Lipid Nanoparticle Assisted mRNA Delivery for Potent Cancer Immunotherapy


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Supporting Information

ABSTRACT: The induction of a strong cytotoxic T cell response is an important prerequisite for successful immunotherapy against many viral diseases and tumors. Nucleotide vaccines, including mRNA vaccines with their intracellular antigen synthesis, have been shown to be potent activators of a cytotoxic immune response. The intracellular delivery of mRNA vaccines to the cytosol of antigen presenting immune cells is still not sufficiently well understood. Here, we report on the development of a lipid nanoparticle formulation for the delivery of mRNA vaccines to induce a cytotoxic CD 8 T cell response. We show transfection of dendritic cells, macrophages, and neutrophils. The efficacy of the vaccine was tested in an aggressive B16F10 melanoma model. We found a strong CD 8 T cell activation after a single immunization. Treatment of B16F10 melanoma tumors with lipid nanoparticles containing mRNA coding for the tumor-associated antigens gp100 and TRP2 resulted in tumor shrinkage and extended the overall survival of the treated mice. The immune response can be further increased by the incorporation of the adjuvant LPS. In conclusion, the lipid nanoparticle formulation presented here is a promising vector for mRNA vaccine delivery, one that is capable of inducing a strong cytotoxic T cell response. Further optimization, including the incorporation of different adjuvants, will likely enhance the potency of the vaccine.

KEYWORDS: mRNA, lipid nanoparticles, vaccines, immune response, cancer immunotherapy, cytotoxic T cells

Cancer immunotherapy is based on the ability of the immune system to recognize and kill cancer cells. Recent clinical trials testing checkpoint blockers or adoptive T cell transfer have shown that antigen specific T cells can control cancer. To harness the immune system to treat cancer, one needs to develop strategies to neutralize tumor-promoting inflammation, to modify the tumor microenvironment that regulates T cell activity, and to broaden the T cell repertoire by vaccination. The adaptive immune system acts to protect us from recurring infections through its two arms, the humoral arm, consisting of antibodies, and the cellular arm, consisting of T cells. Antibodies are a great tool to clear extracellular pathogens and toxins. However, for certain intracellular pathogens and tumors, specialized T cells, known as cytotoxic T Cells (CTLs) or cluster of differentiation 8 (CD 8) T cells, are needed. Nucleotide vaccines with their ability to induce a strong Major Histocompatibility Complex I (MHC-I) mediated CD 8 T cell response are very attractive. However, their delivery to target cells with minimal toxicity remains difficult. Challenges for mRNA vaccine delivery include: mRNA has to (a) be protected from degradation by omnipresent endonucleases, (b) reach the target cells, and (c) be both endocytosed and induce endosomal escape before degradation. Various strategies have been advanced for successful mRNA vaccine delivery, such as encapsulation of mRNA in viral and nanoparticle vectors, or simply sequence optimization for increased stability and tailored immunogenicity.

Our laboratory recently developed a library of lipid nanoparticles (LNPs) for the delivery of mRNA to hepatocytes. Vectors for the intracellular delivery of oligonucleotides have been developed in various shapes and sizes. However, nanoparticles in a size range of up to about 200 nm may be particularly well-suited for the delivery of mRNA vaccines. Professional antigen presenting cells (APCs), especially dendritic cells (DCs), are important targets to induce T cell immunity. APCs are enriched in the lymph nodes and
Our formulation consists of an ionizable lipid, a phospholipid, cholesterol, a polyethylene glycol (PEG) containing lipid, and an additive for the delivery of mRNA vaccines. The ionizable lipid is positively charged at low pH to allow complexation with the negatively charged mRNA and may also help with cellular uptake and endosomal escape.24 The phospholipid and cholesterol are both important for the stability of the LNPs and may also help with endosomal escape.25,26 The PEGylated lipid hinders LNP aggregation, aids in vivo biodistribution, and reduces nonspecific interactions.27 We hypothesized that LNPs made from these components can be optimized for mRNA vaccine delivery for induction of a potent CD 8 T cell immune response.

To test the potential of expanding antigen specific T cell populations, there is no adequate in vitro assay. This follows because both T cell counts and antibody titers are “second-order” effects that do not just depend on transfection efficiency of a particular type of immune cell, but also on the complex immunological signaling cascade which is necessary for an immune response to take place. Accordingly, a library of LNP formulations was optimized to induce a potent T cell response in vivo.

