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Design and preclinical assessment of mRNA-1345 prefusion F glycoprotein-encoding mRNA vaccine for respiratory syncytial virus

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Abstract

Respiratory syncytial virus (RSV) is a significant cause of lower respiratory tract disease in young children and older adults. We designed a codon optimized mRNA vaccine, mRNA-1345, that encodes the RSV F-glycoprotein stabilized in the prefusion (preF) conformation and with a deletion at the cytoplasmic tail. mRNA-1345 protein expression was higher in vitro versus previous mRNA-based RSV vaccine candidates evaluated clinically. In rodent models, mRNA-1345 induced a robust neutralizing and preF-biased antibody response, a T helper 1-biased cellular response, and demonstrated dose-dependent protection against RSV challenge with no evidence of enhanced respiratory disease. These findings underscored the potential of mRNA-1345 as an effective RSV vaccine and are substantiated by clinical data demonstrating efficacy of mRNA-1345 in older adults.

Introduction

Respiratory syncytial virus (RSV) is a common seasonal virus that can cause mild to severe respiratory tract disease in people of all ages[1, 2]. Older adults, persons with underlying cardiopulmonary disease or immunocompromised, and pediatric populations are at increased risk for serious RSV-associated complications, which can lead to hospitalization and death[3, 4]. In 2019, an estimated 5.2 million cases of RSV-associated acute respiratory infection (RSV-ARI) were reported across high-income countries in older adults (aged ≥ 60 years), leading to 470,000 hospitalizations and 33,000 in-hospital deaths[5]. Among children aged ≤ 5 years, approximately 33 million episodes of RSV-ARI were estimated to have occurred globally in 2019, leading to 3.6 million hospitalizations, and 26,300 in-hospital deaths[6].

While treatment for RSV disease mainly involves supportive care[7], the number of licensed prophylactic options has increased in recent years[8]. In pediatric populations, two monoclonal antibodies are approved to provide passive immunity against RSV: palivizumab for children at high risk of RSV disease[9, 10] and nirsevimab for neonates and infants during their first RSV season as well as high-risk children (≤ 24 months) in their second RSV season[11, 12]. Both antibodies bind the RSV envelope fusion glycoprotein (RSV F)[9, 11], and were shown to prevent severe RSV disease in children[13, 14]. In adults aged ≥ 60 years, three RSV vaccines have been approved to date for the prevention of lower respiratory tract disease (LRTD) caused by RSV (RSV-LRTD)[8, 15, 16], including the first approved mRNA-based RSV vaccine, mRNA-1345 (mRESVIA, Moderna, Inc., Cambridge, MA, USA)[17]. Additionally, one of these vaccines (ABRYSIVO; Pfizer, Inc., New York, NY, USA) is also indicated for active immunization of pregnant women for the prevention of RSV-LRTD in infants from birth until 6 months of age[16]. There are no licensed RSV vaccines for active immunization of children.

These licensed RSV vaccines each contain, or encode in the case of mRNA-1345, the RSV F glycoprotein stabilized in the prefusion (preF) conformation[15–17]. The F glycoprotein mediates membrane fusion during the virus entry process and is conserved across RSV strains and antigenic subtypes (RSV-A and RSV-B)[18, 19]. It exists in two primary conformational states, preF and postfusion (postF)[18]. The preF

conformation is the main target of the protective neutralizing antibody response induced by natural RSV infection, displays all the known sites of neutralization (whereas many are absent in the postF conformation), and is associated with a greater neutralizing antibody response than the postF conformation[18, 20–22].

While the prefusion-stabilized form of the F protein has supported the development and approval of adult RSV vaccines[7], development of pediatric RSV vaccines has not yet been successful in part because of the experience with a formalin-inactivated RSV (FI-RSV) vaccine candidate in the 1960s[23]. In a clinical study, immunization of RSV-naïve infants with an FI-RSV vaccine caused an increased frequency of severe RSV disease (enhanced respiratory disease [ERD]) after subsequent natural RSV infection, leading to more frequent hospitalizations and resulting in two infant deaths[23]. The ERD outcome following RSV infection has been attributed to several reasons including the lack of antibody affinity maturation resulting in low-avidity, non-protective antibodies, failure to elicit cytotoxic T lymphocytes, and an exacerbated T helper type 2 (Th2) response[7, 24]. Therefore, investigational pediatric RSV vaccines must be evaluated in preclinical animal models and in clinical studies in adults and RSV seropositive children prior to evaluation in RSV-naïve infants[25–28]. In these animal models, the candidate vaccine should induce RSV neutralizing antibodies, avoid induction of excess non-neutralizing antibodies, avoid a Th2-biased response, and avoid lung inflammation after live RSV challenge[26]. Induction of CD8 T cell response may also be desirable[26]. These characteristics will discriminate the preclinical profile of the candidate vaccine from that of FI-RSV[26].

Previously, the preclinical and clinical evaluations of two other mRNA-based RSV vaccine candidates (mRNA-1777, mRNA-1172) encoding prefusion-stabilized RSV F glycoprotein were described[29–31]. mRNA-1172, which incorporated interprotomer disulfides and linked F1 and F2 subunits in addition to the preF-stabilizing mutations used in mRNA-1777, appeared more immunogenic in animal models and in Phase 1 clinical studies in healthy younger (18–49 years) and older (60–79 years) adults[31]. Both vaccine candidates were generally well tolerated in these clinical studies[30, 31].

