Supporting Information

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SI Materials and Methods

Synthesis of Microparticles (MPs) and Nanoparticles (NPs). All lipids were obtained from Avanti Polar Lipids. Lipid formulations were comprised of 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000], and 1,2-dioleoyl-3-trimethylammonium-propane in a 60:20:20 mol ratio. Lipid-coated particles were prepared by a double emulsion/solvent evaporation synthesis (1). Briefly, 80 mg poly(DL-lactide-*co*-glycolide) (PLGA, M_r = 40,000–75,000), with a 50:50 lactide:glycolide ratio was codissolved in 5 mL dichloromethane with 6.0 mg lipids. For microparticle synthesis, the solution was added to 40 mL distilled water over a period of 1 min while homogenizing at 12,000 rpm using a T-25 Digital Homogenizer (IKA). For nanoparticle synthesis, the lipid-polymer solution was added to 40 mL distilled water over a period of 1 min while sonicating at 12 W using a Microson XL probe tip (Microson). In both cases, emulsification (oil/water) was performed for a total of 3 min, and solvent was removed by stirring overnight. After solvent removal, particles were collected by centrifugation at 4 °C, washed 3× with distilled water, and resuspended in PBS buffer for immediate use, or frozen in liquid nitrogen and lyophilized. Fluorescently labeled particles were synthesized by adding 50 ug of 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl], 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Invitrogen) to the lipid composition. Particles encapsulating polyinosinic-polycytidylic acid (polyIC) (Invivogen) were synthesized by modifying the above procedure to include an initial water/oil emulsion step. A specified amount of polyIC (typically 0.1–3% target load with respect to PLGA mass) was dissolved in 500 μL distilled water and added to the polymer/lipid-containing organic phase while sonicating on ice at 12 W. Sonication was performed for a total of 30 s and the resulting water/oil emulsion was then either sonicated (NPs) or homogenized (MPs) as described above to form a double emulsion (water/oil/water). For studies involving fluorescently labeled polyIC, polyIC was labeled with FITC or Cy5 (Mirus Bio) and labeling density was determined according to the manufacturer's recommended protocol.

Particle Characterization. Particle size was determined using an LA-950 laser diffraction size analyzer (Horiba Jobin Yvon). Surface charge was determined using a 90Plus/ZetaPals ξ-potential analyzer (Brookhaven Instruments). Cofocal Laser Scanning Microscopy (CLSM) was performed using an LSM 510 confocal microscopy system (Carl Zeiss) equipped with a Mai Tai two-photon laser source. For studies involving encapsulated polyIC, a known particle mass containing the specified cargo was hydrolyzed overnight in 0.2 M NaOH and the UV-visible absorbance (λ_{max} = 251 nm) was measured and converted to concentration by comparison to a standard curve. PolyIC loading level was calculated as the mass of polyIC per mass of hydrolyzed PLGA particles. PolyIC release kinetics were determined by incubating a known mass of polyIC-containing particles in PBS buffer at 37 °C. At specified intervals, incubation solutions were centrifuged, and the supernatant was analyzed for polyIC content as described above. Particles were resuspended in a fresh aliquot of PBS, and returned to 37 °C. Kinetic release curves were constructed by normalizing the cumulative release at each interval to the measured total load.

Animal Care and Injections. Animals were cared for in the US Department of Agriculture-inspected Massachusetts Institute of Technology Animal Facility under federal, state, local, and National Institutes of Health guidelines for animal care. One day before injections, female C57Bl/6 mice in groups of four to eight were shaved on the hindquarter and treated with a mild depilatory cream to remove fur. Mice were then injected subcutaneously on each side of the tail base with 10 μL of a 0.1% (wt/vol) solution of Evans blue tracer dye (VWR). In immunization experiments, all mice received tracer injections at the tail base, regardless of vaccine formulation or injection route. For experiments involving fluorescently labeled particles and in vivo imaging, female C57Bl/6-albino mice were placed on an alfalfa-free diet (PharmaServ) 3 d prior to particle injections. On the day of injection, intramuscular injections were administered at each caudal thigh muscle using an injection volume of 25 μL (50 μL total). Intranodal injections were administered at each inguinal lymph node using an injection volume of 10 μL (20 μL total).