We prepared and optimized a LNP library complexed with mRNA coding for the model immunology protein ovalbumin (OVA). Each formulation was tested in vivo in groups of five C57BL/6 mice by subcutaneous injection in the lower back (dorsal posterior) at a dose of 10 μg of mRNA per mouse (Figure 1A–C). In the first phase of the optimization (Library A), we tested different lipids for the individual components: ionizable lipid, phospholipid, cholesterol, PEGylated lipid, and additive at a constant molar ratio (Table 1). The mice were bled 7 days after a single injection; the red blood cells were lysed, and the monocytes were stained using a tetramer conjugate for the OVA-epitope SIINFEKL to determine the percentage of OVA specific CD8 T cells (Figure 1D). A list of the tested formulations and the corresponding CD8 T cell levels are provided in Table S1. Among the ionizable lipids tested, C12-200, cKK-E12, S03013, DOTAP, and DODAP, only cKK-E12 performed better than in the original
We also replaced cholesterol with DC-cholesterol. By testing different phospholipids, 5 of the 6 tested, performed better than DOPE did in the original formulation. However, using either phospholipid, the molar composition did not perform as well at all (Figure 1D). Based on the Library A screening, we decided to further investigate the properties of formulation B-11 yielding the highest CD 8 T cell levels. To evaluate the locations of mRNA transfection and protein synthesis, we formulated firefly luciferase (FFL) mRNA in B-11 LNPs and injected 10 μg of mRNA per mouse, as used in the vaccine study. Twenty-four hours later, we used bioluminescence to detect the location of protein expression (Figure 2A). We found FFL expressed at the injection site and in the draining lymph nodes, in the inguinal lymph nodes, as well as in some axillary lymph nodes. Luminescence was not detected in the liver, spleen, lung, or intestines. We then monitored the protein expression of the 3 formulations A-1, A-6, and B-11 at the injection site over time (Figure 2B and C). It is noteworthy that all three formulations reached the expression maximum of about 4 orders of magnitude after 24 h and declined slowly afterward. Formulation B-11 that elicited the highest CD 8 T cell levels did not translate into a higher maximal FFL concentration, but a higher percentage of OVA-specific mRNA coding for Cre-recombinase.

To determine whether we are transfecting APCs, we used the Aii14D reporter mouse and LNPs containing mRNA coding for Cre-recombinase (Figure 2D). These mice harbor a mutation in the Gt(ROSA)26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter driven tdTomato red fluorescent protein. Cells express tdTomato upon Cre-mediated recombination. We have chosen the Aii14D reporter mouse because expression levels using commercially available mRNA coding for fluorescent proteins, such as GFP, tdTomato, or cyan fluorescent protein, were below the detection levels for analysis using flow cytometry. The draining lymph nodes, the inguinal lymph nodes in this case were removed, digested, and the monocytes were stained with secondary antibodies. Flow cytometry revealed that 4.6% of DCs, 1.2% of macrophages, 3.3% of neutrophiles, and 0.06% of B cells expressed the Cre-recombinase.

Unmodified RNA has the potential to activate endosomal Toll-like receptors 3 (TLR3), TLR7, and TLR8. Activation of these receptors induces an inflammatory response, transcription of pro-inflammatory cytokines, as well as up-regulation of

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[a] Lipid abbreviations: DOPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; POPE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPS: 1,2-dioleoyl-sn-glycero-3-phospho-seryl-serine; DC-cholesterol: 3′-[N-(N,N-dimethylamino)carbamoyl]cholesterol hydrochloride; C14-PEG2000: 1,2-di-myristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt); C14-PEG300: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-300] (ammonium salt); C14-PEG1000: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000] (ammonium salt); C14-PEG3000: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000] (ammonium salt); C14-PEG2000: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000].