In this study, we further optimized the mRNA RSV vaccine through structural engineering of the preF protein, with the aim to enhance immunogenicity. We present the stepwise rational design and preclinical evaluation of mRNA-1345, consisting of a single mRNA sequence that encodes the membrane-anchored RSV F glycoprotein stabilized in the preF conformation. Recently, a single dose of mRNA-1345 vaccine (50 µg) was shown to be efficacious in protecting adults aged ≥ 60 years from RSV-LRTD in a pivotal phase 2–3 trial (NCT05127434)[32]. Consistent with the demonstrated efficacy, immunogenicity analysis of the single-dose mRNA-1345 (50 µg) from this phase 2–3 trial demonstrated increased neutralizing antibodies (nAb) against RSV-A by 8.4-fold and against RSV-B by 5.1-fold at day 29 relative to baseline among adults aged ≥ 60 years, including those at risk for severe disease[33]. Clinical studies of mRNA-1345 are ongoing in other vulnerable populations including children and infants[33–36].

Results

Screening candidate constructs

mRNA constructs based on mRNA-1172 (encoding RSV preF) with additional features such as codon redesign, deletion of the cytoplasmic tail (ΔCT), interprotomer and intraprotomer disulfide bonds, cavity-filling hydrophobic amino-acid mutations[22], modification of the 5' untranslated region and some of their combinations were screened for preF protein expression in Expi293 cells at 24 hours post-transfection. Codon redesign was deployed to further increase protein expression[37], and deletion or replacement of the viral CT was implemented to alter cellular membrane distribution of the modified F proteins[38]. Three lead candidates with the highest F protein expression in vitro were selected for further characterization: codon_mRNA-1172 (codon redesign), ΔCT_mRNA-1172 (ΔCT), and mRNA-1345 (codon redesign and ΔCT) (Fig. 1a-b).

The preF conformation of the protein expressed by mRNA-1345 was experimentally confirmed by transfecting Expi 293T cells and staining with a panel of RSV-F monoclonal antibodies known to target antigenic sites on preF, postF or both conformations (**Supplementary Fig. 1a**). Interprotomer disulfide bond formation was confirmed by western blot analysis of reduced and non-reduced cell lysates (**Supplementary Fig. 1b**). mRNA-1345 showed enhanced cell surface protein expression compared to mRNA-1172 and the other lead designs at days 1 to 7 post transfection (Fig. 1c, **Supplementary Fig. 1c**). In mRNA-transfected HeLa cells, mRNA-1345 showed increased protein expression at 48 hours compared with mRNA-1172, codon_mRNA-1172, and ΔCT_mRNA-1172, particularly on the cell membrane (**Supplementary Fig. 1d, f**).

To assess whether increased protein expression translated to increased immunogenicity, BALB/c mice were vaccinated and boosted with increasing doses (0.2 μg, 1 μg, or 5 μg) of each candidate vaccine at days 1 and 22. RSV neutralizing antibody titers increased in a dose-dependent manner across different groups. However, despite the enhanced in vitro protein expression observed with mRNA-1345, RSV neutralization titers at day 36 in mice were comparable to those induced by mRNA-1172 (Fig. 1d).

On the basis of increased in vitro protein expression and comparable immunogenicity to mRNA-1172 in BALB/c mice, mRNA-1345 was selected as the lead mRNA vaccine design.

Immunogenicity in BALB/c mice

We sought to further characterize the antibody and T-cell responses elicited by mRNA-1345 in mice, as well as to compare these responses to those induced by control vaccines FI-RSV and adjuvanted preF protein to derisk ERD (Fig. 2).

Neutralizing and binding antibody responses

mRNA-1345 induced RSV-neutralizing antibody as well as preF and postF immunoglobulin G (IgG) binding antibody that were similar or higher in magnitude than that induced by preF protein adjuvanted with alhydrogel (AH) (Fig. 2a). Induction of postF binding antibody with a preF-stabilized vaccine was expected, given there are regions of structural homology between the preF and postF conformation. FI-RSV induced high levels of RSV postF IgG, but no detectable RSV neutralizing or preF binding antibodies (Fig. 2a). The antibody response to mRNA-1345 was primarily functional (neutralizing), as evidenced by the low ratio of RSV postF IgG to RSV neutralizing antibody, whereas the response to FI-RSV was primarily non-functional (non-neutralizing), as evidenced by higher ratios of RSV postF IgG to RSV neutralizing antibody (Fig. 2a).

In BALB/c mice, the IgG antibody subclasses induced by a vaccine can be an indicator of a T helper type 1 (Th1) or Th2 response[39]; induction of only the IgG1 subclass is associated with a Th2-biased response, whereas IgG2a is also induced in the presence of the type 1 cytokine interferon gamma (IFNγ)[40]. mRNA-1345 induced a balanced RSV preF IgG1 and IgG2a response (Fig. 2b), while preF protein + AH induced mostly IgG1, indicating a Th2 response (Fig. 2b). FI-RSV also trended toward a IgG1 Th2 bias, but the response magnitude was low given the low RSV preF IgG response (Fig. 2b). Because the IgG response to FI-RSV was almost exclusively detected against the RSV postF antigen, postF IgG1 and IgG2a were measured to better characterize the IgG antibody subclass response. mRNA-1345 induced RSV postF IgG1 and IgG2a, again indicating a Th1 response (Fig. 2c). In contrast, FI-RSV and preF protein + AH induced mostly postF IgG1, indicating a Th2 response (Fig. 2c).

Serum from mRNA-1345-vaccinated mice showed reduced binding to an RSV preF site Ø mutant protein, demonstrating that mRNA-1345 induces antibodies to the immunodominant site Ø epitope (**Supplementary Fig. S2**). Additionally, mRNA-1345 did not induce binding antibodies to the related human metapneumovirus (hMPV) fusion protein stabilized in the preF conformation, indicating specificity of the antibodies to RSV and no or limited cross-reactivity with hMPV preF.

