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.

vaccine platform | SAM vaccine | respiratory syncytial virus | HIV

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. 1*A*). 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 (25). These single-cycle alphavirus vectors were used as the viral delivery benchmark in our studies. Similarly, EP-mediated delivery of pDNA has been shown to be

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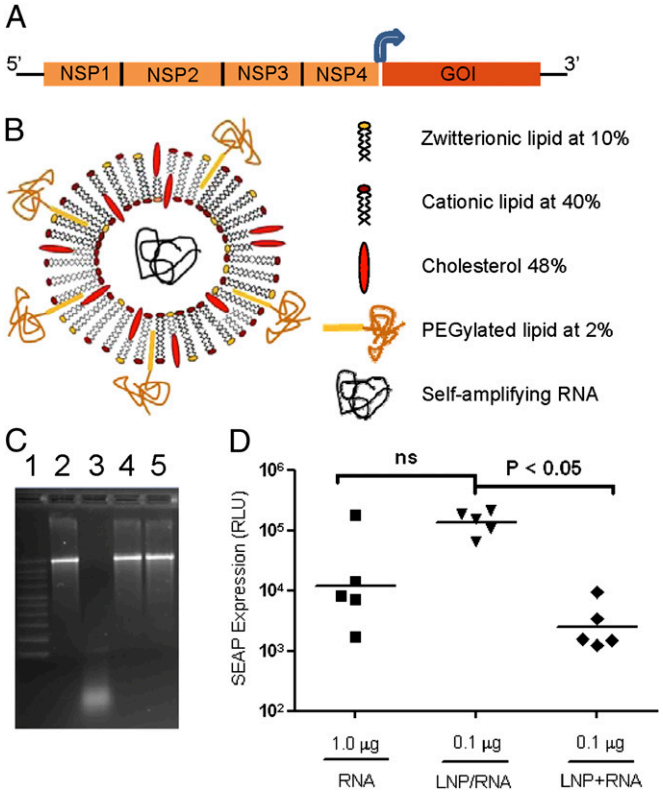

Fig. 1. Characterization of self-amplifying RNA vaccines. (*A*) Schematic illustration of a self-amplifying RNA derived from an alphavirus contains a 5′ cap, nonstructural genes (NSP1–4), 26S subgenomic promoter (grey arrow), the gene of interest (GOI), and a 3′ polyadenylated tail. (*B*) Schematic illustration of a lipid nanoparticle (LNP) encapsulating self-amplifying RNA, with the percent molar ratios of lipid components as indicated. (*C*) RNA agarose gel electrophoresis: RNA ladder (lane 1), self-amplifying RNA (lane 2), self-amplifying RNA after exposure to RNase A (lane 3), phenol-chloroform extraction of self-amplifying RNA from an LNP (lane 4), phenol-chloroform extraction of self-amplifying RNA from an LNP after exposure to RNase A (lane 5). (*D*) In vivo expression of secreted alkaline phosphatase (SEAP) 6 d after intramuscular (i.m.) injection of 1 µg self-amplifying RNA in PBS (RNA), 0.1 µg self-amplifying RNA encapsulated in an LNP (LNP/RNA), and 0.1 µg self-amplifying RNA mixed (not encapsulated) with LNP (LNP + RNA). Data are from individual mice (five per group, depicted as dots), and the geometric mean is represented by a solid line. NS, not significant.

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. 1*B*). The ionizable cationic lipid 1,2-dilinoleyloxy-3-dimethylaminopropane (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. 1*C*, lane 4) and that the LNPs protected the RNA from degradation by RNase A (Fig. 1*C*, lane 5). In contrast, the naked control RNA was degraded by RNase A (Fig. 1*C*, 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. 1*D*). 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. 2*A*), relative to the encapsulated LNP/RNA. Bioluminescence intensity induced by LNP/RNA was substantially greater than naked RNA, pDNA, and pDNA launched RNA, and was comparable to the bioluminescence produced by 1×10^6 infectious units (IU) of VRPs. In an extended study (Fig. 2 *B–D*), mice injected with LNP/RNA (1 µg) showed measurable bioluminescence that was already high on day 3, peaked on day 7, and decreased to background by day 63 (Fig. 2*C*). These kinetics contrasted with those observed after administration of pDNA (Fig. 2*D*) or VRP delivery of RNA (Fig. 2*B*). 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. 2*B*). 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. 2*D*).