Immunization Studies. Doses for immunization experiments were 50 μg ovalbumin protein (purified OVA, Worthington) and 5–50 μg polyIC prepared in PBS buffer. For particle doses, a known amount of particles containing the desired dose of polyIC was mixed with OVA solution to yield a final mixture containing the OVA and polyIC dose in the appropriate final injection volume [2 × 25 μL for i.m. injections or 2 × 10 μL for intralymph node (i.LN) injections]. Immunizations were carried out by giving half of the total dose on each flank, either i.m. (2 × 25 μL) or i.LN (2 × 10 μL). Prior to conducting studies comparing i.m. and i.LN injections, we confirmed that i.m. injection at the caudal thigh muscle resulted in drainage to inguinal LNs. At specified intervals, 100 μL of blood was collected for flow cytometric analysis (see above). For experiments studying antibody response, anti-OVA IgG titers (e.g., the dilution of sera at which 450-nm OD reading was 0.5) were determined by ELISA analysis of sera from immunized mice at indicated time points. For experiments involving a boosting injection, soluble OVA and polyIC were administered i.m. (2 × 25 μL) at the doses and times indicated in the text and figure legends.

In Vivo Imaging. Live animal and direct lymph node imaging were performed using a Xenogen IVIS Spectrum whole-animal bioluminescence/fluorescence imaging system (Caliper Life Sciences). Fluorescence data was spectrally demixed for each fluorescent channel and analyzed using the Living Image 4.0 software package (Caliper). Quantification was performed using region of interest (ROI) analysis and all values were background subtracted against control ROIs applied to unimmunized mice.

Flow Cytometry Experiments. Particle and polyIC uptake by specific cell populations was investigated by removing inguinal lymph nodes 24 or 96 h after injection of fluorescent MPs and/or FL-polyIC. Single cell suspensions were prepared by treating lymph nodes with collagenase D (Roche Applied Science) for 10 min at 37 °C and straining through a 40-μm membrane. Cells were washed, blocked (FcBlock, Becton Dickinson), and stained with DAPI (Invitrogen) and antibodies against CD11c, F4/80, and B220 (Becton Dickinson). Values were reported as mean fluorescent intensity following background subtraction against mice treated with empty MPs. Dendritic cell activation studies were carried out in a similar manner, but antibodies against CD40, CD80, and MHCII were used to characterize the

frequencies of expression for each of these markers amongst DAPI$^-$, CD11c$^+$ cell populations. Frequencies of CD8$^+$ T cells, and OVA-specific CD8$^+$ T cells elicited by immunization were determined by flow cytometry analysis of peripheral blood mononuclear cells or splenocytes at selected time points following staining with DAPI, anti-CD8α, and phycoerythrin-conjugated SIINFEKL/H-2K^b peptide-MHC tetramers (Becton Dickinson). For experiments involving splenocytes, spleens were first minced, passed through a 40-μm cell strainer, purified of red blood cells by lysis, then washed in PBS. Intracellular cytokine staining was conducted by pulsing peripheral blood lymphocytes or splenocytes with SIINFEKL peptide (10 μg/mL) for 6 h in the presence of brefeldin A (final 4 h only). Cells were stained with surface markers as above, then fixed, permeabilized, and stained with antibodies against IFN-γ and TNF-α. Lymphadenopathy was measure by performing total lymphocyte counts on LNs processed in equivalent suspension volumes and analyzed for equal data collection intervals. All data was acquired using a FACSCANTOII equipped with a high throughput sipper system (Becton Dickinson), and analyzed using FlowJo 7.5.5 (TreeStar).

Histological Analysis. Lymph node morphology and particle uptake was investigated by removing lymph nodes 24 or 96 h after injection of fluorescently particles and/or polyIC, followed by embedding in optimal cutting temperature media, and controlled freezing in dry ice. Lymph nodes were sectioned onto glass slides at 5-μm intervals using a cryotome, then equilibrated to room temperature. Slides were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and blocked for 15 min, then stained with antibodies against CD3. Slides were washed twice with PBS and treated with VECTASHIELD Hardset Mounting Media (VWR), followed by coverslip addition. Sections were imaged using CLSM.

