Nonviral delivery of self-amplifying RNA vaccines


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Despite more than two decades of research and development on nucleic acid vaccines, there is still no commercial product for human use. Taking advantage of the recent innovations in systemic delivery of short interfering RNA (siRNA) using lipid nanoparticles (LNPs), we developed a self-amplifying RNA vaccine. Here we show that nonviral delivery of a 9-kb self-amplifying RNA encapsulated within an LNP substantially increased immunogenicity compared with delivery of unformulated RNA. This unique vaccine technology was found to elicit broad, potent, and protective immune responses, that were comparable to a viral delivery technology, but without the inherent limitations of viral vectors. Given the many positive attributes of nucleic acid vaccines, our results suggest that a comprehensive evaluation of nonviral technologies to deliver self-amplifying RNA vaccines is warranted.

In the 21st century, vaccines will play a major role in safeguarding the world’s health. However, with increasing life expectancy in high-income countries and newly emerging infections and poverty in low-income countries, new technologies will be required to address changing vaccine needs (1). Nucleic acid vaccines have the potential to address these needs, but despite decades of research there is still no commercial product for human use. Although plasmid DNA (pDNA) vaccines have proven to be a flexible platform and are broadly effective in small animal models, they have generally lacked potency in human clinical trials (2). Recombinant viral vector technologies have the advantage of efficient delivery of the nucleic acid payload, but their utility is often hampered by antivector immunity, production limitations, and safety concerns (3, 4). In 1990, Wolff et al. (5) demonstrated that direct injection (“naked delivery”) of messenger RNA (mRNA) or pDNA into the skeletal muscle of a mouse resulted in expression of the encoded protein. At the time, development of mRNA vaccines was considered unrealistic because of instability in vivo and during storage, and these concerns were compounded by difficulties in manufacturing at large scale. Hence, much of the subsequent development of nucleic acid vaccines focused on pDNA. However, many of the obstacles to mRNA vaccine development have been surmounted, and recently there has been a revival in the use of nonamplifying mRNA vaccines for cancer (6), allergy (7), and gene therapy (8). Naturally transient and cytosolically active mRNA can now be produced at sufficient quantity and quality for human clinical trials (6) and is seen by many (9) as a safer and more potent alternative to pDNA for vaccination. However, to be commercially competitive as a platform technology, mRNA-based vaccines must match the potency of viral vectors at doses of RNA that are not cost prohibitive. To this end, we evaluated the utility of a synthetic lipid nanoparticle formulation of self-amplifying RNA (LNP/RNA) as a means to increase the efficiency of antigen production and immunogenicity in vivo, without the need for a viral delivery system.

For these studies, we used a self-amplifying RNA based on an alphavirus genome (10), which contains the genes encoding the alphavirus RNA replication machinery, but lacks the genes encoding the viral structural proteins required to make an infectious alphavirus particle (Fig. L). The structural protein genes were replaced with genes encoding protein antigens, which are abundantly expressed from a subgenomic mRNA in the cytoplasm of cells transfected with these self-amplifying RNAs (3, 4, 10, 11). The RNA was produced in vitro by an enzymatic transcription reaction from a linear pDNA template using a T7 RNA polymerase, thereby avoiding safety concerns and complex manufacturing issues associated with cell culture production of live viral vaccines, recombinant subunit proteins, and viral vectors. After immunization, replication and amplification of the RNA molecule occurs exclusively in the cytoplasm of the transfected cells (Fig. S1), thereby eliminating risks of genomic integration and cell transformation, which pose safety hurdles for recombinant DNA, viral vectors, and pDNA vaccines (3, 4). Moreover, the barrier of nuclear delivery, which is thought to be a rate-limiting step for nonviral delivery of pDNA, is circumvented.

Viral vector-based technologies are generally regarded as the most efficient means to deliver nucleic acids into cells, but their utility can be restricted by preexisting or vaccine-induced antivector immunity that can decrease vaccine potency (12). To avoid this limitation, to provide protection from degradation, and facilitate entry into cells, nonviral delivery of nucleic acids has been explored extensively. Approaches include administration of nucleic acids in a naked form (simply formulated in buffer); in combination with lipids, polymers, or other compounds; and by physical techniques such as gene gun and electroporation (EP) (2). Injection of naked mRNA or self-amplifying RNA in vivo induces gene expression and generates immune responses (4, 11–13), with self-amplifying RNA being more efficient for gene expression in situ (13, 14). However, naked RNA vaccines suffer from limited potency, in part due to RNA instability in vivo, related to the presence of degradative enzymes in tissues (15). Hence, mRNA vaccines have been formulated with synthetic delivery vehicles such as liposomes (16) and cationic polymers (17) to increase potency. There are limited published data on the in vivo delivery of self-amplifying RNA using nonviral delivery strategies (3, 14, 18), and none has taken advantage of the recently developed, clinically suitable delivery systems for siRNA (19, 20). There has been extensive work on viral delivery of self-amplifying RNA using viral replicon particles (VRPs) (4, 11, 21–24). VRPs are potent vaccines in mice (10, 11), nonhuman primates (11, 22), and humans (15). These single-cycle alphavirus vectors were used as the viral delivery benchmark in our studies.


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broadly effective (26) and thus was used as the benchmark for pDNA delivery.