CD4 and CD8 T-cell response

Cellular responses, including a direct assessment of Th1/Th2 balance, were assessed in mice following mRNA-1345 vaccination. mRNA-1345 induced RSV-specific $CD4^+$ T cells that produced interleukin 2 (IL-2) and the Th1 cytokines IFNγ and tumor necrosis factor alpha (TNFα), but produced low amounts of the Th2 cytokines IL-13, IL-5, or IL-4 (Fig. 3a). mRNA-1345 also induced RSV-specific $CD8^+$ T cells that showed increased surface expression of CD107a, a marker for degranulating cytotoxic T cells, and increased levels of IFNγ, TNFα, and IL-2 (Fig. 3b). Together, these results indicate that mRNA-1345 induced a Th1-biased response as well as a CD8 + T cell response. In contrast, FI-RSV and preF protein + AH induced a lower RSV-specific $CD4^+$ T cell response that was primarily Th2-biased; neither induced a $CD8^+$ T cell response (Fig. 3).

Immunogenicity and challenge in cotton rats

Neutralizing and binding antibody response

A cotton rat challenge model was used to further evaluate the immunogenicity and protection against RSV challenge of single (0.3 μg) or two-dose (3 ng, 30 ng, 0.3 μg, 3 μg, or 30 μg) regimens of mRNA-1345. Cotton rats are generally accepted as the preferred animal model to evaluate the potential of an investigational RSV vaccine to promote ERD and has become the standard preclinical evaluation prior to the clinical evaluation of RSV vaccines in RSV-naïve infants[41]. This mRNA-1345 cotton rat study was conducted according to guidance for preclinical assessment of investigational RSV vaccines[26–28].

As shown in Fig. 4, mRNA-1345 induced RSV-neutralizing and binding (preF and postF) antibodies in a dose-dependent manner. A single dose of 0.3 μg mRNA-1345 induced low levels of F-binding antibodies; this response was boosted by a second dose of vaccine. Infection with RSV also induced an RSV-neutralizing, preF- and postF-binding antibody response; the neutralizing antibodies induced by RSV infection were comparable to those induced by a two-dose series of at least 0.3 μg mRNA-1345. Different lots of FI-RSV (FI-RSV lot #100 [FI-RSV #1] and FI-RSV [#2]) induced mostly postF-binding antibodies that were not neutralizing and did not bind to preF. Injections with non-RSV materials (FI-mock, LNP control, and phosphate-buffered saline [PBS]) did not elicit an RSV antibody response. The postF-IgG to RSV-neutralizing antibody ratio for mRNA-1345 was approximately two- and ten-fold lower than for FI-RSV #1 and FI-RSV #2, respectively. The ratios of RSV F-binding to RSV-neutralizing antibody were largely consistent across mRNA-1345 dose levels.

These results indicate that the antibody response induced by mRNA-1345 in cotton rats was largely functional (neutralizing), whereas the antibody response to FI-RSV was primarily non-functional (non-neutralizing) and directed to the postF conformation.

Challenge in cotton rats

Vaccination with mRNA-1345 protected cotton rats from RSV challenge in a dose-dependent manner relative to control animals; no detectable virus was found in the lungs of cotton rats dosed twice with 30-μg or 3-μg mRNA-1345, and partial protection was observed in cotton rats dosed once or twice with 0.3 μg mRNA-1345 (Fig. 5a). Both lots of FI-RSV afforded partial protection from RSV challenge in the lung, whereas RSV infection on day 0 provided complete protection against RSV challenge both in the lung (Fig. 5a) and the nose (**Supplementary Fig. 3**). Partial protection in the nose of cotton rats was also observed with two mRNA-1345 injections of 30 μg or 3 μg (**Supplementary Fig. 3**).

After RSV challenge, cotton rats vaccinated with FI-RSV had elevated levels of mRNA in the lungs for the Th2 cytokines IL-4, IL-5, and IL-13, whereas mRNA levels for these cytokines were not elevated in the lungs of cotton rats vaccinated with mRNA-1345 (**Supplementary Fig. 4a-c**). Low levels of the Th1 cytokine IFNγ mRNA were detected in all groups except the RSV infection and naïve groups

(**Supplementary Fig. 4d**). IL-2 was detected in all groups, but was elevated in both FI-RSV groups (**Supplementary Fig. 4e**).

Cotton rats vaccinated with all dose levels of mRNA-1345, including those that induced low or undetectable RSV antibody responses, did not develop enhanced lung inflammation after RSV challenge, as evidenced by the histopathology scores, which were in line with those from RSV infection (Fig. 5b). By contrast, vaccination with FI-RSV induced the characteristic enhanced lung inflammation after RSV challenge relative to RSV infection, as indicated by elevated histopathology scores across four evaluated parameters (alveolitis, interstitial pneumonia, perivasculitis, and peribronchiolitis; **Supplementary Fig. 5a-d**).

Discussion

The mRNA-1345 vaccine was developed to reduce the burden of RSV disease. In mice, mRNA-1345 induced a robust neutralizing humoral immune response directed to the RSV preF antigen as well as a Th1-biased CD4 and CD8 cellular response. A similar immune response was observed in cotton rats, which were protected from RSV challenge at higher dose levels with no evidence of increased lung pathology at any dose level.

The mRNA and RSV F protein sequence was selected from a screen of mRNA constructs with various modifications on the backbone of the previous mRNA-1172 RSV vaccine for higher protein expression in Expi293 cells. Of the candidate sequences evaluated, mRNA-1345, which included codon redesign and deletion of the cytoplasmic tail, demonstrated the greatest preF protein expression. Furthermore, fluorescent microscopy indicated that mRNA-1345 showed increased preF localized on the cell surface. The addition of more modifications, such as additional cavity filling mutations or changes to the 5' untranslated region, did not further increase preF protein expression (data not shown). Surprisingly, the increase in protein expression with mRNA-1345 relative to mRNA-1172 did not translate to an increase in mouse humoral immunogenicity.

mRNA-1345 was further assessed in rodent immunogenicity and challenge models. In both BALB/c mice and cotton rats, the antibody response induced by mRNA-1345 was largely functional. Additionally, the RSV preF to postF IgG titer ratios demonstrated that the RSV antibody response induced by mRNA-1345 was preF-biased, as expected given the vaccine was rationally designed to encode the fusion protein stabilized in the preF conformation. A portion of serum antibodies elicited by mRNA-1345 vaccination was directed against antigenic site Ø, a potent neutralizing epitope present only on the preF conformation of the RSV fusion protein[18, 42]. mRNA-1345 also induced a Th1-biased response in BALB/c mice, indicated by type 1 cytokine-producing $CD4^+$ and $CD8^+$ T-cell responses and a balanced IgG2a to IgG1 antibody response. Further, vaccination with mRNA-1345 afforded cotton rats protection from RSV challenge, with no detectable virus in the lungs at higher mRNA-1345 dose levels. mRNA-1345 also did not promote enhanced lung inflammation after RSV challenge, even at dose levels that induced suboptimal immunity and permitted breakthrough virus replication.