Statistical Analysis. Student's t tests were used to compare two groups. One-way ANOVA with Bonferroni posttests were used when comparing more than two groups. Probabilities less than 0.05 were considered significant with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

1. Bershteyn A, et al. (2008) Polymer-supported lipid shells, onions, and flowers. *Soft Matter* 4:1787–1791.

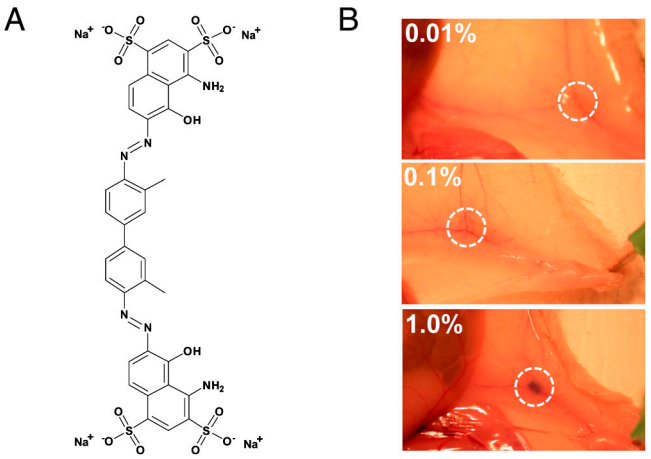

Fig. S1. Tail base injection of tracer dye allows nonsurgical identification of inguinal LN. (*A*) Structure of Evans blue dye. (*B*) Drainage of tracer dye to inguinal LN 2 h after s.c. tail base injection of 10 μL of 0.01% (*Top*), 0.1% (*Middle*), or 1.0% (*Bottom*) wt/vol solutions. Tracer solutions were prepared in sterile saline and 0.1% solutions were used for subsequent experiments described in the main text (mass equivalent = 9.6 μg/injection)

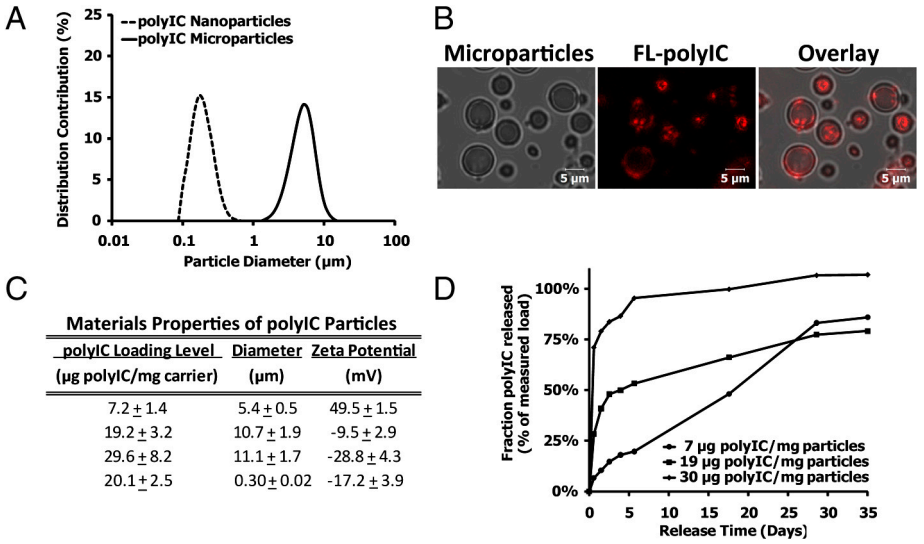

Fig. S2. Materials characterization of polyIC-loaded microparticles and nanoparticles. (*A*) Size distributions of MPs and NPs encapsulating polyIC (7 μg polyIC/mg particles) measured by laser diffraction. (*B*) CLSM imaging of MP-polyIC. Images correspond to brightfield channel (*Left*), fluorescently labeled polyIC (red channel, *Center*), and overlay (*Right*). (Scale bar: 5 μm.) (*C*) Material properties of MP-polyIC and NP-polyIC prepared by several synthesis protocols. Loading levels are expressed as μg polyIC/mg particles. Particle size was measured by laser diffraction, and ζ-potential by electrophoretic mobility. All data correspond to mean values ± SEM and are representative of at least three independent experiments. (*D*) Kinetics of polyIC release from MP-polyIC synthesized by the protocols characterized in *C*. MP-polyIC were incubated in PBS at 37 °C and release was measured over 35 d (see *Materials and Methods*).