Results

Vaccine Formulation Characterization. Proof of concept for LNP-facilitated delivery of self-amplifying RNA vaccines was obtained using a subset of LNPs, called stable nucleic acid lipid particles (SNALPs) (19, 27) (Fig. 1B). The ionizable cationic lipid 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine-N-Dma(DLinDMA) (27), which is highly effective at delivering siRNA systemically in rodents and nonhuman primates, was used as the main component of the lipid nanoparticles. An ethanol dilution process (28) was used to produce small uniform lipid particles with a high RNA encapsulation efficiency. Mean particle size and polydispersity was determined by dynamic light scattering for all six LNP/RNA formulations used in the studies described (Table S1). The number-weighted mean diameters ranged from 79 to 121 nm and Z-average diameters from 130 to 164 nm with a polydispersity index from 0.09 to 0.14. The size distributions were characterized by a single peak with a low polydispersity index, indicating a relatively monodisperse size distribution. A fluorescence-based (Ribogreen) assay to detect free RNA in solution after LNP encapsulation demonstrated that 85–98% of the RNA was encapsulated (Table S1). These particle size and RNA encapsulation data indicate that the ethanol dilution process produces consistent LNP/RNA particles. Agarose gel electrophoresis showed that RNA integrity was maintained during formulation (Fig. 1C, lane 4) and that the LNPs protected the RNA from degradation by RNase A (Fig. 1C, lane 5). In contrast, the naked control RNA was degraded by RNase A (Fig. 1C, lane 3 compared with lane 2). Encapsulation was also required for efficient delivery of functional RNA into muscle. Six days after intramuscular (i.m.) injection of 1 μg naked self-amplifying RNA encoding secreted alkaline phosphatase (SEAP), gene expression, as determined by serum SEAP concentration, was measurable but highly variable (Fig. 1D). Encapsulation of a 10-fold lower dose of RNA (0.1 μg) in LNPs increased the serum SEAP concentration and reduced the variability between animals. Addition of empty LNPs (without RNA) to naked RNA (0.1 μg) decreased the serum SEAP concentration relative to LNP-encapsulated RNA, indicating that RNA encapsulation was necessary for efficient RNA delivery and reporter gene expression.

In Vivo Gene Expression at the Site of Injection. To monitor the efficiency of the self-amplifying RNA as a gene delivery system, expression of a firefly luciferase reporter gene was measured in mice after i.m. administration (Fig. 2). A single bilateral i.m. injection of a low dose (1 μg) of naked self-amplifying RNA resulted in measurable but highly variable bioluminescence at day 7 (Fig. 2A), relative to the encapsulated LNP/RNA. Bioluminescence intensity induced by LNP/RNA was substantially greater than that observed after administration of pDNA (Fig. 2D) or VRP delivery of RNA (Fig. 2B). The same self-amplifying RNA, when delivered using a VRP (1 × 10^6 IU), produced twice the bioluminescence at day 3 compared with LNP/RNA, but expression decayed much more rapidly and reached background levels by day 28 (Fig. 2B). On the other hand, mice administered a pDNA-encoding luciferase (10 μg) delivered using EP in situ displayed the highest measurable bioluminescence at all time points tested, and this level remained high for at least 63 d (Fig. 2D).

Immunogenicity of Candidate Vaccines Encoding Respiratory Syncytial Virus Fusion Glycoprotein (RSV-F). To test the LNP/RNA formulation as a vaccine, self-amplifying RNAs encoding viral antigens were evaluated for immunogenicity in mice. The F protein of RSV is a conserved target of neutralizing antibodies and a promising antigen for RSV vaccine development (29). The immunogenicity of i.m.-injected self-amplifying RNA encoding RSV-F was compared with i.m.-injected VRPs and with pDNA delivered by EP in situ. After two immunizations, LNP/RNA (0.1 μg) elicited anti-F IgG geometric mean titers (GMTs of 10,478). When pDNA was formulated in the LNPs at an equivalent dose of 0.1 μg (Fig. 3), IgG titers (GMT <25) were substantially lower than those elicited by LNP/RNA. EP delivery of pDNA (at a high dose of 20 μg) elicited anti-F IgG titers (GMTs of 6,712) on average 3.9-fold less than those elicited by 0.1 μg of LNP/RNA, although not statistically different. Given that electroplorated pDNA produced the highest and most prolonged levels of gene expression in situ (Fig. 2D), these data
suggested that factors other than antigen load and persistence govern immune responses.

In an additional experiment, in which a broader dose range of the LNP/RNA vaccine candidate was explored (Fig. 4), 10 μg of RNA elicited higher F-specific IgG titers than 1 × 10⁶ IU of VRPs. Seroconversion was established after a single vaccination with LNP/RNA at doses as low as 0.01 μg of RNA (Fig. S3). This contrasted with what was observed for naked RNA, which required two vaccinations and microgram doses of RNA. The LNP/RNA (Fig. 4 B and C) elicited slightly elevated titers of F-specific IgG2a relative to IgG1, consistent with a T helper type 1 phenotype. Consistent with this phenotype, the LNP/RNA was a potent inducer of antigen-specific IFN-γ producing CD4⁺ and CD8⁺ T cell responses (Fig. 4D and Fig. S4).