The preclinical assessments described here supported clinical development of mRNA-1345. A single dose of mRNA-1345 (50 μg) has demonstrated efficacy in protecting against RSV-associated LRTD in adults aged ≥ 60 years in a pivotal phase 2–3 trial[32] and is now licensed for the active immunization against RSV-LRTD in this population[17, 43, 44]. Clinical development of this vaccine began with a Phase 1 study in both younger (18–49 years) and older adult (65–79 years)[45, 46]. Compared with the previous phase 1 studies of mRNA-1172[31] or mRNA-1777[30], mRNA-1345 elicited greater neutralizing antibody geometric mean fold rises against RSV-A and RSV-B subtypes in both older and younger adults (**Supplementary Table 1**)[45, 46]. Furthermore, neutralizing antibody titers have been shown to remain above baseline levels through 12 months after a single dose of mRNA-1345, demonstrating persistence of the immune response[45]. These data suggest that the optimized design of mRNA-1345 results in increased protein expression, which translates to greater immunogenicity in the clinic, even though enhanced immunogenicity was not observed preclinically. In addition to modifications to the mRNA construct, the clinical studies of mRNA-1777, mRNA-1172, and mRNA-1345 utilized different LNPs, which may have contributed to the differences in immunogenicity observed between the vaccines[31].

This study demonstrated the utility of stepwise rational mRNA and protein design in vaccine development to increase antibody responses. In animal models, mRNA-1345 induced a neutralizing, preF-biased antibody response, a Th1-biased cellular response, and protected from RSV challenge without the immunological or pathological featured associated with ERD. The results obtained in these preclinical studies compliment the demonstrated efficacy of mRNA-1345 in the pivotal phase 2–3 clinical trial[32] and supported clinical evaluation of mRNA-1345 in other vulnerable populations, including RSV-naïve infants[34–36].

Methods

Next-generation RSV mRNA vaccine design and synthesis

Membrane-anchored prefusion-stabilized RSV F glycoprotein vaccine candidates were encoded as mRNA constructs, with or without the cytoplasmic tail (ΔCT) ending at position 559. Mutations were S155C, S290C, V207L, and S190F for mRNA-1777 and S155C, S290C, V207L, S190F, A149C, Y458C, and F2-F1 linker T103-GS-G145 for mRNA-1172. Each mRNA sequence was synthesized using codon-optimized nucleotides in vitro through an optimized T7 RNA polymerase-mediated transcription reaction, with complete replacement of uridine by N1-methyl-pseudouridine. The DNA template contained the antigen coding sequence within the open reading frame, flanked by 5' and 3' untranslated region sequences, and terminated with an encoded polyA tail. The resulting mRNA products were capped with a 5’-Cap 1 structure using vaccinia capping enzyme and Vaccinia 2' O-methyltransferase (New England Biolabs, Ipswitch, MA). The mRNA was then purified using oligo-dT affinity chromatography and encapsulated in LNP through a modified ethanol-drop nanoprecipitation process, as described previously[47, 48]. Briefly, mRNA was encapsulated in LNPs by microfluidic mixing of ionizable, structural, helper, and polyethylene glycol lipids with mRNA in acetate buffer (pH 5.0), followed by neutralization,

cryoprotectant addition, filtration, and storage at -70°C until further use. Analytical characterization including determining particle size and polydispersity, encapsulation efficiency, mRNA purity, osmolality, pH, endotoxin levels, and bioburden was performed to ensure suitability for in vivo studies.

In vitro protein expression

HEK239T cells (ATCC CRL-11268), cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, were maintained at 37°C with 5% CO_2. Cells were transfected with mRNAs at various concentrations using a TransIT mRNA transfection kit (Mirus Bio, Madison, WI). Cells were collected and resuspended in fluorescence-activated cell sorting (FACS) buffer (1 × PBS supplemented with 2% FBS) at days 1–7 post transfection. Cell surface expression of the RSV F protein was detected by staining resuspended cells with specific monoclonal antibodies (e.g. D25 at 10 µg/mL; specific for site 0) in FACS buffer for 30 minutes on ice. Cells were washed twice with FACS buffer and then incubated with Alexa Fluor 647 goat anti-human IgG in FACS buffer for 30 minutes on ice. Cell viability was assessed using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Waltham, MA; cat. L34966). Data were collected using an LSR Fortessa flow cytometer (Becton Dickinson) and analyzed with FlowJo software (version 10.7.1).

Immunofluorescent imaging

Fluorescent images were captured using a fluorescence microscope (Opera Phenix, Revvity) with appropriate filter sets for DAPI and Alexa Fluor 488. Images were acquired at 24 hours and 48 hours post transfection to assess surface expression of the RSV F protein. All images were captured at 63 times magnification. Green fluorescence indicated RSV F protein expression, and blue fluorescence indicated nuclear staining. Image analysis was performed using Harmony software (Revvity). Mean fluorescence intensity of RSV F protein expression was quantified and normalized to the mRNA-1172 control. The results are presented as mean ± standard error.

