antibodies against classical DC activation markers and analyzed by flow cytometry, as above. To test if DCs isolated from immunized mice present peptides from iPEM capsules in a manner that can expand antigen-specific $CD8^+$ T cells (OT-I), DCs isolated from iPEM-immunized mice on Day 3 were co-cultured with $CD8^+$ T cell from OT-I mice for 48 hrs. Proliferation and cytokine secretion were then assessed by fluorescence dilution assays and ELISA as described above. For immunohistochemical analysis, lymph nodes were removed on day 3, frozen, sectioned at 10 μm intervals, then the tissue was fixed. Fixed sections were blocked with 5% donkey serum (Sigma) and 5% goat serum (Sigma) in PBS for 30 min. After a PBS wash, samples were stained for T cells with a purified rabbit anti-mouse antibody (CD3e, Abcam) for 1 hr at room temperature, then washed twice and stained with a fluorescently-conjugated antibody for B cells (rat anti-mouse B220 APC, eBioscience) and a goat anti-rabbit antibody (Dylight 405, Jackson Immunoresearch). Stained sections were washed then fixed in 4% paraformaldehyde before quenching in 1% glycerol. Sections were then mounted with Prolong Diamond Antifade Mountant (Life Sciences), and imaged.

Statistical Analysis. Statistical analysis was carried out using one way analysis of variance (ANOVA) with a Tukey post test in GraphPad Prism v.6.02. Survival analysis was carried out using a Logrank test. Statistical significance was defined at p values ≤ 0.05 (95% confidence interval) and indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Supporting Figures

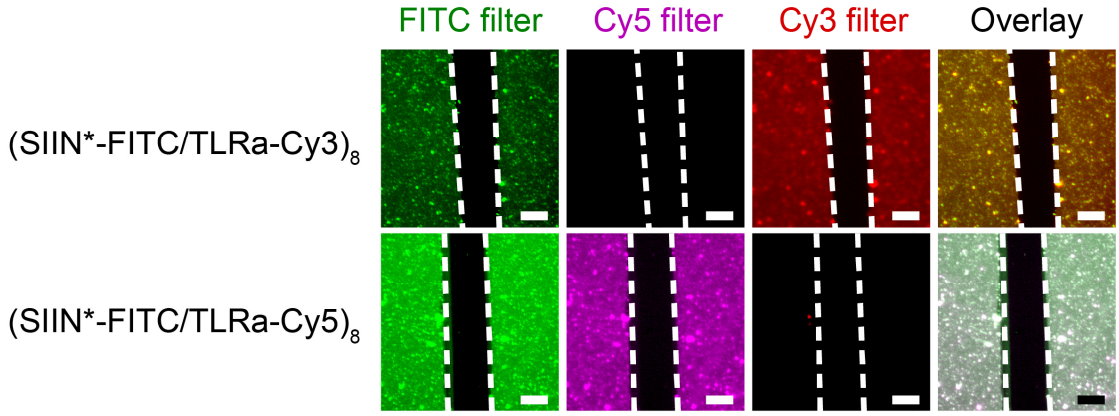

Figure S1. Fluorescently-labeled antigen (FITC, green) and adjuvant (Cy3, red; Cy5, magenta) can be independently visualized without signal overlap between filter sets. iPEMs were assembled on quartz microscope slides using the indicated components, then a needle was used to remove of portion of the film to provide contrast for imaging (white lines).