Protection from Virus Challenge. To test the efficacy of the LNP/RNA vaccine candidate, a cotton rat intranasal RSV challenge model (Fig. 5) was used to compare LNP/RNA, naked self-amplifying RNA, VRP RNA delivery, and a RSV-F subunit (29) adsorbed onto the adjuvant aluminum hydroxide (alum). As described for other naked self-amplifying RNA vaccines (12), the unformulated RNA vaccine elicited serum F-specific IgG and RSV neutralizing antibodies after two vaccinations (Fig. 5A and B). The LNP/RNA formulation boosted F-specific IgG titers approximately 8-fold (GMTs 4,355) and neutralization titers 10-fold (GMTs 1,493) relative to the same dose of naked RNA (GMTs of 526 (F-specific IgG) and 154 (neutralization)]. The LNP/RNA was statistically superior to naked RNA and equivalent to the 5 × 10⁶ IU of VRPs [GMTs 5,861 (F-specific IgG) and 1,690 (neutralization)]. The neutralization titers elicited by LNP/RNA are above the titer of 380 that correlates with protection in cotton rats (30) and similar to passively acquired serum neutralizing titers that correlate with protection of human infants from severe RSV disease (31, 32). All self-amplifying RNA vaccines provided protection from a nasal RSV challenge, reducing the lung viral load 1,000-fold compared with control animals (Fig. 5C) and these data are in agreement with previous reports for naked RNA (12). Importantly, the immunogenicity and protective efficacy generated by 1 μg LNP/RNA was equivalent to the responses elicited by the VRP delivery technology. A recombinant F protein subunit vaccine formulated with alum was shown to be the most potent vaccine for induction of total and protective antibody responses. However, for infant RSV vaccine development, safety considerations favor replicating (or self-amplifying) vaccine candidates over subunit approaches (33).

Discussion

This report demonstrates that the LNP delivery system, extensively explored for systemic delivery of siRNA, can be used for delivery of self-amplifying RNA vaccines. The ethanol dilution process produces small uniform particles with high encapsulation efficiency. The potency of this unique LNP/RNA vaccine in mice and cotton rats was comparable to a single-cycle alphavirus vector (VRP, 1 × 10⁶ IU) at a reasonable dose of RNA (1 μg) and was generally comparable to pDNA delivered using EP at higher doses. VRPs have been shown to be potent in nonhuman primates and humans at a 100-fold higher dose (1 × 10⁸ IU) (22, 25), and thus we anticipate that this unique RNA vaccine will be immunogenic at submilligram doses in larger species, but this remains to be directly tested. For pDNA the immune responses in larger species have been generally lower than in small animals, with the amount of
pDNA required for effective immunization of larger animals being 1,000-fold higher than for small species (milligrams versus micrograms) (2). The current costs of clinical manufacturing of pDNA are on the order of $50–100 per milligram (1–10 g scale) (26). The current projection for the cost of mRNA manufacture is comparable (9), which makes a very compelling commercial case for extensive evaluation of nonviral delivery of self-amplifying RNA and testing in larger animal species.

The major limitation for delivery of pDNA is thought to be the nuclear barrier, and transport across the nuclear membrane seems to be particularly inefficient in nondividing cells, such as mature myocytes. This barrier can be overcome by using relatively high pDNA doses and EP to facilitate pDNA delivery to the nucleus (34). RNA vaccines avoid this rate-limiting step by using cytoplasmic amplification and expression, resulting in more efficient transfection of different cell types, including quiescent or slowly proliferating cells, such as vascular endothelia or myocytes.

In addition, because the replication cycle of the self-amplifying RNA is strictly cytoplasmic, the need for codon modification is diminished, and the risks of splicing and destruction of the transcript are avoided. The lack of immune response elicited by LNP delivery of pDNA (Fig. 2) versus the response to i.m. injection of LNP/RNA and EP delivery of pDNA could potentially be attributed to inefficient transport of pDNA across the nuclear membrane. Facilitated delivery of RNA, either by VRPs or LNPs, resulted in higher levels of reporter gene expression (SEAP and luciferase) than naked RNA delivery and considerably lower variability of expression between animals. The LNP delivery system may achieve these effects by a combination of increased transfection efficiency and protection of the RNA from enzymatic degradation at the site of injection. The shorter duration of expression in situ after RNA immunization compared with pDNA may be related to the induction of apoptosis of the transfected cell, which could occur during RNA amplification, leading to transient gene expression (12, 13). Studies are underway to identify the transfected cells at the site of injection (Fig. 2C) and to determine why LNP delivery leads to longer gene expression than VRP delivery.

LNP delivery systems with low surface charge are sequestered by antigen presenting cells after s.c. administration (35, 36). For
an LNP/RNA vaccine, this sequestration might result in antigen production and/or stimulation of innate immune pathways within these immune cells. Further studies will be needed to elucidate the mechanism of action of this unique vaccine, which can then be used to rationally design enhancements to the vaccine. In other studies, the LNP/RNA vaccine also elicited functional immune responses against antigens from HIV (Fig. S5). Therefore, the LNP formulation markedly enhances the potency of the self-amplifying RNA, thereby generating a robust and potentially generic vaccine technology. Recently, there has been an exponential growth in the development of clinically suitable nonviral delivery systems for siRNA (19, 20); we have taken advantage of these innovations to develop the LNP/RNA formulation and have now embarked on a more extensive evaluation of other nonviral delivery systems.