Immunogenicity of Candidate Vaccines Encoding Respiratory Syncytial Virus Fusion Glycoprotien (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 RNA) was significantly more immunogenic than naked RNA (1 µg), as measured by the F-specific IgG geometric mean titers (GMT of 26,170 vs. 2,292; Fig. 3). The F-specific IgG response to 1 µg of LNP/RNA was equivalent to that elicited by 1×10^6 IU of VRPs (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 electroporated pDNA produced the highest and most prolonged levels of gene expression in situ (Fig. 2*D*), these data

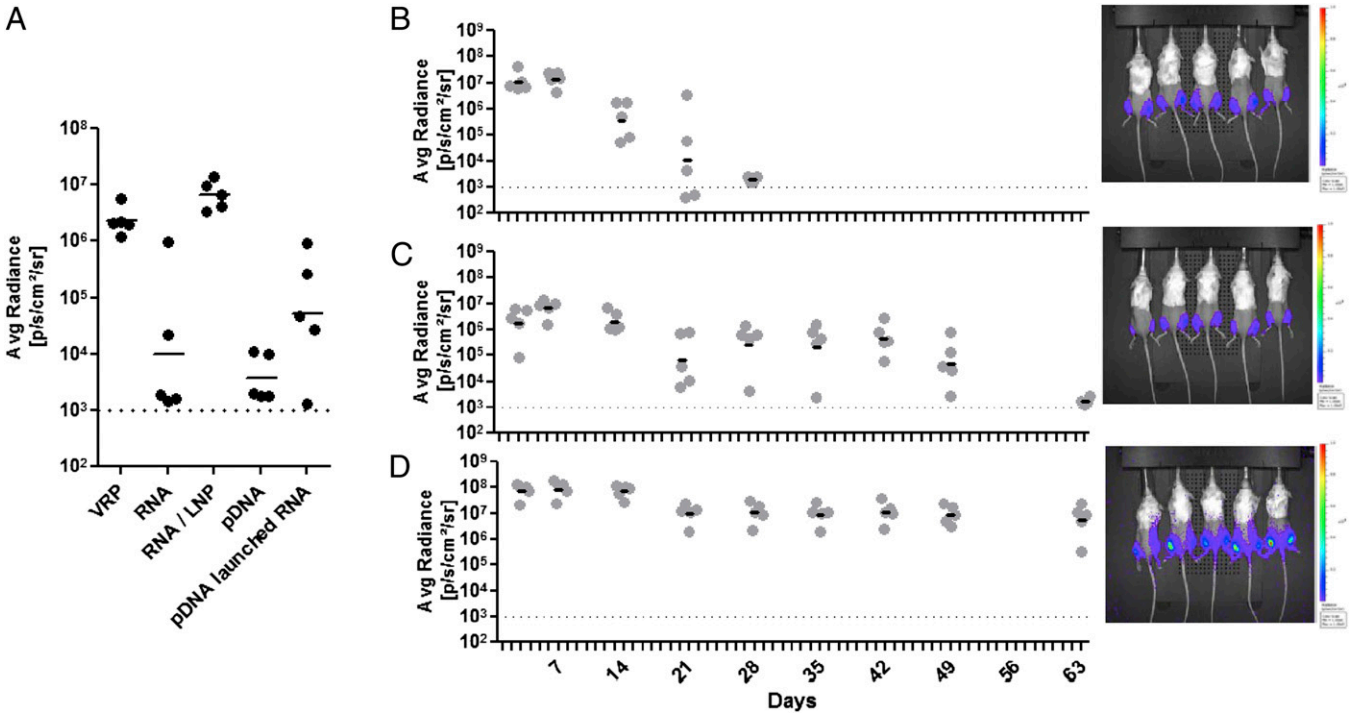

Fig. 2. Whole-mouse in vivo bioluminescence imaging after bilateral i.m. injection with RNA or DNA encoding firefly luciferase. (*A*) Bioluminescence recorded from individual animals on day 7 after administration of viral replicon particles (VRPs, 1×10^6 infectious units) (IU), self-amplifying RNA (1 μg), self-amplifying RNA encapsulated in lipid nanoparticles (LNP/RNA, 1 μg), plasmid DNA (pDNA, 10 μg), or a pDNA-launched self-amplifying RNA (10 μg). Each dot represents the whole-mouse photon count expressed as photons per second per square centimeter per steradian (p/s/cm^2/sr); the solid line represents the geometric mean; and the dotted line represents the limit of detection (1×10^3 p/s/cm^2/sr). (*B*) Average radiance over 63 d after administration of VRPs (1×10^6 IU), with representative bioluminescence images at day 7. (*C*) Average radiance over 63 d after administration of self-amplifying RNA (1 μg) encapsulated in LNP, with representative bioluminescence images at day 7. (*D*) Average radiance over 63 d after administration of electroporated pDNA (10 μg), with representative bioluminescence images at day 7. Data are from individual mice (five per group, gray circles); the geometric mean is represented by a solid line; the limit of detection is indicated by the dashed line (1×10^3 p/s/cm^2/sr). Additional images from the 63-d time course can be found in Fig. S2.