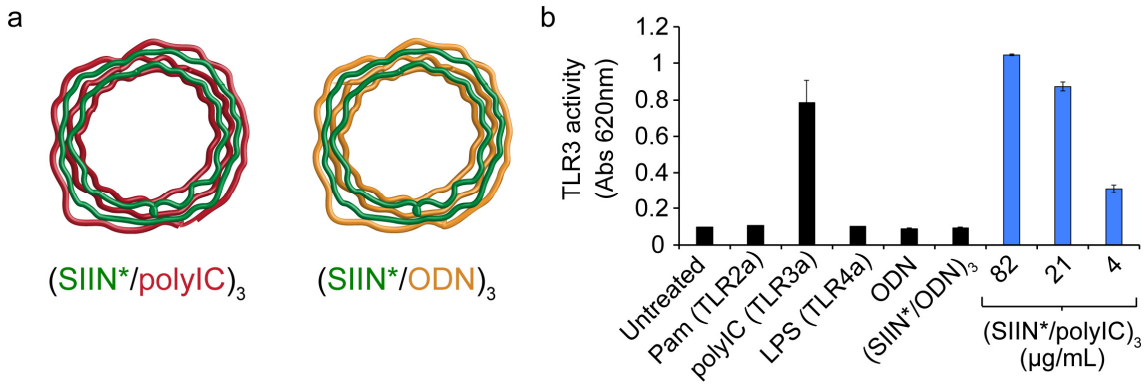

Figure S2. iPEM capsules efficiently trigger TLR-specific signaling. a) Schematic illustration of iPEM capsules assembled from SIIN* and polyIC (immunogenic) or SIIN* and a non-immunogenic control oligonucleotide, ODN. b) Quantification of TLR3-specific signaling in reporter cells following treatment using iPEM capsules designed with an architecture of $(SIIN*/polyIC)_3$ or $(SIIN*/ODN)_3$, or controls of Pam3CSK4 (TLR2a), LPS (TLR4a), or ODN. Data are representative of 2-3 studies conducted in triplicate. Values for all panels indicate the mean ± s.e.m.

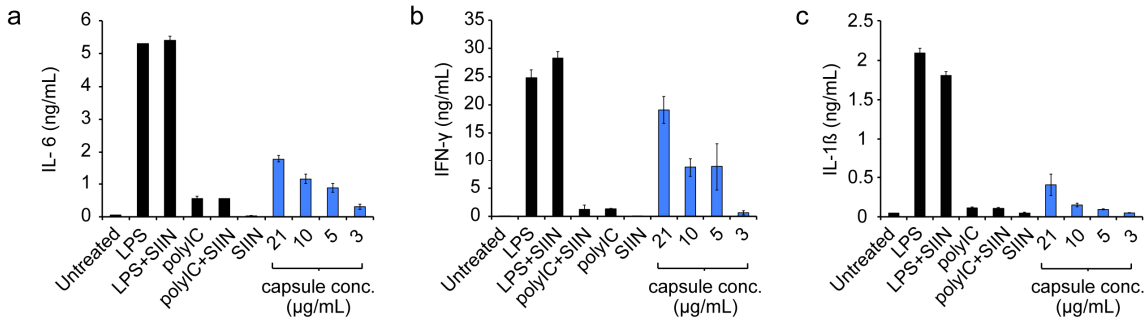

Figure S3. iPEM capsules induce proinflammatory cytokines. ELISA was used to measure the secretion of the pro-inflammatory cytokines (a) IL-6, (b) IFN-γ, and (c) IL-1β during primary DC culture measured by ELISA. Values for all panels indicate the mean ± s.e.m. Data are representative of 2-3 experiments each conducted in triplicate.

