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Supporting Information for

Biodegradable lipophilic polymeric mRNA nanoparticles for ligand-free targeting of splenic dendritic cells for cancer vaccination

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Expanded Materials and Methods

Monomer and polymer synthesis. The bioreducible monomer 2,2′-disulfanediylbis(ethane-2,1-diyl) diacrylate (R) was synthesized as previously described (22, 71). In brief, 2-hydroxyethyl disulfide (Sigma-Aldrich) was acrylated in dichloromethane (DCM) with acryloyl chloride as the acrylation reagent and in presence of triethylamine (TEA) (Sigma-Aldrich) overnight at room temperature. The TEA HCl precipitate was removed by filtration and the product washed with water and dried with sodium sulfate, and the solvent was removed by rotary evaporation. Bioreducible lipophilic PBAEs were synthesized *via* Michael addition reaction. In the first step, the diacrylate backbone monomer R and a combination of the hydrophilic side chain amine-containing monomer 4-amino-1-butanol (S4) and a lipophilic amine-containing side chain monomer (1-dodecylamine [Sc12], tetradecylamine [Sc14], hexadecylamine [Sc16], or oleylamine [Sc18]) were dissolved in anhydrous dimethylformamide (DMF), and the reaction proceeded for 24 h at 85 °C with stirring. The diacrylate monomer to total amine monomer molar ratio was 1.05:1 at a total monomer concentration of 500 mg/mL, and the hydrophilic to lipophilic side chain molar ratio was 1:1 unless otherwise noted. In the synthesis of bioreducible PBAEs without a lipophilic subunit, only S4 was used as side chain monomer. In the second step, the obtained acrylate-terminated polymer was dissolved together with an endcapping monomer (2-(3-aminopropylamino)ethanol [A], N,N-Bis(2-hydroxyethyl)ethylenediamine [B], Diethylentriamine [C], N-(2-Hydroxyethyl)ethylenediamine [D], or N-(3-Aminopropyl)piperidine [E]) (0.5 M endcap monomer and 200 mg/mL of base polymer) in tetrahydrofuran (THF), and the reaction proceeded for 1 hour at room temperature to form the final polymer. The obtained polymers were purified by precipitation in a 1:1 mixture of diethyl ether and hexane and two washes. The polymers were dried under vacuum for 48 hours and then dissolved in anhydrous dimethyl sulfoxide (DMSO) at 100 mg/mL and stored as aliquots at −80°C with desiccant.

NP preparation. NPs were formed by first separately dissolving polymer and nucleic acid cargo, including mRNA, CpG oligodeoxynucleotide (ODN 1826; InvivoGen), and/or poly(I:C) high molecular weight (HMW) (InvivoGen), in 25 mM sodium acetate buffer (NaAc, pH 5) at specified concentrations. The polymer and nucleic acid cargo were then mixed together at specified w/w ratio (100-300 w/w) and allowed to self-assemble into NPs for 6 min at room temperature.

Polymer and NP characterization. Polymer molecular weight was measured using gel permeation chromatography (GPC) relative to linear polystyrene standards using a refractive index detector (Waters). Prior to measurements, polymers were dissolved in butylated hydroxytoluene (BHT)-stabilized THF and filtered through 0.2-μm polytetrafluoroethylene (PTFE) filters. The hydrodynamic diameter of the NPs in 1× PBS was measured by dynamic light scattering (DLS) using a Zetasizer Pro (Malvern Panalytical). Zeta potential was measured *via* electrophoretic mobility using same instrument to characterize the surface charge of the NPs. NP size and morphology was visualized by TEM using a Talos L120C microscope (Thermo Scientific). NP samples of 20 μL were added to carbon-coated copper TEM grids (Electron Microscopy Sciences) for 10 min. The grids were then washed three times for 10 seconds each with MilliQ water and blotted in between and after and allowed to dry at room temperature for 20 min before imaging.

Cell culture and cell line preparation. Murine DC2.4 cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 10 mM HEPES, 1X non-essential amino acids, and 50 μM beta-mercaptoethanol. Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow isolated from C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME). On day 0, bone marrow was flushed from femurs and tibias of mice, filtered through 70-μm sterile nylon mesh and then resuspended in 5 mL ACK lysis buffer. Cells were plated in a 6-well plate at $1x10^6$ cells/mL in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, 50 μM beta-mercaptoethanol, and 20 ng/mL recombinant murine GM-CSF (Peprotech; Cranbury, NJ). On day 3, an equal volume of media

with 40 ng/mL GM-CSF was added. Cells were harvested on day 6 by collecting loosely adhered cells. B16-F10 and B16-F10-OVA cells were cultured in DMEM high glucose with sodium pyruvate (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin, with the addition of 5 mg/mL G418 for B16-F10-OVA cells. MC38-OVA cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

***In vitro* mRNA transfection.** Unless stated otherwise, all mRNA used was purchased from TriLink Biotechnologies with 5-Methoxyuridine modification and CleanCap technology. Cells were plated at 15,000 cells per well in 100 µL of medium in 96-well plates (unless stated otherwise) and allowed to adhere overnight. NPs were formulated as described above with eGFP mRNA (and CpG or Poly(I:C) if indicated) and then added to the cells. After a 2 hour incubation at 37 °C, medium with NPs was replaced with 100 µL of fresh medium. Lipofectamine MessengerMAX (Thermo Fisher Scientific) and jetMESSENGER (Polyplus) were used for comparison to commercial mRNA transfection reagents following manufacturer protocols. For cellular uptake studies, 20% of the total mRNA was replaced with Cy5-labeled eGFP mRNA (Trilink Biotechnologies). After 6 hours (for uptake experiments) or 24 hours (for transfection experiments), transfection/uptake was evaluated via flow cytometry using an Attune NxT Flow Cytometer (Thermo Fisher). The MTS CellTiter 96 Aqueous One (Promega) cell proliferation assay was performed 24 hours post-transfection according to the manufacturer's instructions as a measure of cell viability. The metabolic activity of treated cells was normalized to that of untreated cells.