The two phospholipids (DSPC and DOPS), out of the six tested, performed better than DOPE did in the original formulation. However, using either phospholipid, 5−10 days after the immunization, more than 20% of the mice tested developed inflammation at the injection site. For this reason, we stopped using DSPC and DOPS. Formulations without phospholipid did not perform as well at all (Figure 1D, Formulation A-1). We also replaced cholesterol with DC-cholesterol, because it has been successfully used to formulate lipid nanoparticles for DNA plasmid and siRNA delivery. However, we did not observe a higher percentage of OVA-specific CD 8 T cells using DC-cholesterol. By testing different PEG chain lengths, we found that the PEG length as well as the molar composition did not perform as well at all. The two phospholipids (DSPC and DOPS), out of the six tested, performed better than DOPE did in the original formulation. However, using either phospholipid, 5−10 days after the immunization, more than 20% of the mice tested developed inflammation at the injection site. For this reason, we stopped using DSPC and DOPS. Formulations without phospholipid did not perform as well at all (Figure 1D, Formulation A-1). We also replaced cholesterol with DC-cholesterol, because it has been successfully used to formulate lipid nanoparticles for DNA plasmid and siRNA delivery. However, we did not observe a higher percentage of OVA-specific CD 8 T cells using DC-cholesterol. By testing different PEG chain lengths, we found that the PEG length as well as the anchor lipid greatly influence the LNP diameter: C14-PEG350 (232 nm), C14-PEG1000 (121 nm), C14-PEG2000 (67 nm), C18-PEG2000 (110 nm), and C14-PEG3000 (96 nm), with the smallest LNP, C14-PEG2000, yielding the highest T cell levels. Arachidonic acid has been used to deliver LNPs carrying mRNA for Cas9. However, in the case of vaccines, the removal of the arachidonic acid additive almost tripled the CD 8 T cell count. Interestingly, the SLS additive performed better than the no additive case. Based on the Library A screening, we identified cKK-E12 from formulation A-2, C14-PEG2000 from formulation A-12, and SLS from formulation A-18 as promising components for further investigation in Library B.

In the second phase of the optimization (Library B), we combined the different individual components that we identified in the first phase as being beneficial (Library A) and investigated the effect of altering the molar compositions of the components. We found that varying the molar composition of cKK-E12 correlated with detected CD8 T cell levels. Lower molar compositions of cKK-E12 led to increased T cell levels until 10 mol %, beyond which the deviation around the mean concentration of antigen specific CD 8 T cell levels increased significantly (Figure S1). The particle sizes in the tested formulations ranged between 50 and 150 nm. In this particle size range, we could not establish a correlation between particle size and CD 8 T cell expansion (Figure S3A). The measured formulations had a negative zeta potential, and the highest CD 8 T cell expansions were observed with formulations having zeta potentials between −15 and −3 mV (Figure S3B). For either the molar compositions of DOPE and cholesterol, we found no clear correlations between their molar compositions and the number of CD 8 T cells present. We then decided to further investigate the properties of formulation B-11 yielding the highest CD 8 T cell levels. To evaluate the locations of mRNA transfection and protein synthesis, we formulated firefly luciferase (FFL) mRNA in B-11 LNPs and injected 10 μg of mRNA per mouse, as used in the vaccine study. Twenty-four hours later, we used bioluminescence to detect the location of protein expression (Figure 2A). We found FFL expressed at the injection site and in the draining lymph nodes, in the inguinal lymph nodes, as well as in some axillary lymph nodes. Luminescence was not detected in the liver, spleen, lung, or intestines. We then monitored the expression levels using the 3 formulations A-1, A-6, and B-11 at the injection site over time (Figure 2B and C). It is noteworthy that all three formulations reached the expression maximum of about 4 orders of magnitude after 24 h and declined slowly afterward. Formulation B-11 that elicited the highest CD 8 T cell levels did not translate into a higher maximal FFL concentration, compared to the other two formulations, but exhibited less drop-off in FFL concentration. All three formulations produced FFL for at least 10 days. The injection of the same amount of unformulated mRNA led to an increase of 1 order of magnitude and dropped off rapidly.