Currently there is no vaccine for RSV and, because of a history of disease enhancement upon natural infection after immunization with an RSV vaccine candidate that elicited Th12-biased (and non-neutralizing) RSV-specific immune responses in RSV-naïve infants, elicitation of a non-Th12-biased RSV-specific response is considered an essential attribute for infant RSV vaccine candidates (33). The LNP/RNA provides this benefit (Fig. 4) in addition to strong cellular immune responses. The ratio of F-specific IgG titer to RSV neutralization titer (Table S2) is low for the LNP/RNA vaccine relative to the RSV-F subunit adsorbed on alum; thus it may be particularly suitable for development into a safe and effective infant RSV vaccine candidate.

Various RNA vaccines have been evaluated in human clinical trials, including naked and formulated non-amplifying mRNA (37–39) and self-amplifying RNA packaged in VRPs (25). Those studies showed RNA vaccines to be well tolerated and immunogenic. The recent pioneering work of others (6) on the clinical production of smaller (~2 kb) nonamplifying mRNA has established the feasibility of large-scale production of mRNA using an enzymatic transcription reaction. Whereas production of larger (~9 kb) RNA will add additional challenges, these are not insurmountable. In addition, long-term stability of RNA during storage has been demonstrated (6, 40). There are considerable advantages to producing the self-amplifying RNA from a cell-free transcription reaction and achieving delivery in vivo with a synthetic delivery system. These include (i) ease and speed of a generic production methodology, (ii) elimination of theoretical risks of generating infectious virus through recombination during production, (iii) avoidance of anti-vector immunity that currently limits the general utility of viral vectors, and (iv) the generation of a humoral and cellular immune responses (both CD4 and CD8).

Thus, this technology has potential as a platform to address multiple disease targets. Our preliminary work in rodents suggests that it will be fruitful to pursue a more extensive evaluation of nonviral delivery of self-amplifying RNA in larger species and the application of this technology as a platform: The SAM vaccine platform. This unique nucleic acid vaccine technology could enable a new generation of potent, versatile, and easily produced vaccines to address the health challenges of the 21st century.

Materials and Methods

RNA Synthesis. DNA plasmids encoding the self-amplifying RNAs were constructed using standard molecular techniques. Plasmids were amplified in _Escherichia coli_ and purified using Qiagen Plasmid Maxi kits (Qiagen). DNA was linearized immediately following the 3’ end of the self-amplifying RNA sequence by restriction digest. Linearized DNA templates were transcribed into RNA using the MEGAscript T7 kit (Life Technologies) and purified by LiCl precipitation. RNA was then capped using the Vaccinia Capping system (New England BioLabs) and purified by LiCl precipitation before formulation.

**LNP/RNA Formulation.** DLinDMA was synthesized as previously described (27). The 1,2-Diastearoyl-sn-glycerol-3-phosphocholine (DSPC) was purchased from Genzyme. Cholesterol was obtained from Sigma-Aldrich. 1,2-dimyr-istoyl-sn-glycerol-3-phosphoethanolamine-4-[methoxy(polyethylene glycol)]-2000] (ammonium salt) (PEG DMG 2000) was obtained from Avanti Polar Lipids. A modified ethanol dilution process (28) was used to produce the LNP formulation with the following molar ratios of lipid components: DSPC: cholesterol: PEG-DMG 2000: DLnDMA 10:48:2:40 molar percent. An 8.1 N:P molar ratio (nitrogen on DLnDMA to phosphate on RNA) and 100 mM citrate buffer (pH 6) were used for the formulations. In the first step of the in-line mixing, equal volumes of lipid (in ethanol) and RNA in buffer were mixed, through a T-junction via a KDS-220 syringe pump (kdScientific), and a third syringe with equal volume of buffer was added simultaneously to the lipid/RNA mixture. After 1 h equilibration at room temperature, the mixture was further diluted with 1:1 vol/vol citrate buffer. Next, the LNPs obtained were concentrated and dialyzed against 1× PBS using tangential flow filtration (TFF) (Spectrum Labs) with polyethersulfone (PES) hollow fiber membranes with a 100-kDa pore size cutoff and 20 cm² surface area. For in vitro and in vivo experiments, formulations were diluted to the required RNA concentration with 1× PBS (Teknova). Formulations were characterized for particle size, RNA concentration, encapsulation efficiency, and ability to protect from RNase digestion as described in SI Materials and Methods.

**Preparation of Other Vaccine Candidates.** pDNA preparation and delivery using EP, production of VRPs, and production of the RSV-F subunit absorbed on alum are described in detail in SI Materials and Methods.