Encapsulation efficiency and NP stability. mRNA encapsulation efficiency was assessed using the RiboGreen RNA assay (Invitrogen) following manufacturer's protocols. In brief, NPs were added to a 96-black-well plate and incubated in either PBS or 10 mg/mL heparin solution. Ribogreen reagent was added and fluorescence readings were performed using a Biotek Synergy 2 fluorescence multiplate reader (BioTek) to compare encapsulation efficiency for NPs in PBS compared to free mRNA (NPs in presence of heparin). The gel electrophoresis assay was performed to examine NP stability when incubated in 10% serum for 4 h. NPs were formed with Cy5-labeled mRNA (TriLink Biotechnologies) alone or Cy5-labeled mRNA and FITC-labeled CpG ODN (ODN 1826; InvivoGen) or Rhodamine-labeled poly(I:C) HMW (InvivoGen). Nucleic acid cargos were incubated alone as controls for calculation of % dissociation of the NP cargo. Samples were loaded in an 1% agarose (UltraPure Agarose, Invitrogen) gel, and the gel was run for 20 min at 100 V and imaged with iBright FL1500 Imaging System (Thermo Fisher).

Characterization of intracellular trafficking. Endosomal escape of NPs was studied in DC2.4 cells using immunofluorescence staining. Cells were plated onto coverslips in 12-well plates and grown overnight. NPs were prepared with 20% Cy5-mRNA and 80% unlabeled mRNA and incubated with cells for 6 h. Cells were washed with PBS and then stained for 30 min with Hoechst 33342 (Thermo Fisher Scientific) nuclear stain at 1:5000 dilution and Cell Navigator Lysosome Staining dye (AAT Bioquest) at 1:2500 dilution in complete media. Stained cells were washed twice in PBS and then fixated in 10% formalin. Fixated cells were washed twice in PBS and once in MilliQ H_2O and mounted on slides with SlowFade Diamond antifade mounting medium (Life Technologies) and sealed. A Zeiss Z2 Axio Observer with Apotome microscope (Zeiss) and a 63× oil immersion lens was used to visualize transfection, cellular uptake, and endosomal escape of NPs. Colocalization of Cy5-NPs and lysotracker stain was quantified by calculating the Manders' coefficient in acquired images using ImageJ.

NP formulation for *in vivo* transfection. All animal work was performed in strict adherence of the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee (ACUC). NPs for *in vivo* mRNA delivery were formulated at 100 w/w unless stated otherwise. All *in vivo* transfections utilized the R18D polymer (BR6-S4, Sc18 [50:50]-D). R18D and mRNA/adjuvants were diluted separately in NaAc and then mixed at a 4:1 volume ratio. VacciGrade poly(I:C) (HMW) and CpG ODN 1826 were used for *in vivo* studies (Invivogen). NPs were allowed to assemble at room temp for 6 minutes, and then a 500 mg/mL sucrose solution was used to bring the solution to isotonicity. NPs were administered to animals via 200 µL

intravenous injections (by tail vein injection for Ai9 studies or by retro-orbital injection for luciferase studies and tumor studies).

***In vivo* bioluminescence transfection studies.** NPs encapsulating fLuc mRNA were formulated as described above and administered to 6- to 7-week-old male BALB/c or C57BL/6J mice *via* retro-orbital injection. For comparison of routes of administration, NPs were administered via either retro-orbital (200 µL), intramuscular (50 µL), or subcutaneous (200 µL) injections. Whole-body bioluminescence of BALB/c or shaved C57BL/6J mice was assessed at 6 hours post-injection (or at prespecified timepoints up to 96 hours for time course study). D-Luciferin potassium salt solution (25 mg/mL in PBS; Cayman Chemical Company) was administered to mice via 150 µL intraperitoneal injections, and mice were imaged using the IVIS Spectrum Imager (Perkin Elmer) after 10 min. For timecourse studies, animals were euthanized immediately after whole-body imaging via cervical dislocation, and selected organs were extracted, submerged in D-luciferin solution (250 µg/mL), and imaged with IVIS.

Cre mRNA delivery to Ai9 mice. Ai9 mice were purchased from Jackson Laboratory (JAX stock #007909) and bred in the Johns Hopkins animal facility (40). NPs encapsulating Cre mRNA and adjuvants (if indicated) were administered to Ai9 mice via tail vein injections, and tdTomato expression following Cre-Lox recombination was allowed to accumulate for 24 hours, at which point animals were euthanized via cervical dislocation. Spleens were extracted and dissociated by a 1-hour incubation in collagenase D (2 mg/mL) at 37°C, followed by mechanical pressing through a 70-µm cell strainer. Cells were pelleted by centrifugation, the supernatant was removed, and red blood cells in the cell pellet were lysed by incubating in ACK lysis buffer (Quality Biological) for 1 min at room temperature. Cells were diluted in PBS, passed through a 100-µm cell strainer, pelleted by centrifugation, and resuspended in fluorescence-activated cell sorting (FACS) buffer (2% FBS in PBS with 0.02% sodium azide). Surface staining of cells with fluorescent antibodies was then performed using the antibodies and dilutions listed in **SI Appendix Table S1** in FACS buffer for 30 min at 4°C, at which time cells were washed twice and resuspended in FACS buffer for further analysis. Flow experiments were performed using an Attune NxT flow cytometer and analyzed using FlowJo software (FlowJo).