To determine whether we are transfecting APCs, we used the Aii14D reporter mouse and LNPs containing mRNA coding for Cre-recombinase (Figure 2D). These mice harbor a mutation in the Gt(ROSA)26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter driven tdTomato red fluorescent protein. Cells express tdTomato upon Cre-mediated recombination. We have chosen the Aii14D reporter mouse because expression levels using commercially available mRNA coding for fluorescent proteins, such as GFP, toTomato, or cyan fluorescent protein, were below the detection levels for analysis using flow cytometry. The draining lymph nodes, the inguinal lymph nodes in this case were removed, digested, and the monocytes were stained with secondary antibodies. Flow cytometry revealed that 4.6% of DCs, 1.2% of macrophages, 3.3% of neutrophiles, and 0.06% of B cells expressed the Cre-recombinase.

Unmodified RNA has the potential to activate endosomal Toll-like receptors 3 (TLR3), TLR7, and TLR8. Activation of these receptors induces an inflammatory response, transcription of pro-inflammatory cytokines, as well as up-regulation of
chemokines and type I interferons. This effect is desirable for vaccine application, and activation of the immune system is necessary to initiate an immune response. However, other cytoplasmatic RNA sensors, such as cytoplasmic retinoic acid-inducible gene I (RIG-I) or protein kinase RNA-activated (PKR), may hinder translation and enhance RNA degradation. In contrast to the immune activation, this effect of unmodified mRNA would be counter-productive for a strong immune response. Kariko et al. showed that by incorporating naturally occurring modified nucleosides, such as 5-methyl-cytidine, 5-methyluridine, 2-thiouridine, or pseudouridine, activation of the pattern recognition receptors can be suppressed. In contrast to the immune activation, this effect of unmodified mRNA would be counter-productive for a strong immune response. However, other cytoplasmatic RNA sensors, such as cytoplasmic retinoic acid-inducible gene I (RIG-I) or protein kinase RNA-activated (PKR), may hinder translation and enhance RNA degradation. In contrast to the immune activation, this effect of unmodified mRNA would be counter-productive for a strong immune response. Kariko et al. showed that by incorporating naturally occurring modified nucleosides, such as 5-methyl-cytidine, 5-methyluridine, 2-thiouridine, or pseudouridine, activation of the pattern recognition receptors can be suppressed. We then compared the capacity of modified and unmodified mRNA formulations to elicit CD8 and CD4 T cell proliferations in vivo. We measured the CD8 and CD4 T cell levels in blood, 6–11 days after immunization, of mRNA coding for OVA unmodified and mRNA with the same nucleotide sequence, fully substituted with pseudouridine and 5-methylcytidine (Figure 3A and B).

For both modified and unmodified mRNA, when compared with irrelevant mRNA, similarly formulated, we did not find much difference in the CD 4 T cell levels at any time point. On the other hand, the CD8 T cells present a very different picture. Specifically, while the CD 8 T cell levels for modified mRNA differ from the control only on day 7, mice treated with unmodified mRNA LNP s exhibited much higher CD 8 T cell levels at all time points measured. Modified mRNA containing LNP-treated mice reached a 5.7% (P < 0.003) significantly higher level of CD 8 T cells on day 7. This increase may be attributed to the activation of the innate immune system pattern recognition receptors, inducing inflammation, such as type I interferon. In this regard, our data are in agreement with two recently published articles investigating the role of type I interferon after intravenous injection of LNP mRNA vaccines. Both studies suggest that type I interferon is necessary for a protective CD8 T cell response. We also measured the antigen specific IgG titers 7 weeks after a single immunization (Figure S2). Only at a serum dilution of 1:16 or smaller, OVA specific IgG serum antibody titers were more than a standard deviation different from those in the control mice that were immunized with LNP s containing an irrelevant control mRNA.