BALB/c mice models

All mouse experiments were conducted in compliance with US regulations and policies and were reviewed and approved by the Moderna, Inc. Animal in Vivo Pharmacology Review Committee. Mice were euthanized using carbon dioxide (CO_2) inhalation, introduced at 45% of the chamber volume per minute.

For immunogenicity assessments, female BALB/c mice were given either two-dose (days 1 and 22) or three-dose (days 0, 21, and 49) regimens of intramuscular vaccinations of mRNA-1345. Control animals that received two-dose series were vaccinated with mRNA-1777, mRNA-1172, codon mRNA-1172, or ΔCT_mRNA-1172 on days 1 and 22; control animals that received three injections were vaccinated on days 0, 21, and 49 with PBS (n=5), recombinant RSV preF protein plus alhydrogel adjuvant (preF protein +

AH; 10 µg; n=8), formalin-inactivated RSV (FI-RSV; 1:100; n=8), a or LNP-formulated irrelevant mRNA (mRNA encoding human factor-IX with mutations to block mRNA translation, LNP control; 5 µg; n=8). Neutralization titers were assessed on day 36 in animals that received two-dose series of mRNA-1345 or control treatments; blood collection was submandibular without anesthesia. Animals that received three injections of mRNA-1345 or control treatments were anesthetized via aerosolization with 3% isoflurane, then retro-orbitally bled for serology on days 35 (14 days post second dose) and 56 (7 days post third dose).

RSV neutralization assay

Two-fold serial dilutions of heat-inactivated serum samples were mixed with a fixed amount of RSV for 1 hour at 37°C and transferred to a HEp-2 cell culture monolayer. After an incubation period of 1 day at 37°C, the culture supernatants were removed and monolayers washed once with PBS and fixed with 50%/50% methanol/ethanol. After fixation, the plates were stained using a mouse monoclonal antibody against RSV, secondary horseradish peroxidase (HRP)-labelled anti-mouse antibody and TrueBlue. Stained plates were scanned using the Immunospot analyser and 50% plaque (spot) reduction titers were calculated using the formula as described previously[49].

PreF and postF IgG assays

96-well microtiter plates coated with 1 µg/mL of preF or postF RSV F protein diluted in PBS were incubated at 4°C overnight and washed four times with PBS/0.05% Tween-20 and blocked with SuperBlock (ThermoFisher #37515) for 1.5 hour at 37°C. After washing, six five-fold serial dilutions (in PBS + 5% goat serum + 0.05% Tween-20 [0.05% Tween-30 in cotton rat model]) of individual test serum were added, as well as six five-fold dilutions of the Assay Standard (a pool of serum with a high titer of RSV antibody from animals vaccinated twice with mRNA-1345) that had pre-defined anti-RSV titers. Each plate also contained wells of assay diluent without serum for blank subtraction. Plates were incubated for 2 hours at 37°C and washed. For BALB/c mice models, HRP-conjugated anti-mouse IgG, IgG1, or IgG2a (Southern Biotech #1030-05, #1070-05, #1079-05, respectively) was added at a 1:10,000 dilution in assay diluent. For cotton rat model, 100 µL/well of a 1:5000 dilution of HRP-conjugated chicken anti-cotton rat IgG was added. Plates were incubated for 1 hour at 37°C, washed, and bound antibody was detected with SureBlue TMB Microwell Peroxidase substrate (SeraCare #5120-0077). The reaction was stopped by adding TMB Stop Solution (SeraCare #5150-0021) and the absorbance was measured at OD of 450 nm. Results are presented as geometric mean titers and geometric standard deviations for all animals in a group.

Serum antibody profiling

RSV preF, RSV preF site Ø mutant (K65N-N67T-P205N-V207T-K209N-S211T), and hMPV preF antigens were used to profile the antibody response induced by mRNA-1345. Antigens were coupled to carboxylated magplex-microspheres magnetic Luminex beads (Luminex Corp) by carbodiimide-NHS ester-coupling (Thermo Fisher Scientific). Antigen-coupled microspheres were washed and incubated with serum samples at an appropriate sample dilution at 4°C overnight for 2 h at 37°C in 384-well plates (Greiner Bio-One). Unbound antibodies were washed away, and antigen-bound IgG was detected by using a phycoerythrin (PE)-coupled detection antibody. After 1 hour of incubation, plates were washed, and fluorescence was acquired using an xMAP INTELLIFLEX (Luminex). Flow cytometry was performed with an IQue (Intellicyt), and analysis was performed on IntelliCyt ForeCyt (v8.1). PE median fluorescent intensity (MFI) is reported as a readout for antigen-specific antibody titers.

T-cell stimulation and intracellular cytokine staining

Whole spleens were collected from BALB/c mice on day 56 and homogenized into mononuclear single-cell suspensions. Cells were resuspended in R10 media, assessed for viability with acridine orange/propidium iodide, adjusted to a concentration of 2 x 10^7 viable cells/mL and incubated at 37°C for 6 hours with a protein transport inhibitor cocktail (Invitrogen), CD107a PE (Becton, Dickinson and Company, cat. 558661, clone 1D4B) and 1 µg/mL pool of overlapping peptides (15mers with 11 amino acid overlap) covering the RSV protein (GenScript, custom order) for peptide stimulation. Cells were stained with a surface stain antibody cocktail in Brilliant Stain Buffer (BD) containing the following: FC Block (BD, clone 2.4G2), CD4 BV480 (BD, cat. 565634, clone RM4-5), CD8 BUV805 (BD, cat. 612898, clone 53 – 6.7), CD44 BUV395 (BD, cat. 740215, clone IM7), CD62L APC-R700 (BD, cat. 565159, clone MEL-14), and I-A/I-E Alexa Fluor 488 (BD, cat. 562352, clone M5/114.15.2). Cells were washed, fixed and permeabilized, and intracellular staining was performed the following day at 4°C for 30 minutes with an antibody cocktail, including CD3 BV605 (BD, cat. 564009, clone 17A2), IFN BV421 (BD, cat. 563376, clone XMG1.2), IL-2 BV785 (Biolegend, cat. 503843, clone JES6-5H4), IL-4 PE-CF594 (BD, cat. 562450, clone 11B11), IL-5 APC (BD, cat. 554396, clone TRFK5), IL-13 PE-Cy7 (Invitrogen, cat. 25-7133-82, clone eBio13A), and TNF BV711 (BD, cat. 563944, clone MP6-XT22). Cells were washed and resuspended in 0.5% PFA-FC stain buffer solution and run on a Cytek® Aurora flow cytometer and analyzed using FlowJo software (version 10.7.1).