Tumor vaccination and safety studies. For both B16-F10-OVA and B16-F10 tumor studies, $3x10^5$ cells in 50 µL RPMI 1640 media were inoculated subcutaneously in the right flank of C57BL/6J mice on day 0. For MC38-OVA studies, $1x10^6$ cells in 50 µL RPMI 1640 media were inoculated subcutaneously in the right flank of C57BL/6J mice on day 0. R18D NPs encapsulating fLuc mRNA (as an irrelevant mRNA control) or OVA mRNA were administered intravenously by retro-orbital injections on days 4 and 9 for the B16-F10-OVA study or days 9 and 14 for the MC38-OVA study at 10 µg mRNA/mouse and 2.5 µg CpG or 0.1 µg poly(I:C) for adjuvant groups (n = 7-8 mice/group). For the B16-F10 study, instead of OVA mRNA, a 1:1 mixture (each at 5 µg/mouse) of custom-synthesized TRP2 mRNA (NCBI gene accession number: NM_021882) and GP100 mRNA (NCBI gene accession number: NM_010024) (TriLink Biotechnologies) was used, and NPs were administered intravenously by retro-orbital injection on days 4 and 9. 200 µg of aPD-1 was injected intraperitoneally on day 5 for B16 studies or day 10 for the MC38-OVA study. For all studies, tumor area was measured by caliper every other day starting on day 7 by a blinded researcher, and mice were euthanized by cervical dislocation once the tumor reached 200 mm^2. For the MC38-OVA study, on day 65, mice that had completely cleared their tumors (n = 4 mice for mOVA/CpG NP group, n = 2 mice for aPD-1 group, and n = 1 mouse for mLuc/CpG group) and 7 age-matched mice were rechallenged with subcutaneous inoculation of $1x10^6$ MC38-OVA cells on the left flank. For B16-F10-OVA and MC38-OVA studies, approximately 100 µL of blood was collected from mice via cheek bleeds and assessed for the presence of OVA-specific CD8+ T cells at the time points stated. Blood samples were resuspended in ACK lysis buffer, washed in PBS and resuspended in FACS buffer with TruStain FcX antibody (BioLegend). Cells were then stained for 45 minutes at 4°C with AlexaFluor 488 anti-CD3 (**SI Appendix Table S1**), APC anti-CD8a at 1:80 dilution (BioLegend, catalog no. 100712, clone 53-6.7), PerCP anti-CD4 at 1:80 dilution (BioLegend, catalog no. 100431, clone GK1.5), and BV421 H-2Kb SIINFEKL tetramer or BV421 H-2Kb SSIEFARL tetramer (NIH Tetramer Core Facility) as a negative control

at 1:100 dilutions. Samples were analyzed with the Attune NxT flow cytometer. Any risk of systemic toxicity following treatment with bioreducible lipophilic NPs was examined. Animals received intravenous injections of R18D NPs delivery mRNA alone or mRNA along with CpG or poly(I:C). Untreated animals were used as controls. Blood was collected from each animal 24 and 72 hours post-treatment, and the serum was collected by centrifugation at 1500 rcf for 10 min at 4°C. The serum samples from treated and untreated animals were analyzed for AST and ALT activity (Sigma-Aldrich, key biomarkers for liver health. The collected serum was also analyzed using the 13-plex Mouse Cytokine Release Syndrome LEGENDplex panel (Biolegend) to assess systemic cytokine levels. The AST and ALT activity assays, and the LEGENDplex assay were performed according to manufacturer's instructions.

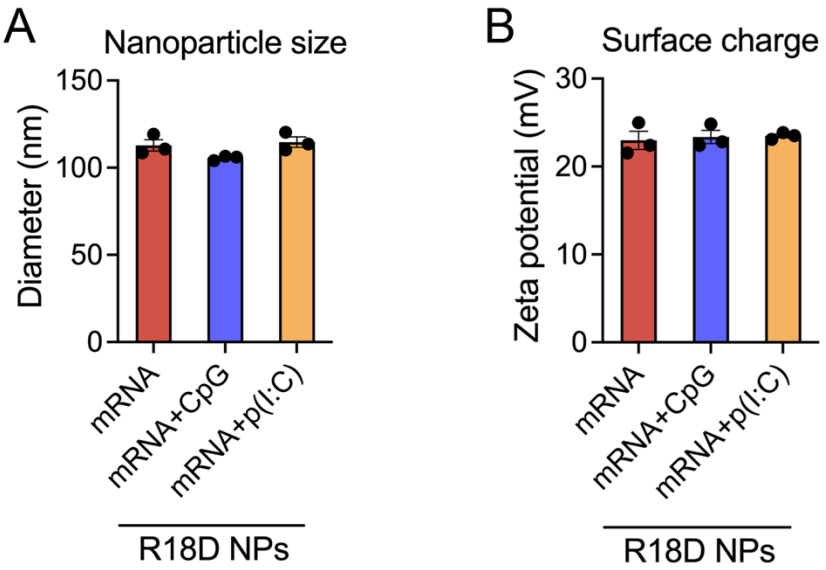


Fig. S1. Physical characteristics of bioreducible lipophilic PBAE nanoparticles (NPs). (*A*) Hydrodynamic diameter and (*B*) surface charge of R18 NPs carrying mRNA or mRNA along with CpG or poly(I:C) assessed *via* dynamic light scattering (DLS) (n = 3). Error bars represent SEM.