To address the functionality of the proliferated CD 8 T cells, we tested formulation B-11 in a transgenic OVA-expressing mouse model. Figure 2A shows the biodistribution of luciferase expression using the B-11 formulation 24 h after subcutaneous injection. The inguinal and axillary lymph nodes emit light 24 h after injection. Importantly, no FFL expression is detected in the liver, kidney, spleen, colon, or lung. A sample set of mouse organs are analyzed 15 min after the injection of D-luciferin. Figure 2B shows the FFL expression was visualized 24 h after injection by optical imaging. Figure 2C shows the quantitative expression of FFL during 12 days. The formulation of mRNA in LNPs increases the FFL expression up to 3 orders of magnitude compared to unformulated mRNA. The FFL expression remains elevated for 10 days. Interestingly, the formulation yielding the highest CD8 T cell levels at day 7 does not exhibit a higher peak FFL expression but exhibits a slower decrease over time. The corresponding antigen specific CD8 T cell levels at day 7 post injection using mRNA coding for ovalbumin (OVA, 10 μg per mouse, n = 5 per group) are 1.1 ± 1.3% for Formulation A-1, 3.1 ± 1.6% for Formulation A-6, and 4.2 ± 1.5% for Formulation B-11. (D) Quantification of the percentage of transfected cells of the indicated type 2 days after the injection of LNP s containing mRNA coding for Cre-recombinase in Ai14D reporter mice, as determined by FACS analysis (n = 3 for control, and n = 4 for Cre LNP). *P < 0.05, **P < 0.01; unpaired student t test. The irrelevant control mRNA used in the figure corresponds to mRNA coding for OVA.
C57BL/6 mice (n = 7) were immunized with mRNA LNP (10 μg mRNA per mouse in 100 μL of PBS; the mRNA is either unmodified or completely substituted with 5-methylcytidine (5meC) and pseudouridine (ψ)), and subsequently, mice were bled at specific time points. The red blood cells were lysed, and the monocytes were stained with tetramer, live–dead stain, and CD4 and CD8 antibody conjugates. (A) Representative FACS profiles of mice treated with the indicated conditions. The CD8 T cell response in peripheral blood is much stronger from unmodified mRNA LNP vaccines. (B) The percentage of OVA specific CD8 T cells peaks at day 7 after subcutaneous injection. Compared to unmodified mRNA, the substitution with 5meC and ψ induces an immune response only slightly higher that in the group treated with irrelevant control mRNA LNPs. mRNA coding for β-galactosidase was used as the irrelevant control (***P < 0.01 by the ordinary one-way ANOVA Bonferroni’s multiple comparisons test). (C) No significant increase in circulating antigen specific CD 4 T cells could be detected. (D) mRNA LNP formulation B-11 induces potent in vivo antitumor immunity. Mice (C57BL/6j, n = 10 for the control group and n = 5 for the treated mice) were injected subcutaneously in the upper back with 1 × 10^6 B16-OVA melanoma cells on day 0. Treatment began when tumors were clearly visible in all mice (day 3) with LNP formulation B-11 containing OVA mRNA either modified or unmodified (days 3, 6, and 10, 10 μg of total mRNA per mouse and injection). Both treatment groups slow down tumor growth after the second treatment and shrink the tumor after the third treatment. Mice that reached the maximal allowed tumor area of 250 mm^2, or that developed ulceration, were euthanized and recorded as having tumor areas of 250 mm^2. (E) Overall survival is increased for both treatment groups. Statistical analysis was done using a log rank analysis (***P < 0.001, as compared with the untreated control group, two-way ANOVA with Bonferroni posthoc). (F) The percentage of SIINFEKL specific CD 8 T cells that were analyzed on day 17; the difference in CD 8 T cells was not statistically significant.
We then tested the hypothesis if enhanced TLR activation could increase the potency of the immune response by incorporating an adjuvant in the LNP formulation. We replaced 1% of the molar composition of PEG in the optimized LNP formulation with lipopolysaccharide (LPS, 10 μg per mL), consisting of a lipid A anchor, an inner core, an outer core, and an O-antigen repeat.\(^4\) LPS is a very potent TLR4 agonist.\(^4\) We envisioned that the LPS anchors in the outer membrane of the LNPs via the lipid A anchor and points the highly hydrophobic O-antigen repeat outward. An additional benefit of replacing some of the shielding PEG with O-antigen repeat carbohydrates may be that the LNPs bind to APCs via carbohydrate recognizing lectin receptors that are omnipresent on APCs and are endocytosed more efficiently.\(^4\) Dendritic cells express a number of lectins on their surfaces that allow ligand capture and endocytosis. These include the mannose receptor, Langerin also known as CD207,\(^4\) Dec-205,\(^3\) DC-SIGN also known as CD209,\(^4\) Dectin-1, and Dectin-2.\(^3\) The CD 4 T cell kinetics was not different from those observed in either treatment group (Figure S4). The observed CD 8 T cell levels peaked 1 day after the non-LPS LNPs on day 8 at 6.3% antigen specific CD 8 T cells (Figure S5A). It is noteworthy that LPS-containing LNPs may induce local inflammation at LPS concentrations of more than 1.0 μg per mouse. We then added LPS to LNPs containing TRP2 mRNA and tested them in the B16F10 melanoma model. We found that the mice receiving the LPS containing TRP2 mRNA LNPs survived significantly longer compared to the controls and mice receiving TRP2 mRNA LNPs (Figure S5B and C).