Cotton rat RSV challenge model

Cotton rats were assigned to experimental groups and immunized via intramuscular injection with mRNA-1345 vaccine formulations or control treatments. Immunizations were administered at volumes of 100 µL (50 µL injected into each hind leg). mRNA-1345 was administered on days 0 and 28 (3 ng, 30 ng, 0.3 µg, 3 µg, or 30 µg) or on day 0 only (0.3 µg). Control animals were injected on days 0 and 28 with PBS; two different lots of FI-RSV in a 1:125 dilution (“Lot 100” evaluated in early clinical studies [FI-RSV #1][23], and the FI-RSV lot which has been used for preclinical studies [FI-RSV #2]); FI-mock; LNP control

(30 µg); or RSV infection (intranasal, day 0 only; 10^5 PFU). Naïve cotton rats were uninfected or unvaccinated.

On day 56, serum for antibody assays was collected retro-orbitally from animals anesthetized via aerosolization with 3% isoflurane. All vaccinated and control animals were challenged intranasally under anesthesia on day 56 with 10^5 PFU of live RSV A2 strain in a 100 µL volume. At 5 days post-challenge (day 61), cotton rats were euthanized (using CO_2 inhalation, introduced at 45% of the chamber volume per minute); nasal tissues and lungs were collected for viral load assessment (day 61) and lung tissues were harvested for histopathology and cytokine mRNA analysis.

RSV lung and nose viral titration

Lung and nose homogenates were clarified by centrifugation. Confluent HEp-2 monolayers were infected in duplicate with the diluted homogenates in 24-well plates, incubated and overlaid with 0.75% methylcellulose medium. After 4 days of incubation, the overlays were removed and the cells fixed with 0.1% crystal violet. Visible plaques were counted and virus titers expressed as PFU per gram of tissue. Lung samples with viral titers below the lower limit of quantification (200 PFU/g) were assigned a titer of 200 PFU/g; nose samples with viral titers below the limit of quantification (100 PFU/g) were assigned a titer of 100 PFU/g. Viral titers are presented as mean log10 PFU/g and standard deviation for all animals in a group.

Pulmonary histopathology

Lungs were dissected and inflated with 10% neutral buffered formalin to their normal volume, then immersed in the fixative, after which the lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were analyzed by an analyst which evaluated peribronchiolitis, perivasculitis, interstitial pneumonia, and alveolitis. Slides were scored blindly on a 0–4 severity scale and subsequently converted to a 0-100% histopathology scale (0 = 0%, 1 = 5%, 2 = 25%, 3 = 75%, 4 = 100%). The “cumulative inflammation” score was calculated per animal by summing the scores for the four individual parameters. Histopathology is presented as medians for all animals in a group.

Quantification of cytokine RNA

Total RNA was extracted from homogenized lung tissue using the RNeasy purification kit (Qiagen). 1 µg of total RNA was used to prepare cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Real-time polymerase-chain reactions (PCR) were performed with the QuantiFast SYBR Green PCR Kit (Qiagen), with final primer concentrations of 0.5 µM in a final volume of 25 µL. Amplifications were performed on a Bio-Rad iCycler for one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec. The baseline cycles and cycle threshold (Ct) were calculated by the iQ5 software in

the PCR Base Line Subtracted Curve Fit mode. Relative quantification of DNA was applied to all samples, with a standard curve developed using serially diluted cDNA samples from the lungs of formalin inactivated-RSV-immunized animals at 6 hours post RSV infection. Ct values were converted to relative expression units and normalized to the level of β-actin mRNA in the corresponding sample. Samples with β-actin Ct > 20 were considered to be of poor quality and the cDNA would be resynthesized.

Data availability

All data supporting the findings of this study are available within this article and its Supplementary Information.

Declarations

Ethics: All animal studies were conducted in compliance with US regulations and policies. Mouse experiments (2-injection series) were carried out in compliance with approval from Institutional Animal Care and Use Committee of Moderna, Inc. Mouse (3-injection series) and cotton rat experiments were carried out in compliance with approval from Institutional Animal Care and Use Committee of Sigmovir Biosystems.

Competing Interests

C.A.S., G.S.J., P.J., E.N., S.E., P.K., L.M., N.H., E.H., T.K., C.M., S.M., E.O., V.P., A.R., D.R., A.W., and A.C. are current or previous employees of Moderna, Inc. and may hold stock/stock options in the company.

Additional information

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Author Contribution

C.A.S., G.S.J., P.J., E.N., S.M., and A.C. conceived the design of this project. C.A.S., G.S.J., P.J., E.N., S.E., P.K., L.M., N.H., E.H., T.K., C.M., S.M., E.O., V.P., A.R., D.R., and A.W., collected the data. C.A.S., G.S.J., P.J., E.N., S.E., P.K., T.K., C.M., and A.C. analyzed and interpreted the data. All authors wrote, reviewed, and contributed intellectually to the manuscript. All authors approved the final draft.