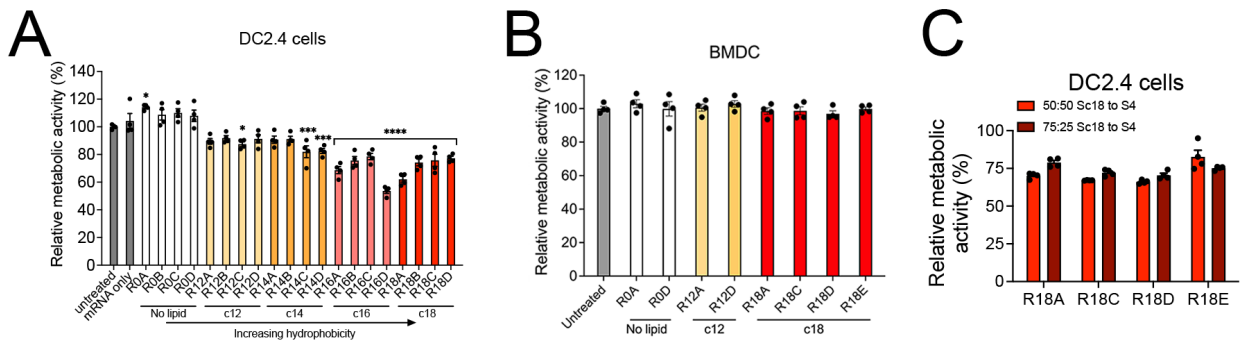


Fig. S2. Cell viability of dendritic cells (DCs) following *in vitro* transfection by bioreducible lipophilic PBAE mRNA nanoparticles (NPs). (*A*) A polymer library was screened for toxicity on the murine dendritic cell line DC2.4. Cells were treated with NPs formed at 200 w/w and a dose of 50 ng mRNA/well and metabolic activity was assessed after 24 h *via* the MTS assay and normalized to untreated cells. Significance indicates comparison to untreated control. (*B*) A subset of the polymer library was screened on murine bone-marrow derived dendritic cells (BMDC). Cells were treated with NPs at a dose of 25 ng mRNA/well and metabolic activity was assessed after 24 h and normalized to untreated cells. (*C*) Polymers with Sc18 monomer were synthesized with 50:50 or 75:25 ratio of lipophilic side chain monomer Sc18 to hydrophilic side chain monomer S4. Viability of DC2.4s following treatment of mRNA NPs with varied lipophilicity was assessed after 24 h *via* MTS assay and normalized to untreated cells. Error bars represent SEM ($n = 4$). *$P <$ 0.05, ***$P < 0.001$, and ****$P < 0.0001$.

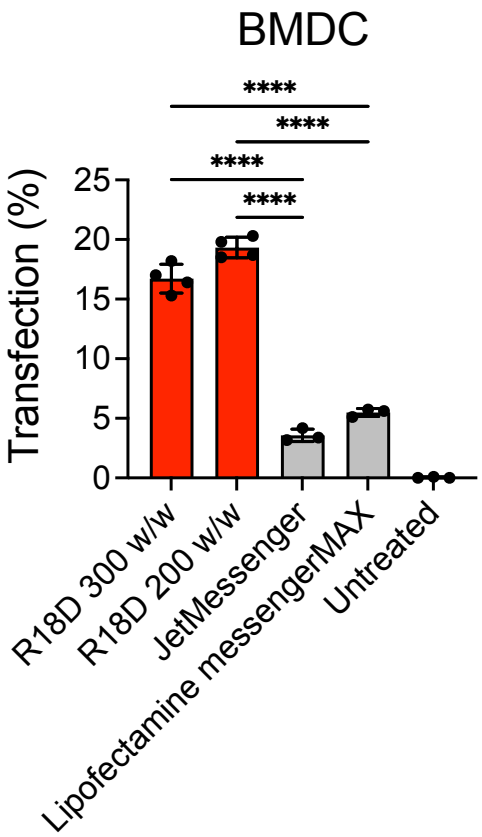


Fig. S3. Transfection of BMDCs with R18D nanoparticles (NPs) compared to leading commercial mRNA transfection reagents. Murine BMDCs were transfected with R18D GFP-mRNA NPs at 250 ng mRNA/well (n = 4 replicates) or with jetMessenger or Lipofectamine MessengerMAX (leading mRNA transfection reagents for hard-to-transfect cells) at 500 ng mRNA/well (n = 3) in a 24-well plate. Error bars represent SEM. ****P < 0.0001.