suggest 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 1×10^6 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_H1 helper T cell 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. 4*D* 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. 5 *A* 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 5×10^6 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. 5*C*) 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, 1×10^6 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 (1×10^8 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

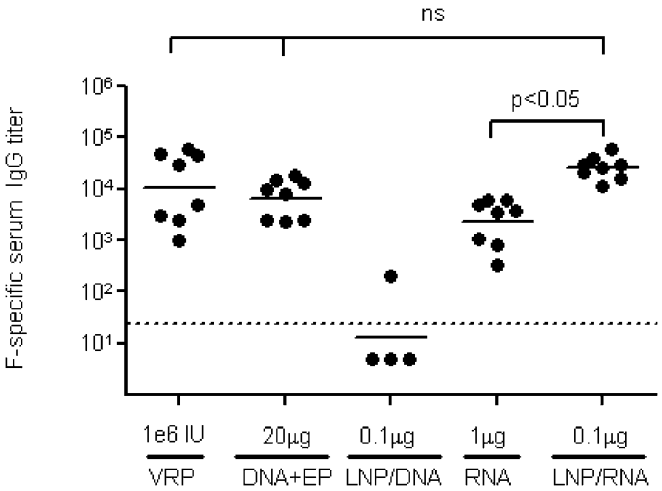

Fig. 3. Comparative mouse immunogenicity studies of a lipid nanoparticle formulated self-amplifying RNA (LNP/RNA) vaccine candidate encoding RSV-F. Groups of eight mice (except LNP/DNA, with four mice per group) were vaccinated intramuscularly (i.m.) on days 0 and 21 with viral replicon particles (VRPs, 1×10^6 IU), plasmid DNA delivered using electroporation (DNA + EP, 20 μg), pDNA formulated with LNP (LNP/DNA, 0.1 μg), naked self-amplifying RNA (RNA, 1.0 μg) or self-amplifying RNA formulated in LNP (LNP/RNA, 0.1 μg). Sera were collected on day 35, and F-specific IgG titers were determined by ELISA. Data are from individual mice (depicted as dots), and the geometric mean titers (GMTs) are solid lines. Dotted line indicates the limit of titer quantification (25 titer limit). To calculate GMTs, titers <25 were assigned a value of 5. F-specific $CD4^+$ and $CD8^+$ T cell frequencies can be found in Fig. S4. NS, not significant.

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. 2*C*) 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