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Figures

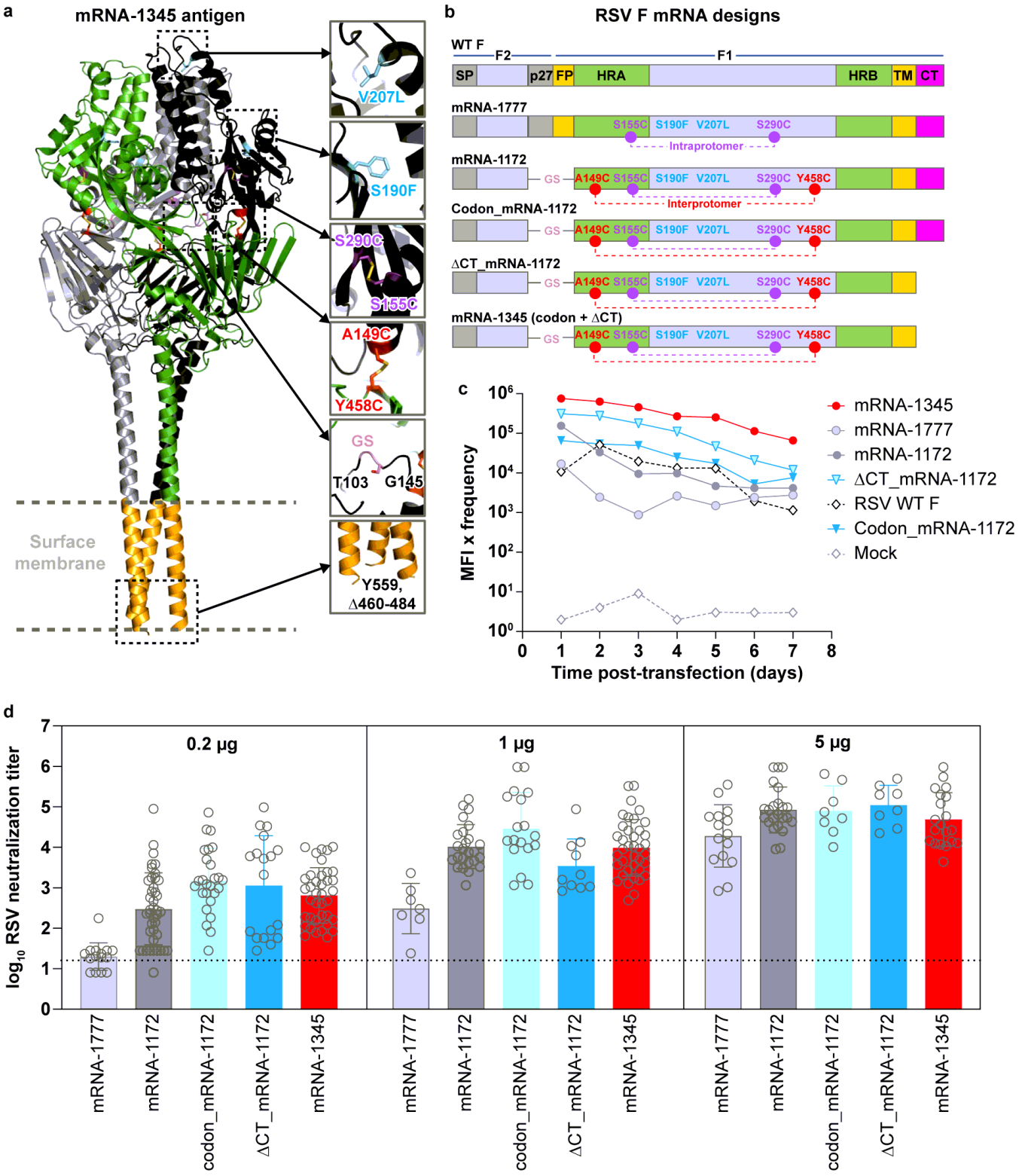
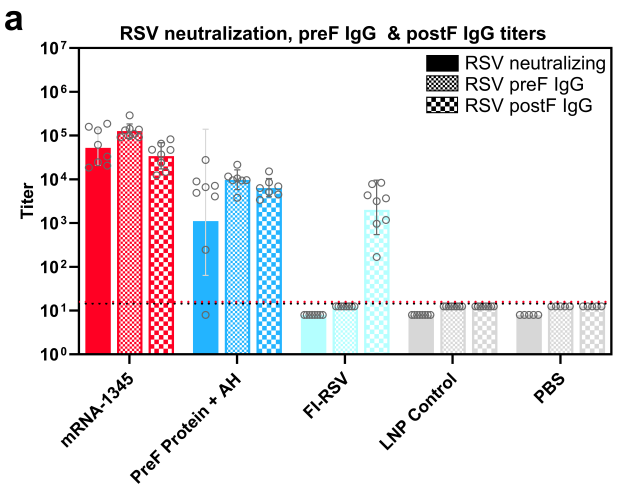
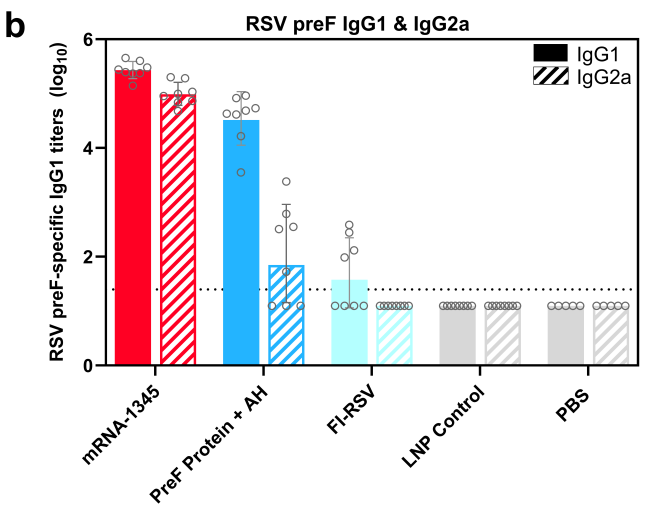