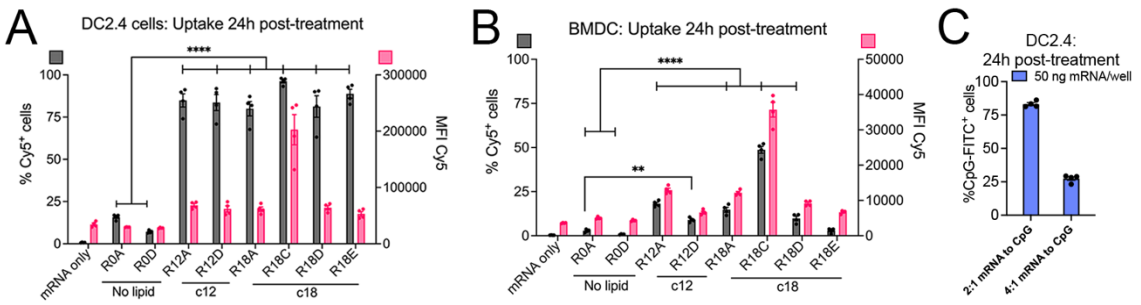
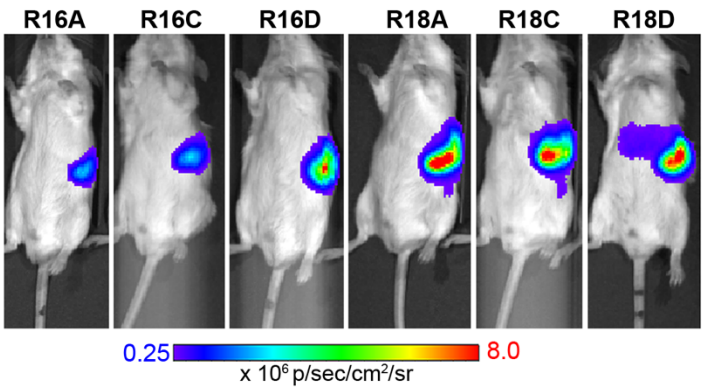


Fig. S4. Cellular uptake of mRNA nanoparticles (NPs) 24 h post-treatment. (*A*) DC2.4 cells and (*B*) murine BMDCs were treated with NPs carrying Cy5-mRNA at a dose of 50 ng mRNA/well, and NP uptake was assessed 24 h post-treatment by flow cytometry (n = 4) (*C*) DC2.4 cells were treated with R18D NPs co-encapsulating mRNA at a dose of 50 ng mRNA/well, and FITC-labeled CpG and uptake of CpG was assessed 24 h post-treatment by flow cytometry (n = 4). Error bars represent SEM. $^{**}P < 0.01$ and $^{****}P < 0.0001$.

A



B

NP formulation	Total flux spleen (p/s)
R16A	0.19×10^7
R16C	0.24×10^7
R16D	0.66×10^7
R18A	1.18×10^8
R18C	0.95×10^8
R18D	1.01×10^8

Fig. S5. *In vivo* transfection following systemic administration of bioreducible lipophilic PBAE nanoparticles (NPs) delivering luciferase mRNA (mLuc) in BALB/c mice. (*A*) Whole animal bioluminescence imaging was performed 6 h post-administration of NPs with varied lipophilic side chains (Sc16 and Sc18) and endcaps (A, C, and D). (*B*) Total bioluminescence intensity in the spleen due to the mLuc transfection was measured.

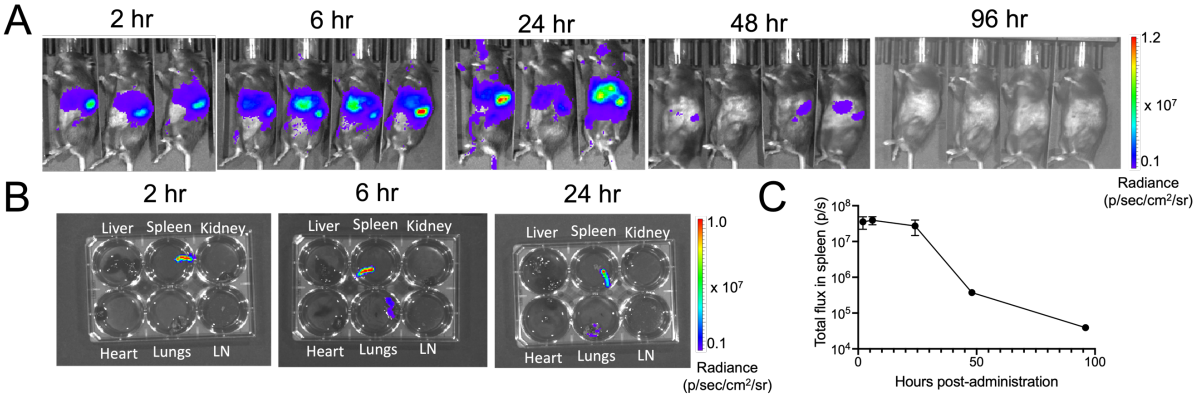


Fig. S6. *In vivo* transfection over time following systemic administration of R18D mRNA nanoparticles (NPs). R18D NPs carrying luciferase mRNA (10 μg/mouse) were administered intravenously in C57BL/6 mice. (*A*) Whole animal bioluminescence imaging was performed 2, 6, 24, 48, and 96 hr post-administration. (*B*) Mice were euthanized at each timepoint and major organs (liver, spleen, kidney, heart, and lungs) and inguinal lymph nodes (LN) were dissected out and imaged by IVIS. Representative organ images at 2, 6, and 24 hr timepoints are shown. (*C*) Total flux in spleen until endpoint of 96 hr post-treatment. Error bars represent SEM.