**In Vivo Models.** Animals were housed in the Novartis Vaccines and Diagnostics Animal Facility. All experiments were approved and conducted according to...
the Novartis Animal Care and Use Committee. Female BALB/c mice, aged 8–10 wk and weighing about 20 g, were obtained from Charles River Laboratories. For the SEAP reporter gene experiment, five mice per group were injected bilaterally i.m. on day 0; blood was obtained at day 6; and a chemiluminescent assay (Phospha-Light System; Applied Biosystems) was used to analyze the serum for SEAP. For the luciferase reporter gene experiments, five mice per group were injected bilaterally i.m. on day 0. Before vaccination, mice were depilated. Mice were anesthetized [2% (vol/vol) isoflurane in oxygen], and their hair was removed with an electric razor followed by Nair. Fifteen minutes before imaging, mice were injected intraperitoneally with 8 mg/kg of luciferin solution (Caliper Life Sciences). Animals were then anesthetized [2% (vol/vol) isoflurane in oxygen] and transferred to the IVIS 200 imaging system (Caliper Life Sciences). Image acquisition times were kept constant as bioluminescence was measured with a cooled CCD camera.

For mouse vaccination experiments, groups of mice were immunized on days 0 and 21. Serum samples were collected 2 wk after each immunization. Immune or unvaccinated control animals were challenged intranasally (i.n.) with 1 × 10^6 plaque forming units (pfus) of RSV 4 wk after the final immunization. Blood collection and RSV challenge were performed under anesthesia with 3% (vol/vol) isoflurane using a precision vaporizer.

Statistical Analyses. We used the one-way ANOVA, Kruskal–Wallis (non-parametric) with Dunn’s posttest on selected groups with a 95% confidence interval. All statistical analyses were performed using Prism 5 software (GraphPad).

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Supporting Information

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SI Discussion

The broader application of the lipid nanoparticle (LNP)-formulated self-amplifying RNA vaccine was tested in mice with an antigen from HIV. LNP/RNA encoding the HIV envelope protein gene (Env, SF162 gp140) were compared with the benchmark of plasmid DNA (pDNA) delivered by electroporation (EP). Intramuscular (i.m.) injection of LNP/RNA (at a dose of 1 μg RNA) produced robust Env-specific serum IgG titers after one or two immunizations (Fig. S5A). These titers were superior to those elicited by i.m. injection of naked RNA (1 μg), and equivalent to those elicited by EP in situ of pDNA (15 μg). Although not statistically significant, the IgG titers after two vaccinations with the LNP/RNA [geometric mean titers (GMT) of 107,204] were 14-fold higher than those after EP of pDNA (GMT of 7,250). The LNP/RNA vaccine candidate was also immunogenic via the intradermal (i.d.) and subcutaneous (s.c.) routes. Serum IgG titers after two i.m. injections of the LNP/RNA (GMT of 107,204) were 10-fold higher than those observed after i.d. delivery (GMT of 9,903) and superior to those after s.c. delivery (GMT of 146). The IgG isotype profile of the antibody responses was dominated by IgG1 for pDNA delivered using EP, but was approximately equal between IgG1 and IgG2a for LNP/RNA (Fig. S5B), suggesting a stronger Th1 phenotype of the helper T cell responses for LNP/RNA compared with the pDNA vaccine. Consistent with this hypothesis, LNP/RNA elicited antigen-specific IFN-γ producing Th1 CD4+ and CD8+ T-cell responses at levels similar to or higher than pDNA delivered using EP (Fig. S5 C and D). In general, it appeared that LNP/RNA was approximately equivalent to pDNA/EP in terms of eliciting Env-specific CD4+ T cells (Fig. S5C) and superior for eliciting Env-specific CD8+ T cells (Fig. S5D).

SI Materials and Methods

pDNA Preparation. For DNA vaccination, plasmids encoding vaccine antigens were constructed using standard molecular techniques. Plasmids were grown in Escherichia coli and purified using Qiagen Plasmid Giga kits (Qiagen).

Particle Size. LNPs/RNA particle sizes were measured using a Zetasizer Nano ZS light scattering instrument (Malvern Instruments) according to the manufacturer’s instructions. Particle sizes are reported as the Z-average (Zve) with the polydispersity index (pdi). All samples were diluted in phosphate buffered saline (PBS) before measurement.

Encapsulation Efficiency and RNA Concentration. The percentages of encapsulated RNA and RNA concentrations were determined by the Quant-iT RiboGreen RNA reagent kit (Life Technologies). For a 75-μg batch size, LNPs were diluted 10-fold in 1× TE buffer (from kit, without Trition X-100), before addition of the dye. To quantify the amount of RNA inside the LNP, the formulation was diluted 10-fold in 1× TE buffer containing 0.5% (vol/vol) Triton X-100 (Sigma-Aldrich) before addition of the dye. Manufacturer instructions were followed to quantify the amount of RNA. For quantification of encapsulation efficiency, RNA concentrations obtained from samples without Triton X were interpreted as “not encapsulated,” because the dye cannot breach lipid membranes, and fluorescence from Triton X-treated samples represented total RNA amount (outside and inside LNPs). Controls of RNA standards with Triton X showed no significant variation in fluorescence. The fluorescence (excitation at 485 nm, emission at 528 nm) was detected with a microplate reader (BioTek Instruments).

All LNP formulations were dosed in vivo based on the encapsulated dose.