In conclusion, we presented evidence that our optimized LNP formulation, B-11, works well for delivering mRNA vaccines. Using the A114D reporter mice and the B-11 LNP formulation, we showed transfection in different immune cell populations, including dendritic cells, macrophages, neutrophils, and B cells. Cytosolic antigen synthesis and degradation by the proteasome enables antigen presentation on MHC-I and, consequently, activation of a potent CD 8 T cell response. We did not only induce CD 8 T cell proliferation, but the killer cells were also functional, as shown by extending the overall survival in a transgenic mouse melanoma model. Even more exciting was the effect on the aggressive B16F10 tumor model, where mRNA coding for the tumor associated self-antigens, TRP2 and gp100, was able to overcome the self-tolerance and to significantly extend the overall mice survival. The fact that adding LPS to the LNP formulation further increased survival is an indication that such additions may increase the potency of mRNA vaccines delivered by LNPs. The proof of concept presented here warrants further investigation of LNPs as potentially useful mRNA vaccine vectors.

**Materials and Methods. Lipid Nanoparticle (LNP) Synthesis.** LNPs were synthesized by mixing an aqueous phase containing the lipids in a microfluidic chip device as described previously.\(^5\) Briefly, the aqueous phase was prepared in 10 mM citrate buffer (pH 3) with corresponding mRNA (OVA, FFL, Cre, gp100, and TRP2, 1 mg/mL in 10 mM TRIS-HCl, from Trilink Biotechnologies, San Diego, CA). The ethanol phase was prepared by solubilizing a mixture of ionizable lipid, phospholipid, cholesterol, lipid-anchored PEG, and additive at predetermined molar ratios. For the LPS containing formulations, the LPS was added to the ethanol phase as a solution in DMSO (1 mg/mL; Lipopolysaccharide from *E. coli* 055:B5, purified by ion-exchange chromatography; Sigma-Aldrich order number L4524). Syringe pumps were used to mix the ethanol and aqueous phases at a 3:1 ratio in a microfluidic chip device. The resulting LNPs were dialyzed against PBS in a 20 000 MWCO cassette at room temperature for 2 h. The lipids used were obtained from 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids, Alabaster, AL), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP, Avanti), C12-200 (prepared as previously described\(^2\)), cKK-E12 (prepared as previously described\(^2\)), S03O13 (prepared as...
previously described\textsuperscript{52}, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, Avanti), cholesterol (Sigma-Aldrich, St. Louis, MO), 3\(\beta\)-[\(N\)-(\(N\)'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-cholesterol, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (C14-PEG2000, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-350] (ammonium salt) (C14-PEG350, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000] (ammonium salt) (C14-PEG1000, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000] (ammonium salt) (C14-PEG3000, Avanti), 1,2-distearyloyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (C18-PEG2000, Avanti), sodium lauryl sulfate (SLS, Sigma-Aldrich), arachidonic acid (Sigma-Aldrich), oleic acid (Sigma-Aldrich), and myristic acid (Sigma-Aldrich).

**LNP Characterization.** The size and polydispersity index (PDI) of the LNPs were measured using dynamic light scattering in 1× PBS (ZetaPALS, Brookhaven Instruments; Table 2). Zeta potentials were measured using the same instrument in a 0.1× PBS solution. Diameters are reported as the largest intensity mean peak average, which constitutes >95% of the nanoparticles present in the sample. To calculate the nucleic acid encapsulation efficiency, a modified Quant-iT RiboGreen RNA assay (Invitrogen) was used as previously described\textsuperscript{53}