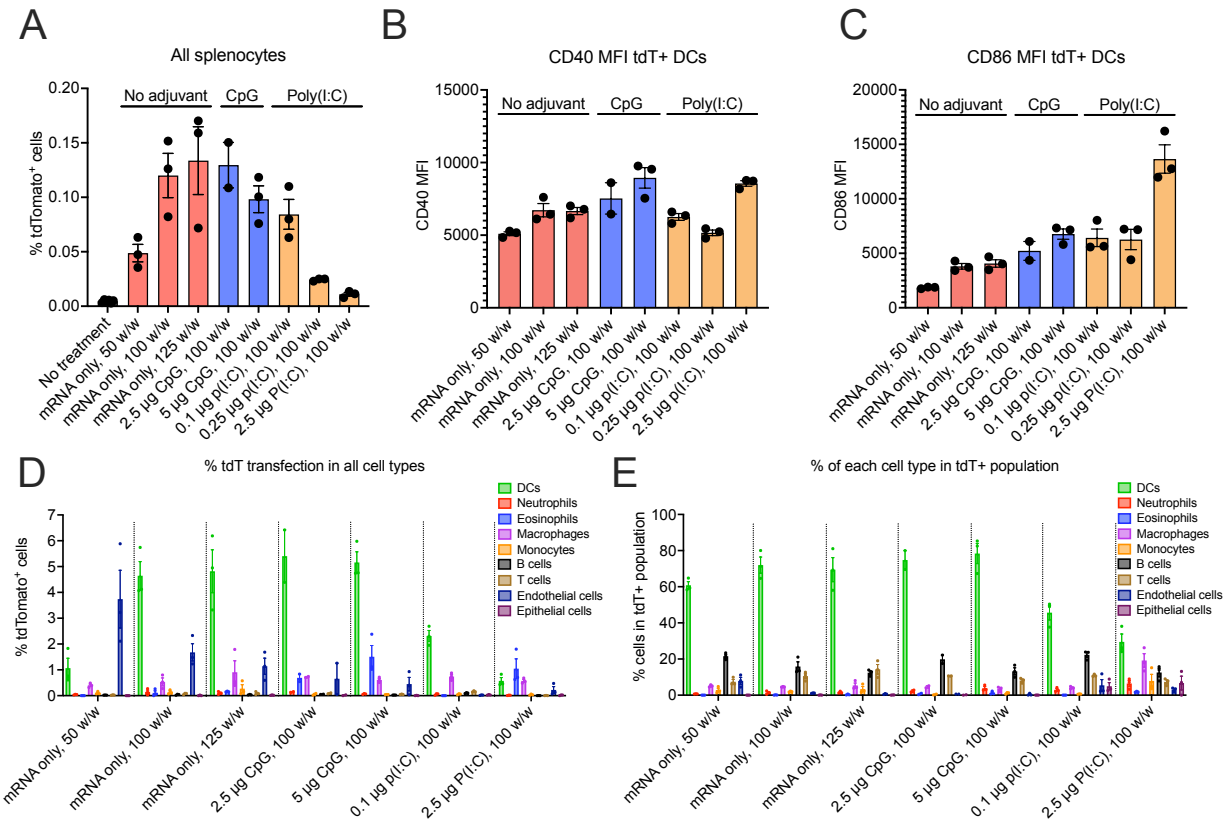


Fig. S7. *In vivo* transfection in splenic cell populations following systemic administration of R18D mRNA nanoparticles (NPs). R18D Cre mRNA NPs were administered intravenously to Ai9 mice at 10 µg mRNA/mouse with CpG and poly(I:C) adjuvants at varying adjuvant doses and w/w ratios of polymer to total nucleic acid. tdTomato expression in key cell populations in the spleen was assessed 24 h post-treatment *via* flow cytometry. (*A*) Percent tdTomato+ cells in all splenocytes. (*B*) CD40 expression in tdTomato+ transfected splenic DCs. (*C*) CD86 expression in tdTomato+ transfected splenic DCs. (*D*) Percent tdTomato transfection in various cell types in the spleen. (*E*) Percent of all transfected tdTomato+ splenocytes that belong to each cell type. Error bars represent SEM.

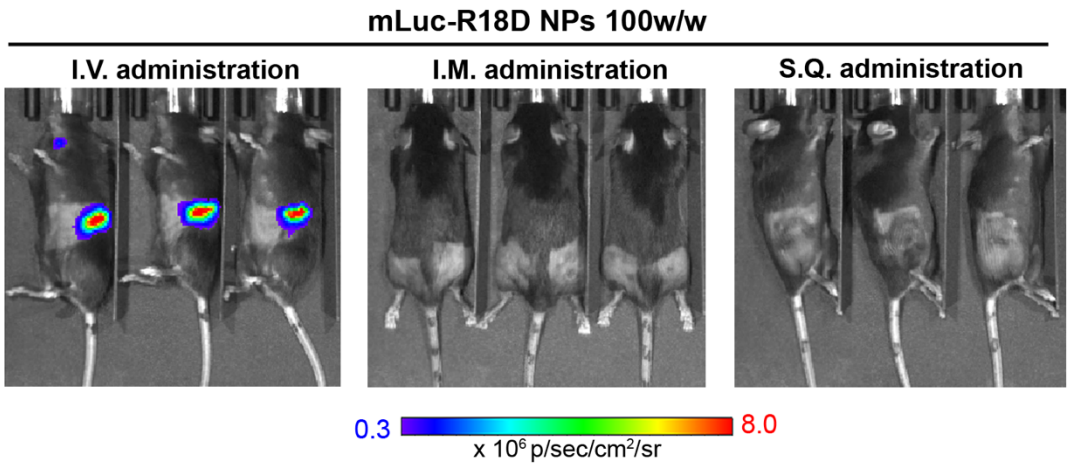


Fig. S8. *In vivo* transfection following intravenous (I.V.), intramuscular (I.M.), and subcutaneous (S.Q) administration of R18D nanoparticles (NPs) delivering luciferase mRNA (mLuc) in C57BL/6 mice. Whole animal bioluminescence imaging was performed 6 h post-administration.