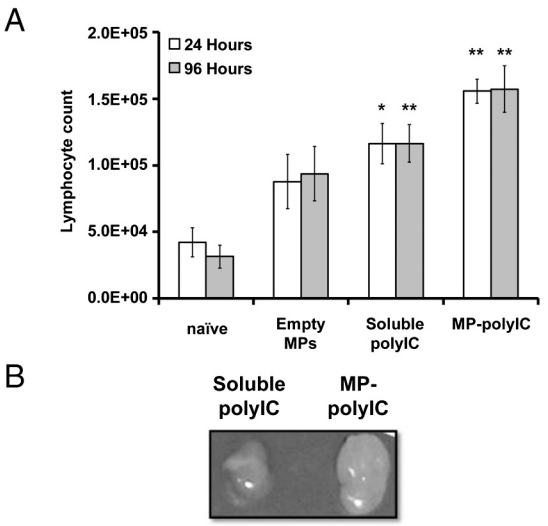

Fig. S3. MP-polyIC and soluble polyIC induce dramatic lymphadenopathy. (*A*) Quantification of LN enlargement 24 and 96 h after i.LN injection of 50 μg soluble OVA and 50 μg polyIC in either soluble or MP-polyIC form, compared with untreated mice and mice receiving empty MPs. LNs were excised and lymphocytes were counted by flow cytometry (see *Materials and Methods*). Data represent mean number of lymphocytes ± SEM for $n = 4$, and are representative of two independent experiments. Statistics are relative to untreated mice at each respective time point (*, $p < 0.05$; **, $p < 0.01$). (*B*) Qualitative comparison of lymphadenopathy in LNs excised from mice 96 h after injection of soluble OVA and the indicated polyIC formulation.

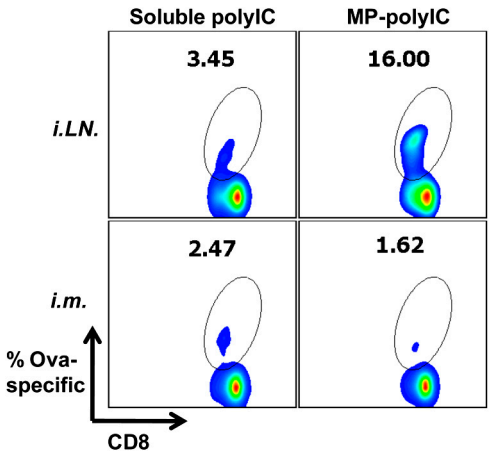

Fig. S4. MP-polyIC synergistically expand antigen-specific T cells when administered i.LN. Flow cytometry scatter plots demonstrating expansion of antigen-specific $CD8^+$ T cells in peripheral blood 7 d after i.LN or i.m. immunization with 50 μg soluble OVA and 50 μg polyIC in soluble or MP form. Plots are representative of three independent experiments each conducted with $n = 4$.

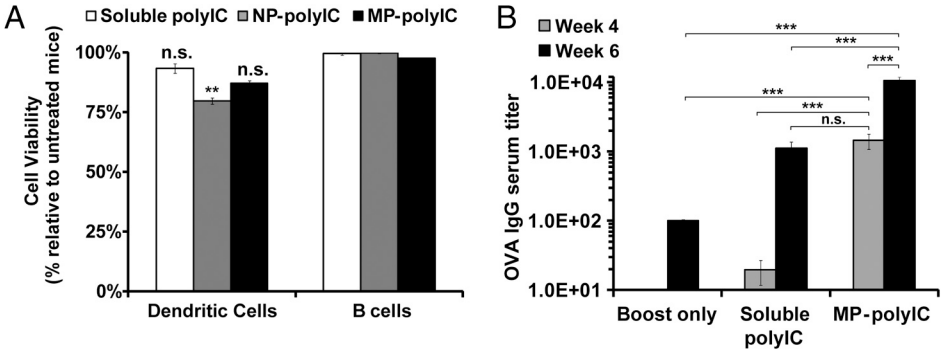

Fig. S5. Intralymph node administration of polyIC is nontoxic and enhances memory recall response. (*A*) Viability of major LN-resident antigen presenting cell populations measured by flow cytometry following staining with DAPI. LNs were excised from mice as described in *Materials and Methods* 4 d after mice were immunized with 50 μg soluble OVA and 50 μg polyIC in soluble, NP, or MP form. Data correspond to mean cell viability percentages ±SEM relative to LNs processed from untreated mice, and are representative of three independent experiments with $n = 4$. Statistics are relative to untreated mice (*, $p < 0.05$; n.s., not significant). (*B*) Groups of mice were primed by i.LN injection of 50 μg OVA mixed with 50 μg soluble polyIC or MP-polyIC, and then 5 wk later were boosted i.m. with 20 μg soluble OVA and 20 μg soluble polyIC. Shown are serum OVA-specific IgG titers before and after boosting at 4 and 6 wk, respectively. The "boost only" group did not receive a priming injection. Results represent mean serum titers ±SEM with $n = 8$ (***, $p < 0.001$; n.s., not significant).