**Cryo-Transmission Electron Microscopy.** To prepare LNPs for cryo-transmission electron microscopy (TEM), they were dialyzed against 0.1× PBS in a 20 000 MWCO cassette for 2 h. A sample of 3 \(\mu\)L of the LNP solution was dropped on a lacey copper grid coated with a continuous carbon film and blotted to remove excess sample without damaging the carbon layer by
Gatan Cryo Plunge III. A grid was mounted on a Gatan 626 single tilt cryo-holder equipped in the TEM column. The specimen and holder tip were cooled down by liquid nitrogen during transfer into the microscope and subsequent imaging. Imaging on a JEOL 2100 FEG microscope was done using minimum dose method. This is essential to avoid sample damage under the electron beam. The microscope was operated at 200 kV and with a magnification in the range of 10 000—60 000 to assess particle diameter and distribution. All images were recorded on a Gatan 2kx2k UltraScan CCD camera.

Mice. All procedures were performed under an animal protocol approved by the Massachusetts Institute of Technology Committee on Animal Care (CAC) and in accordance with the guidelines for animal care in a MIT animal facility. C57BL/6j mice and B6.Cg-Gt(Rosa)24Sortm14(CAG-tdTomato)Hze/J (Ai14D) mice, 6–8 weeks of age, were purchased from Jackson Laboratories and housed in an MIT animal facility.

Immunization. Mice were anesthetized in a ventilated anesthesia chamber with 2.5% isoflurane in oxygen. The lower back of the mice were shaved with a clipper, and the LNPs (0.1 mL containing 10 μg mRNA) were injected subcutaneously in the lower back of the mice. Mice were put back in their cages and monitored for signs of distress and local inflammation at the injection site.

Bioluminescence. Twenty-four hours after the injection of the mRNA LNPs, mice were injected subcutaneously at the injection site with 0.1 mL of D-luciferin (10 mg/mL in PBS). The mice were anesthetized in a ventilated anesthesia chamber with 2.5% isoflurane in oxygen and imaged 20 min after the injection with an in vivo imaging system (IVIS, PerkinElmer, Waltham, MA). Luminescence was quantified using the LivingImage software (PerkinElmer).

Flow Cytometric Analysis. At different time points after the immunization, blood was collected via mouse tail vein, and the red blood cells were lysed using a RBC lysis buffer solution (eBioscience, San Diego, CA). The monocytes were incubated with Fc block (CD16/32, BioLegend, diluted in FACS buffer at 1:9) at 4 °C for 15 min. The monocytes were then incubated for 30 min at room temperature with PE conjugates of MHC class I (eBioscience, diluted in FACS buffer at 1:200), and CD16/32. The samples were analyzed after three washes on a BD LSR II HTS-2 flow cytometer.

Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-Ag-Specific OVA Serum Antibody Detection. Lockwell Maxisorp plates (Thermo Scientific, Waltham, MA) were coated overnight at 4 °C with 44 μL/well of OVA (InvivoGen, San Diego, CA), 5 μg/mL in 100 mMolar carbonate/bicarbonate buffer, pH 9.6, and blocked for 1 h at 37 °C with 200 μL of blocking solution (5% BLOTTO, (Santa Cruz Biotechnology, Dallas, TX), in PBS containing 0.05% Tween20, (Sigma-Aldrich). Serum samples were initially diluted 1:16 in a carrier solution (same as blocking solution), transferred into coated-blocked plates, and serially 2-fold diluted. The plates were incubated for 2 h at 37 °C, washed, and incubated with a detection antibody: goat anti-mouse IgG HRP conjugate (Santa Cruz Biotechnology, diluted 1:1000 in carrier solution) and washed again. Antigen-specific total IgG was detected with HRP substrate ODP (Sigma-Aldrich) and read at 490/630 nm using an infinite M1000 plate reader (Tecan, Switzerland).

Tumor Cell Lines. B16-OVA is a murine B16F10 cell line that stably expresses chicken egg ovalbumin (OVA). The cell line was a kind gift from Dr. Kenneth Rock, Dana-Farber Cancer Institute, Boston. B16F10 melanoma cell line was obtained from ATCC. Both cell lines were maintained in DMEM, supplemented with fetal bovine serum (10%).

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.6b03329.

Additional experimental data and analysis (PDF)

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Notes

The authors declare the following competing financial interest(s): Robert Langer is co-founder and member of the board of directors of Moderna Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in, or financial conflict with, the subject matter or materials discussed in the manuscript apart from those disclosed.

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