RNA Gel Electrophoresis and RNase Protection Assay. Denaturing agarose gel electrophoresis was performed to evaluate the integrity of the RNA after the formulation process and to assess RNase protection of the encapsulated RNA. A denaturing RNA gel was made using 0.4 g agarose (Bio-Rad) dissolved in 36 mL of diethylpyrocarbonate (DEPC)-treated water. A total of 4 mL of 10× denaturing gel buffer (Life Technologies) was added to the agarose solution. The gel was poured and was allowed to set for at least 30 min at room temperature. The gel was then placed in a gel electrophoresis tank and 1× Northernmax running buffer (Life Technologies) was used. A 1:1 (vol/vol) mixture of sample to 25:24:1 (vol/vol/vol), phenol:chloroform:isoamyl alcohol was made to extract the RNA from the lipids into the aqueous phase. The aqueous phase was removed for RNA analysis. For the RNase protection assay, 3.8 milliunit arbitrary units (mAU) of RNase A (Life Technologies) per microgram of RNA were added to the LNP/RNA formulation for 30 min at room temperature. RNase was then inactivated by incubating the sample with 6.4 mAU/μg RNA of proteinase K (Novagen) at 55 °C for 10 min. After RNase inactivation, RNA was extracted from LNPs (as described above). Before loading, the samples were incubated with formaldehyde loading dye, denatured for 10 min at 65 °C and cooled to room temperature. Ambion Millennium marker was used as the molecular weight standard for the RNA construct (Life Technologies). The gel was run at 90 V. The gel was stained using 0.1% (vol/vol) SYBR gold according to the manufacturer’s guidelines (Invitrogen) in water by rocking at room temperature for 1 h. Gel images were taken on a Chemidoc XRS imaging system (Bio-Rad).

pDNA Delivery Using EP. EP was used to deliver pDNA vaccines. Mice were anesthetized [2% (vol/vol) isoflurane in oxygen] and both hind legs were closely shaven to expose the area on the limb to be treated. A dose of 50 μL of vaccine candidate was injected into the quadriceps muscle of the hind limb using a 0.5-cc insulin syringe. The muscle was electroporated using an Elgen DNA Delivery system (Inovio). The instrument parameters were as follows: 60 V, 2 pulses each at 60 ms. Another dose was similarly delivered to the second limb, followed by electroporation.

Production of Viral Replicon Particles (VRPs). To compare RNA vaccines to traditional RNA-vectored approaches for achieving in vivo expression of reporter genes or antigens, we used VRPs produced in baby hamster kidney (BHK) cells by previously described methods (1). In this system, the antigen-expressing (or reporter gene-expressing) replicons consisted of alphavirus chimeric replicons (VCR) derived from the genome of Venezuelan equine encephalitis virus (VEEV) engineered to contain the 3′ terminal sequences (3′ UTR) of Sindbis virus and a Sindbis virus packaging signal (PS) (see figure 2 in ref. 1). These replicons were packaged into VRPs by coelectroporating them into BHK cells together with defective helper RNAs encoding the Sindbis virus capsid and glycoprotein genes (see figure 2 in ref. 1). The VRPs were then harvested and tittered by standard methods and inoculated into animals as solutions in cell culture fluid or other isotonic buffers.

RSV-F Subunit Vaccine Candidate. The RSV-F subunit vaccine candidate was expressed and purified as previously described (2).
ELISA for Antigen-Specific Serum Antibody. ELISA plates (MaxiSorp 96-well; Nunc) were coated overnight at 4 °C with 1 µg/mL purified recombinant RSV-F or SF162 oligomeric (o)-gp140 Env protein in PBS. After washing [PBS with 0.1% (vol/vol) Tween 20], plates were blocked with Superblock blocking buffer in PBS (Thermo Fisher Scientific) for at least 1.5 h at 37 °C. The plates were then washed; fivefold serial dilutions of serum in assay diluent [PBS with 0.1% (vol/vol) Tween 20 and 5% (vol/vol) goat serum] from experimental or control animals were added, and the plates were incubated for 2 h at 37 °C. Antigen-specific antismouse IgG or IgG subclasses were detected by incubating washed plates with HRP-conjugated goat antimouse IgG, HRP-conjugated goat antimouse IgG1, or HRP-conjugated goat antianimouse IgG2a (all from Southern Biotech) for 1 h at 37 °C. Antigen-specific anti-cotton rat IgG was detected with HRP-conjugated chicken anti-cotton rat IgG (ICL). Finally, plates were washed, and 100 µL of tetramethylbenzidine peroxidase substrate solution (KPL) were added to each well. Reactions were stopped by addition of 100 µL of 1 M H₃PO₄, and absorbance was measured at 450 nm using a plate reader (Molecular Devices). Titers were defined as the reciprocal serum dilution at an OD550 of >0.5, normalized to a species-specific standard that was included on each plate. Standards, with previously defined titers, were pooled sera from RSV-infected cotton rats, adjuvanted RSV-F or SF162.ogp140-immunized mice, or adjuvanted SF162.o-gp140-immunized mice. If the titer of a sample was below the first serum dilution tested, 1:25, it was assigned a titer of 5.