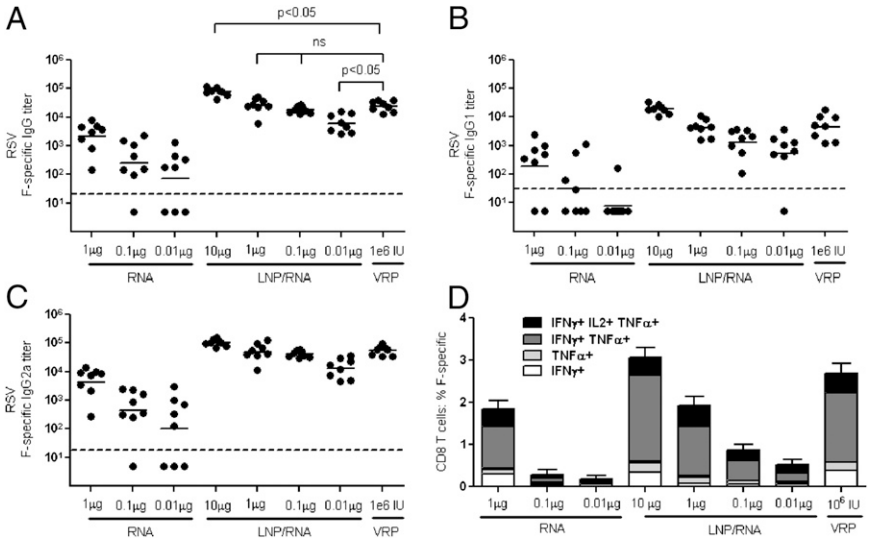

Fig. 4. Mouse immunogenicity studies of a lipid nanoparticle formulated self-amplifying RNA (LNP/RNA) candidate vaccine encoding RSV-F. Groups of eight mice were vaccinated intramuscularly (i.m.) on days 0 and 21 with naked self-amplifying RNA (0.01–1 μg), self-amplifying RNA formulated in lipid nanoparticles (LNP/RNA, 0.01–10 μg), or viral replicon particles (VRPs, 1×10^6 IU). Sera were collected on day 35, and F-specific IgG (*A*), IgG1 (*B*), and IgG2a (*C*) titers were determined by ELISA. Dots depict measurements from individual mice and solid lines, the geometric mean titers of eight mice per group. Dotted lines indicate the limit of 25 for quantification. For determination of GMTs, a titer <25 was assigned a value of 5. (*D*) Frequencies of RSV-F antigen-specific, cytokine-producing $CD8^+4^-$ T cells in spleens of BALB/c mice vaccinated on days 0 and 21 with RNA, RNA/LNP, or VRP. Spleens were collected 4 wk after the second vaccination and pooled (four spleens per pool) before antigen stimulation in vitro and flow cytometry analysis. Error bars indicate the 95% confidence upper limits. No $IL2^+$ $TNF\alpha^+$, $IL2^+IFN\gamma^+$, $IL2^+$, or $IL5^+$ CD8 T cells were detected. Serum IgG titers 2 wk after the first vaccination can be found in Fig. S3. NS, not significant.

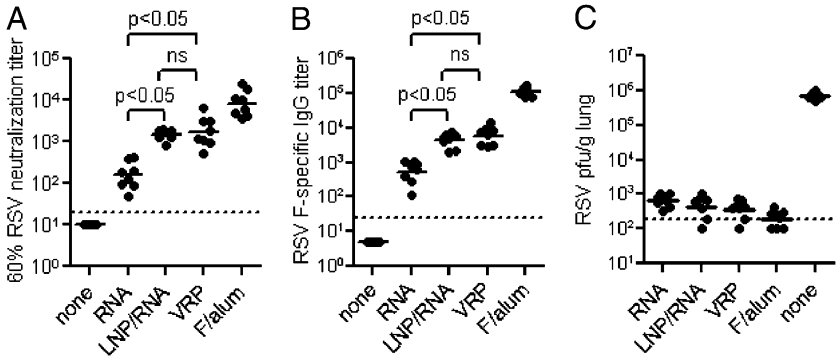

Fig. 5. Cotton rat immunogenicity and protection studies of a lipid nanoparticle-formulated self-amplifying RNA (LNP/RNA) candidate vaccine encoding RSV-F. Groups of eight rats were vaccinated i.m. on days 0 and 21 with naked self-amplifying RNA (1 μg), self-amplifying RNA formulated in LNPs (LNP/RNA, 1 μg), viral replicon particles (VRPs, 5×10^6 IU), or alum-formulated RSV-F subunit (10 μg); or they were not vaccinated. All animals were challenged intranasally with 1×10^5 pfu of RSV on day 49. (*A*) Serum RSV neutralization titers and (*B*) serum F-specific IgG titers 2 wk after the second vaccination (day 35). (*C*) Lung viral load 5 d after the RSV challenge (day 54). Data are from individual rats (depicted as dots), and the geometric mean titers (GMTs) are solid lines. Dotted lines indicate assay limits of detection ($A = 20$, $B = 25$, $C = 195$). For determination of GMTs, a titer below the limit of detection was assigned a value of 10 (*A*), 5 (*B*), or 100 (*C*). NS, not significant.

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 T_H2-biased (and non-neutralizing) RSV-specific immune responses in RSV-naive infants, elicitation of a non-T_H2-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*-glycero-3-phosphocholine (DSPC) was purchased from Genzyme. Cholesterol was obtained from Sigma-Aldrich. 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-[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: DLinDMA 10:48:2:40 molar percent. An 8:1 N:P molar ratio (nitrogen on DlinDMA 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 cm^2 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 Lifesciences). 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. All vaccines were injected into both quadriceps (50 μL per site). When measurement of T cell responses was required, spleens were removed at day 35 or 49. RSV-F–specific IgG, neutralizing antibody titers and T cell responses were determined essentially as described previously (29) and are included in *SI Materials and Methods*.

Female cotton rats (*Sigmodon hispidis*) were obtained from Harlan Laboratories. Groups of animals were immunized i.m. in a single hind leg (100 μL) on days 0 and 21. Serum samples were collected 2 wk after each immunization. Immunized or unvaccinated control animals were challenged intranasally (i.n.) with 1×10^5 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 (nonparametric) 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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