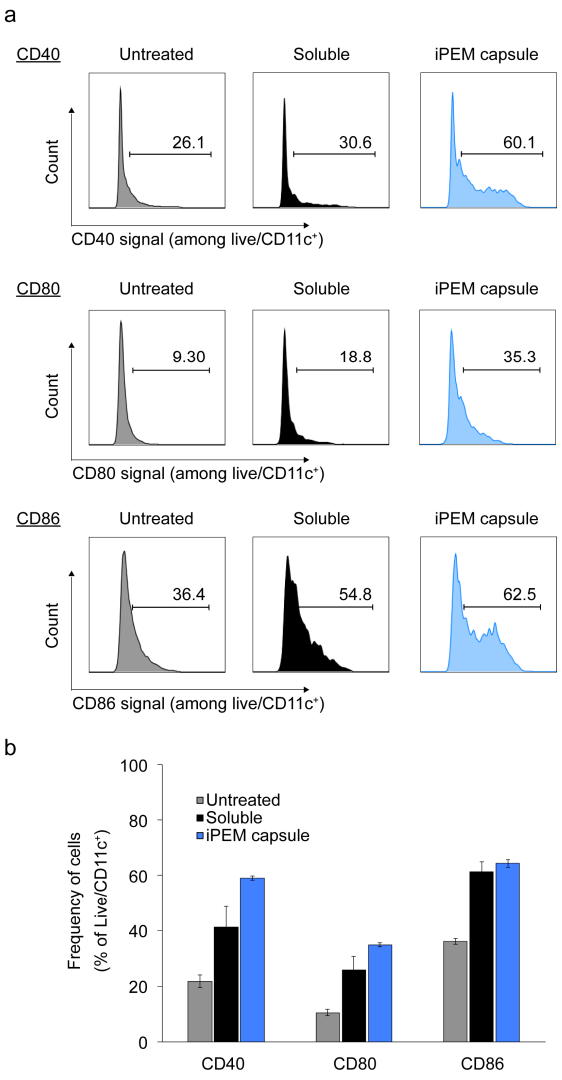

Figure S4. *In vivo* activation of lymph node-resident DCs by iPEM capsules. a) Representative flow cytometry histograms of CD40, CD80, and CD86 expression among DCs isolated from draining lymph nodes of untreated mice, or mice immunized with antigen and adjuvant in soluble form or assembled into iPEM capsules. Lymph nodes were harvested and analyzed three days after immunization. b) Frequencies of CD40, CD80, and CD86 expression in DCs from draining lymph nodes corresponding to the groups described in (a). Values for all panels indicate the mean ± s.e.m. (N=4 mice/group). Data are representative of 3 similar experiments.

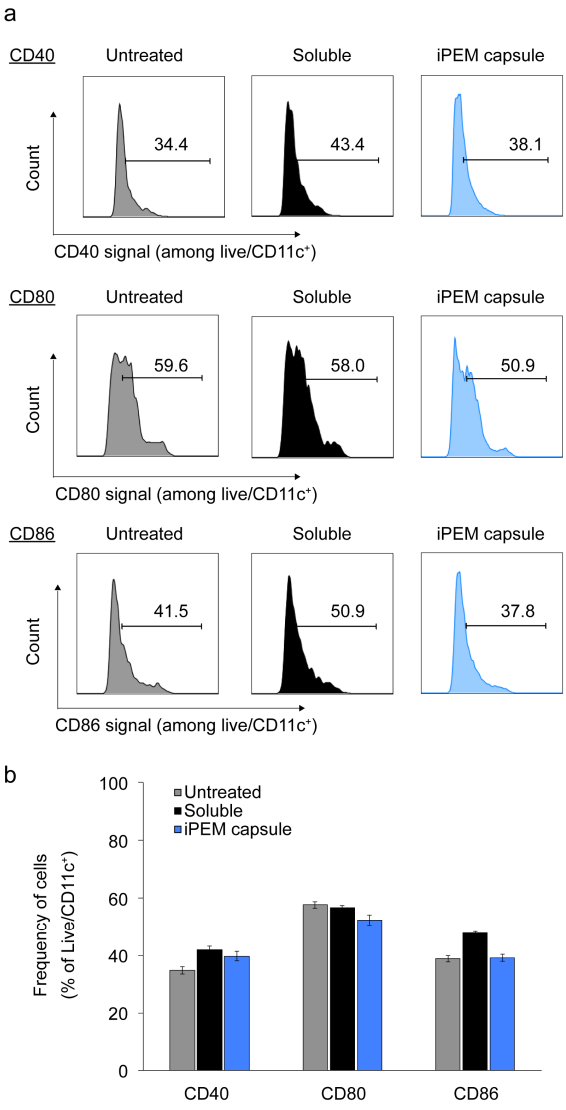

Figure S5. *In vivo* activation of spleen-resident DCs by iPEM capsules. a) Representative flow cytometry histograms of CD40, CD80, and CD86 expression among DCs isolated from spleens of untreated mice, or mice immunized with antigen and adjuvant in soluble form or assembled into iPEM capsules. Spleens were harvested and analyzed three days after immunization. b) Frequencies of CD40, CD80, and CD86 expression in DCs from spleens corresponding to the groups described in (a). Values for all panels indicate the mean ± s.e.m. (N=4 mice/group). Data are representative of 3 similar experiments.

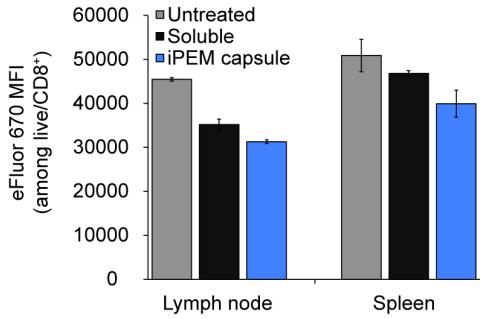

Figure S6. iPEM capsule immunization enhances the ability of DCs to expand antigen-specific T cells. MFI of fluorescent dye used to indicate proliferation (eFluor 670) of OT-I $CD8^+$ T cells following 48 hrs of co-culture with DCs isolated from lymph nodes and spleens of untreated mice, or mice immunized with the indicated formulations. Values indicate the mean ± s.e.m. (N=4 mice/group). Data are representative of 2 similar experiments.

References for Supporting Information

[1] K. A. Hogquist, S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, F. R. Carbone, *Cell* **1994**, *76*, 17.