Intracellular Cytokines Immunofluorescence Assay. Two to five spleens from identically vaccinated BALB/c mice were pooled, and single-cell suspensions were prepared. Two antigen-stimulated cultures and two unstimulated cultures were established for each splenocyte pool. Cultures contained 1 × 10⁶ splenocytes, anti-CD28 mAb, and brefeldin A (BD Biosciences). RSV-F–specific T cells were stimulated with a pool of RSV-F peptides representing amino acid sequences 85–93, 249–258, and 51–66. For HIV-1, Env–specific T cells were stimulated with a pool of Iaα-restricted 23mers (YGVPWKEATTTLFCASDAK, AYDTEYHVNWVWATHACVPTDP, ITQACPKVSEFIPIHYVCAP, NVSTVQCTHGIRPVVSTOLL) and a H-2Dβ-restricted 9mer (IGPPGRAFYA) each at a final concentration of 2.5 µg/mL. Unstimulated cultures did not contain peptides and were otherwise identical to the stimulated cultures. After culturing for 6 h at 37 °C, cells were washed and then stained with Pacific Blue-labeled anti-CD4 and Alexa Fluor (AF) 700-labeled anti-CD8 monoclonal antibodies (mAb; BD Biosciences). Cells were washed again and then fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min. The fixed cells were then washed with Perm-wash buffer (BD Biosciences) and then stained with a mixture of PerCP Cy5.5–labeled anti–IFNγ (eBioscience), AF488–labeled anti–TNFα, allopurinol–labeled anti–IL-2, and phycoerythrin–labeled anti–IL-5 (all from BD Biosciences). Cells were washed and then analyzed on an LSR II flow cytometer (BD Biosciences). FlowJo software (Tree Star) was used to analyze the acquired data. The CD4⁺ 8⁻ and CD8⁺ 4⁻ T cell subsets were analyzed separately. For each subset in a given sample, the percent cytokine-positive cells was determined. The net (%) antigen-specific T cells were calculated as the difference between the percent cytokine-positive cells in the antigen-stimulated cultures and the percent cytokine-positive cells in the unstimulated cultures. For each 96-well plate, 2.5×10⁶ cells were added to each well; 6 h later, Foci were visualized with a mixture of mouse anti-RSV-F and anti-nucleoprotein antibodies (AbD Serotec) and counted using an LSR II flow cytometer (BD Biosciences). The CD4⁺ 8⁻ T cell subset was assigned a titer of 10.

Neutralization. Neutralization was determined using standard statistical methods. RSV neutralization assay. Two- to fivefold dilutions of heat-inactivated (HI) test sera were preincubated with RSV (at a concentration yielding 80–140 syncytia in virus control wells at the assay’s end) for 2 h before adding the virus/serum mixture to HEp-2 cells in 96-well plates. After 2 h, cells were overlaid with medium containing 0.75% (wt/vol) methylcellulose and incubated for ~42 h. Foci were visualized with a mixture of mouse anti-RSV-F and antinucleoprotein antibodies (AbD Serotec) and counted using a CTL Immunospot S5 UV Analyzer (Cellular Technology). The neutralization titer was defined as the reciprocal of the highest serum dilution producing at least a 60% reduction in number of foci relative to control wells without serum. If the titer of a sample was below the first serum dilution tested, 1:20, it was assigned a titer of 10.

RSV viral load. Lungs were harvested and weighed 5 d after infection, homogenized, and clarified. Virus from the processed lung samples was titered by plaque assay on HEp-2 cells by infecting for 2 h, removing inoculum, and overlaying with medium containing 1.25% (wt/vol) SeaPlaque agarose (Lonza). Cells were stained with neutral red 4 d later, and plaques were counted. If the viral load for an individual animal was below the assay limit of detection (~200 pfu/g lung), it was assigned a titer of 100 pfu/g lung.


2. Swanson KA. et al. (2011) Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. Proc Natl Acad Sci USA 108:9619–9624.
Fig. S1. Schematic illustration of the steps in replication and expression of self-amplifying RNA after delivery to a mammalian cell. (1) Delivery of RNA to the cytoplasm. (2) Translation of the ORF encoding the four nonstructural proteins that form the RNA-dependent RNA polymerase (RDRP), which produces a negative-sense copy of the genome. (3) RDRP catalyses production of positive-sense genomes from the negative sense copy. (4) RDRP catalyses transcription of subgenome. (5) Translation of the gene of interest, leading to protein expression.
Fig. S2. Whole-mouse bioluminescence imaging kinetics after bilateral i.m. injection with viral replicon particles (VRPs, \(1 \times 10^6\) infectious units) (IU), self-amplifying RNA formulated in lipid nanoparticles (LNP/RNA, 1 \(\mu\)g), or pDNA delivered using electroporation (DNA + EP, 10 \(\mu\)g) encoding luciferase.

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Fig. S3. Mouse immunogenicity study of a LNP/RNA vaccine encoding RSV-F, data after the first immunization. Groups of eight mice were vaccinated on days 0 and 21 with naked self-amplifying RNA (0.01–1 µg), self-amplifying RNA formulated in lipid nanoparticles (LNP/RNA, 0.01–10 µg), or viral replicon particles (VRPs, $1 \times 10^6$ IU). Sera were collected on day 14 (2wp1), and F-specific IgG titers were determined by ELISA. Measurements from individual mice are depicted as dots, and the geometric mean titers of eight mice per group are depicted as solid lines. The dotted line indicates the limit of titer quantification set at 25. If an individual animal had a titer of <25 (limit of detection), it was assigned a titer of 5.