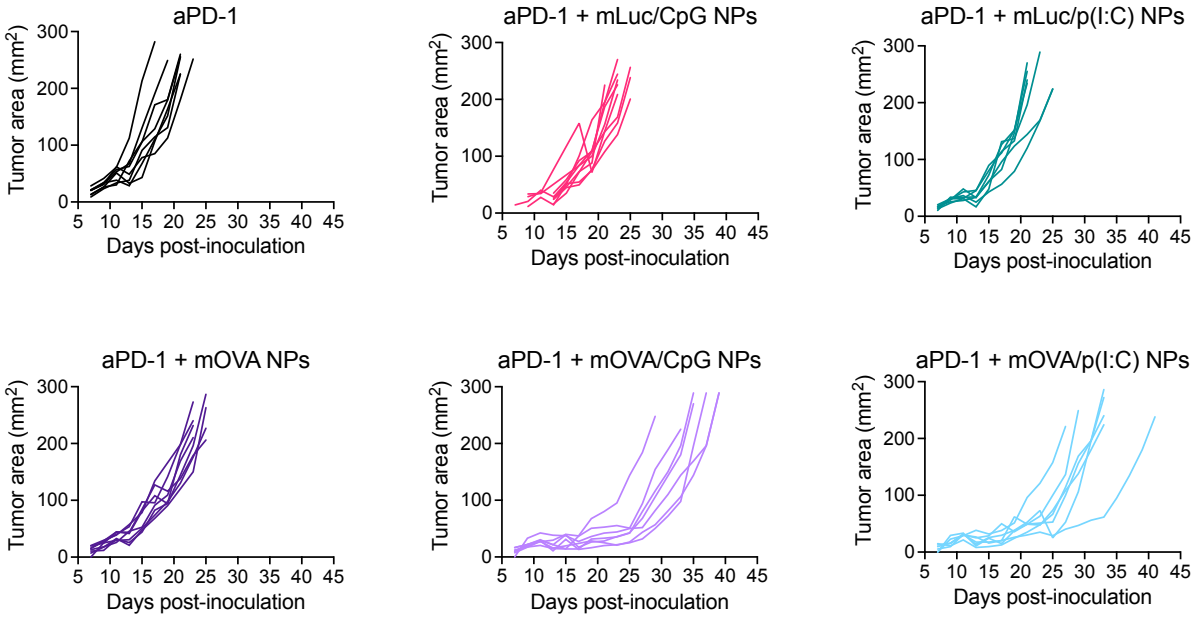


Fig. S9. Individual tumor growth curves for B16-OVA mRNA nanoparticle (NP) vaccination study. $3x10^5$ B16-OVA cells were inoculated subcutaneously in the right flank of C57BL/6 mice on day 0, and R18D NPs encapsulating Luciferase-encoding mRNA (as an irrelevant mRNA control) or OVA-encoding mRNA were administered intravenously on days 4 and 9 at 10 μg mRNA/mouse and 2.5 μg CpG or 0.1 μg poly(I:C) for adjuvant groups (*n* = 7-8 mice/group). 200 μg of aPD-1 was injected intraperitoneally on day 5. Tumor area was measured every other day beginning on day 7 post-inoculation, and individual tumor growth curves for each treatment group are shown.

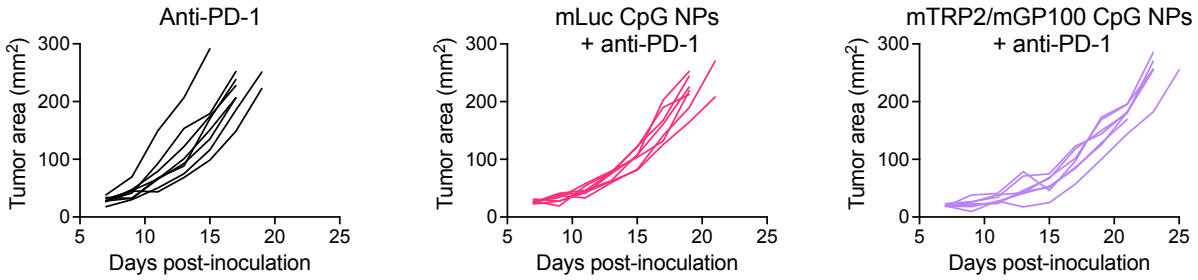


Fig. S10. Individual tumor growth curves for B16-F10 mRNA nanoparticle (NP) vaccination study. $3x10^5$ B16-F10 cells were inoculated subcutaneously in the right flank of C57BL/6 mice on day 0, and R18D NPs encapsulating luciferase-encoding mRNA or a 1:1 mixture of TRP2- and GP100-encoding mRNA (10 μg total mRNA/mouse) and CpG (2.5 μg/mouse) at were administered intravenously on days 4 and 9 (*n* = 7-8 mice/group). 200 μg of aPD-1 was injected intraperitoneally on day 5. Tumor area was measured every other day beginning on day 7 post-inoculation, and individual tumor growth curves for each treatment group are shown.

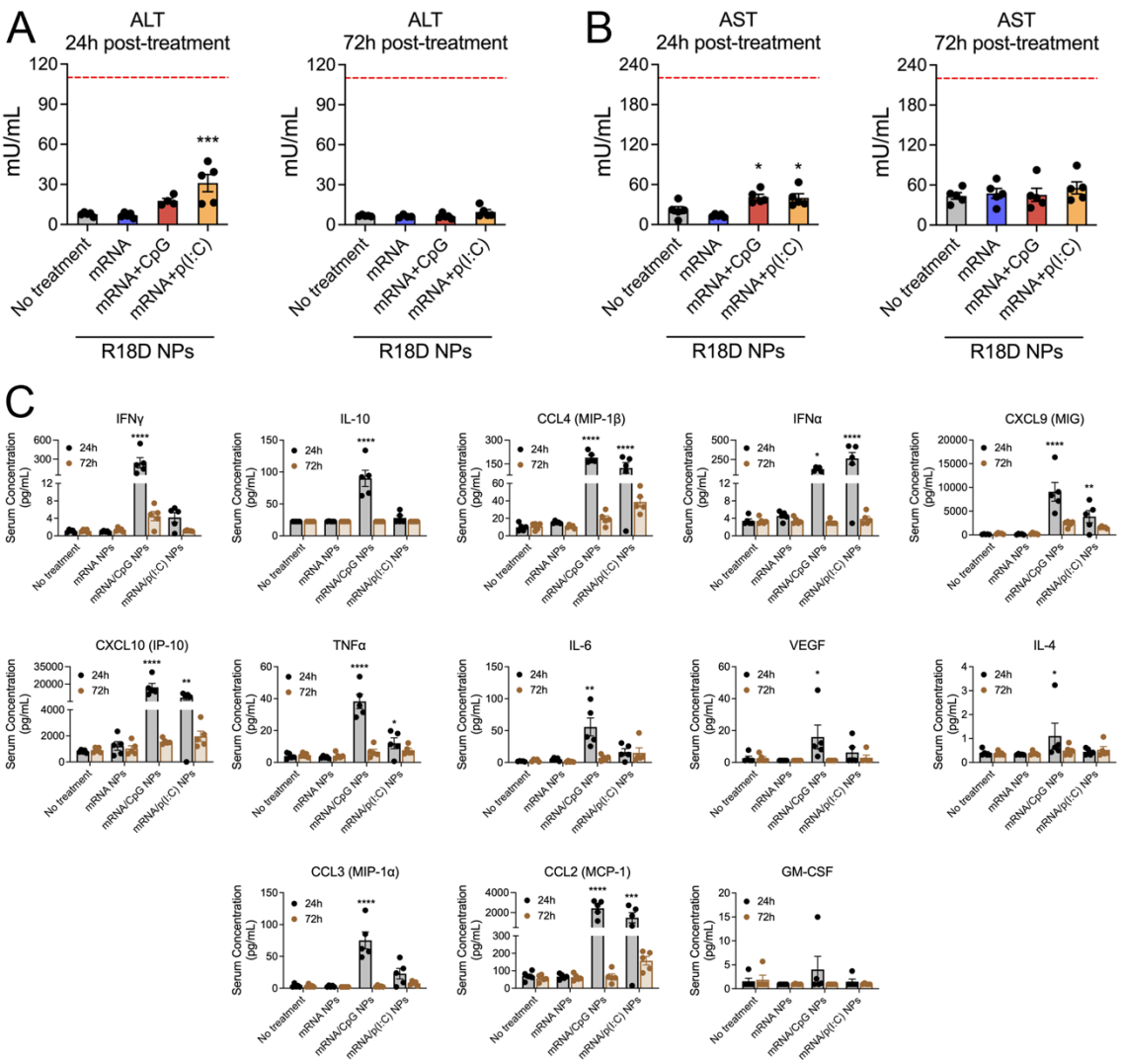


Fig. S11. Liver health and systemic cytokine levels in serum following systemic administration of R18D nanoparticles (NPs) delivering mRNA alone or along with CpG or poly(I:C). The activity levels in blood of the biomarkers (*A*) alanine aminotransferase (ALT) and (*B*) aspartate aminotransferase (AST) for liver health were assessed 24 and 72 hours post NP treatment (n = 4-5). Dashed lines indicate the upper activity levels for normal healthy liver in mice (1). (*C*) A cytokine release syndrome panel was used to assess serum cytokine levels 24 and 72 hours post NP treatment (n = 5). Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Table S1. Antibodies used for Ai9 flow cytometry studies.

Antibody	Dilution	Clone	Supplier	Catalog No.
BV421 anti-CD45	1:100	30-F11	Biolegend	103134
BV605 anti-CD86	1:20	GL-1	Biolegend	105037
AlexaFluor 488 anti-CD11b	1:100	M1/70	Biolegend	101217
PerCP anti-CD40	1:20	3/23	Biolegend	124624
APC anti-CD11c	1:100	N418	Biolegend	117310
AlexaFluor 700 anti-I-A/I-E	1:100	M5/114.14.2	Biolegend	107622
APC/Cy7 anti-Ly6G	1:100	1A8	Biolegend	127624
AlexaFluor 488 anti-CD3	1:80	17A2	Biolegend	100210
APC anti-CD19	1:100	6D5	Biolegend	115512
AlexaFluor 700 anti-CD31	1:100	390	Biolegend	102444
APC/Cy7 anti-CD326	1:80	G8.8	Biolegend	118218

References:

1. Y. Bao *et al.*, Acetaminophen-Induced Liver Injury Alters Expression and Activities of Cytochrome P450 Enzymes in an Age-Dependent Manner in Mouse Liver. *Drug Metabolism and Disposition* **48**, 326-336 (2020).