Fig. S4. Frequencies of F-specific, cytokine-producing T cells in spleens of BALB/c mice vaccinated i.m. on days 0 and 21 with viral replicon particles (VRPs, $1 \times 10^6$ IU), pDNA delivered using electroporation (DNA EP, 20 µg), pDNA formulated with lipid nanoparticles (LNP/DNA, 0.1 µg), naked self-amplifying RNA (RNA, 1.0 µg) or self-amplifying RNA formulated in LNPs (LNP/RNA, 0.1 µg). Spleens were analyzed 4 wk after the second vaccination. Single-cell suspensions from pooled spleens were cultured for 5 h in the presence of brefeldin A and anti-CD28 mAb and in the absence or presence of synthetic peptides representing immunodominant epitopes in the F protein. Cells were stained for cell surface CD4 and CD8 markers and for intracellular cytokines IL-2, IFN-γ, TNF-α, and IL5 and were analyzed by flow cytometry. No IL-5+ cells were detected. (A) Percentage F-specific CD4+ T cells with the Th1 and Th0 phenotypes are shown. (B) Frequencies for the F-specific CD8 T cells that produced one or more of IL-2, TNF-α, and IFN-γ. Error bars indicate the 95% confidence upper limits.
Fig. S5. Mouse immunogenicity study of a lipid nanoparticle (LNP/RNA) vaccine encoding the HIV gp140 surface glycoprotein (SF162-o-gp140). Mice were immunized via intramuscular (i.m.), intradermal (i.d.), or s.c. injection on days 0 and 21 with self-amplifying RNA (RNA, 1 μg), self-amplifying RNA formulated in lipid nanoparticles (LNP/RNA, 1 μg), or pDNA delivered using electroporation (DNA + EP, 15 μg DNA). Sera were collected on day 14 (2wp1) and 35 (2wp2), and gp140-specific IgG(A), IgG1, and IgG2a(B) titers were determined by ELISA. Each symbol in the gp140-specific IgG(A), IgG1, and IgG2a(B) graphs represents an individual mouse titer with the horizontal bar representing the geometric mean for the group. Titers <25 (dotted line) were assigned a value of 5 for calculation of GMTs. On day 35, pooled splenocytes were stimulated with Env-derived antigenic peptides, stained for intracellular cytokines, and subjected to flow cytometry (Materials and Methods). Graphs show the Env-specific (%) frequencies of CD4+ (C) or CD8+ (D) T cells with error bars denoting 95% confidence limits. NS, not significant.

Table S1. Particle size and encapsulation efficiency of the LNP/RNA formulations

<table>
<thead>
<tr>
<th>Study</th>
<th>Gene</th>
<th>Zav, nm (pdi)</th>
<th>Size by intensity, nm</th>
<th>Size by number, nm</th>
<th>% RNA encapsulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNP/RNA SEAP expression (Fig. 1D)</td>
<td>SEAP</td>
<td>162 (0.13)</td>
<td>188.7</td>
<td>118.0</td>
<td>85.3</td>
</tr>
<tr>
<td>Kinetics of expression (Fig. 2C)</td>
<td>Luc</td>
<td>145.8 (0.09)</td>
<td>161.9</td>
<td>105.2</td>
<td>93.6</td>
</tr>
<tr>
<td>LNP/RNA vs. DNA + EP (Fig. 3)</td>
<td>RSV-F</td>
<td>158.6 (0.09)</td>
<td>176.1</td>
<td>116.6</td>
<td>90.7</td>
</tr>
<tr>
<td>LNP/RNA dose–response (Fig. 4)</td>
<td>RSV-F</td>
<td>164.3 (0.14)</td>
<td>177.1</td>
<td>120.9</td>
<td>95</td>
</tr>
<tr>
<td>Cotton rat challenge (Fig. 5)</td>
<td>RSV-F</td>
<td>157.7 (0.10)</td>
<td>174.2</td>
<td>117.9</td>
<td>97.5</td>
</tr>
<tr>
<td>LNP/RNA vs. DNA + EP (Fig. S3)</td>
<td>HIV gp140</td>
<td>129.9 (0.104)</td>
<td>141.8</td>
<td>78.82</td>
<td>92</td>
</tr>
</tbody>
</table>

Data are reported as the Z-average (Zve) with the polydispersity index (pdi), number-weighted mean, and intensity-weighted mean average diameters.

Table S2. Cotton rat immunogenicity and protection studies of a LNP/RNA candidate vaccine RSV-F

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Ratio of F-specific IgG titer to RSV neutralization titer, day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg RNA</td>
<td>3.4</td>
</tr>
<tr>
<td>1 μg LNP/RNA</td>
<td>2.9</td>
</tr>
<tr>
<td>5 x 10⁶ IU VRPs</td>
<td>3.5</td>
</tr>
<tr>
<td>10 μg F/alum</td>
<td>13</td>
</tr>
</tbody>
</table>

Groups of eight rats were vaccinated i.m. on days 0 and 21 with naked RNA (1 μg), LNP/RNA (1 μg), VRP (5 x 10⁶ IU), or alum-formulated RSV-F subunit (10 μg), or were not vaccinated. All animals were challenged intranasally with 1 x 10⁵ pfu RSV on day 49.