Figure 1

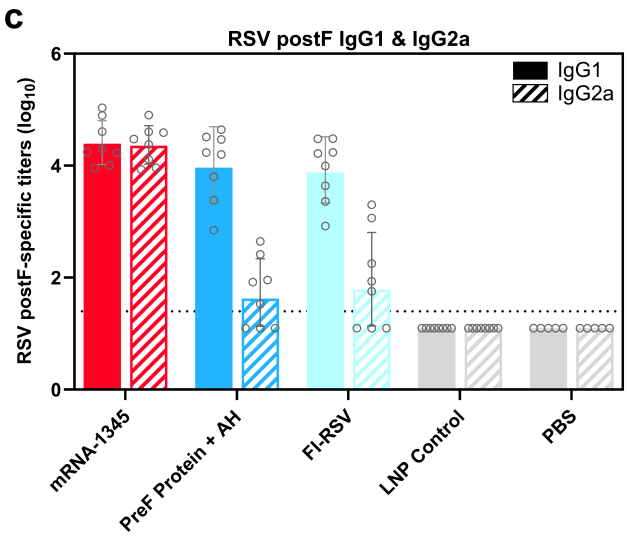
Design and experimental characterization of RSV preF mRNA vaccine candidates



	mRNA-1345, 5 µg	preF Protein + AH, 10 µg	FI-RSV, 1:100	LNP Control	PBS
preF IgG : RSV neut	2.4	8.7	1.6	N/A	N/A
postF IgG : RSV neut	0.6	5.7	249.9	N/A	N/A
preF IgG : postF IgG	3.7	1.5	0.006	N/A	N/A



	mRNA-1345, 5 µg	preF Protein + AH, 10 µg	FI-RSV, 1:100	LNP Control	PBS
IgG1 : IgG2a	2.8	322	N/A	N/A	N/A



	mRNA-1345, 5 µg	preF Protein + AH, 10 µg	FI-RSV, 1:100	LNP Control	PBS
IgG1 : IgG2a	1.1	197	64	N/A	N/A

Figure 2

RSV neutralizing and binding antibody responses to mRNA-1345 in comparison with FI-RSV vaccine and recombinant F protein vaccine in BALB/c mice

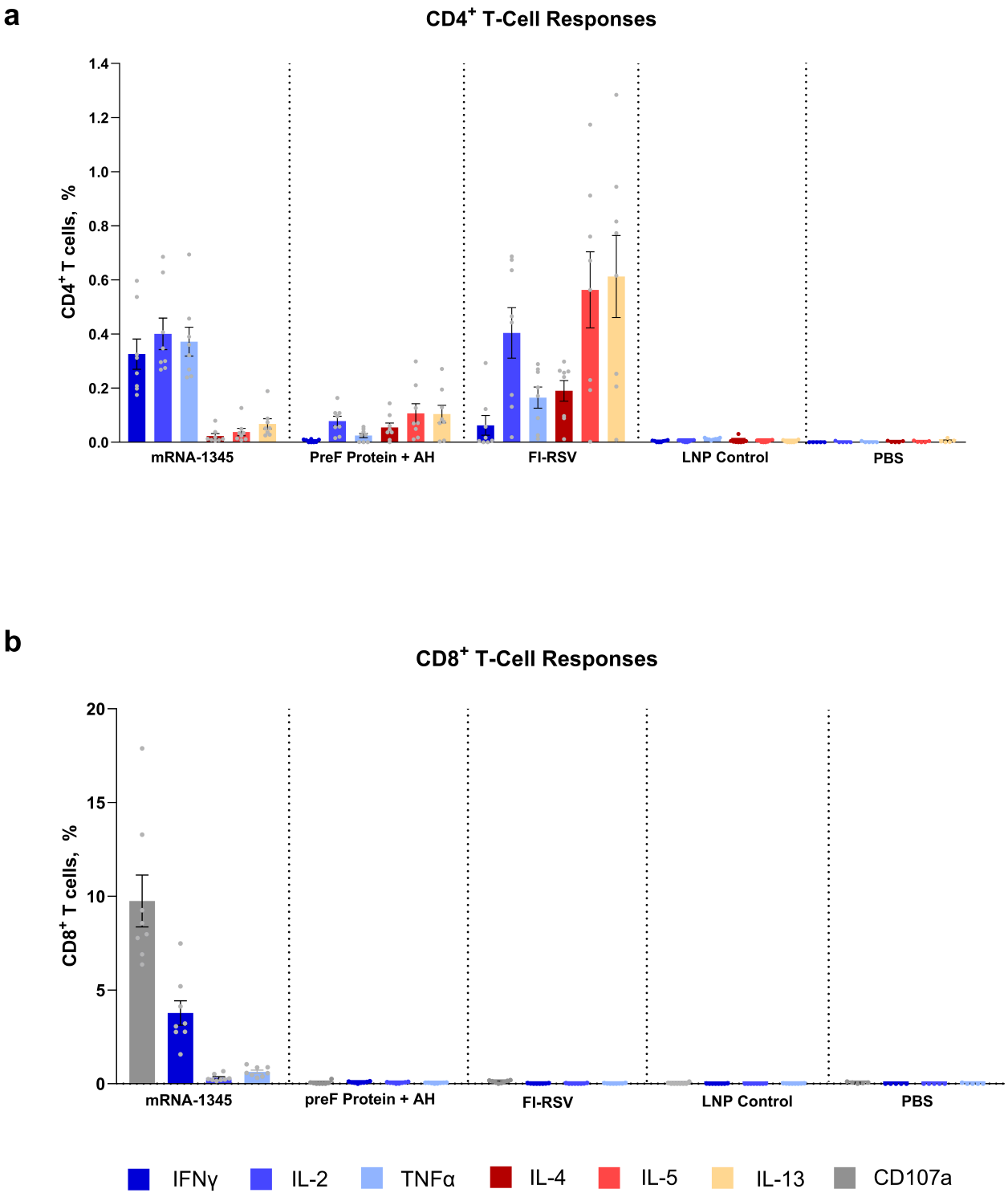
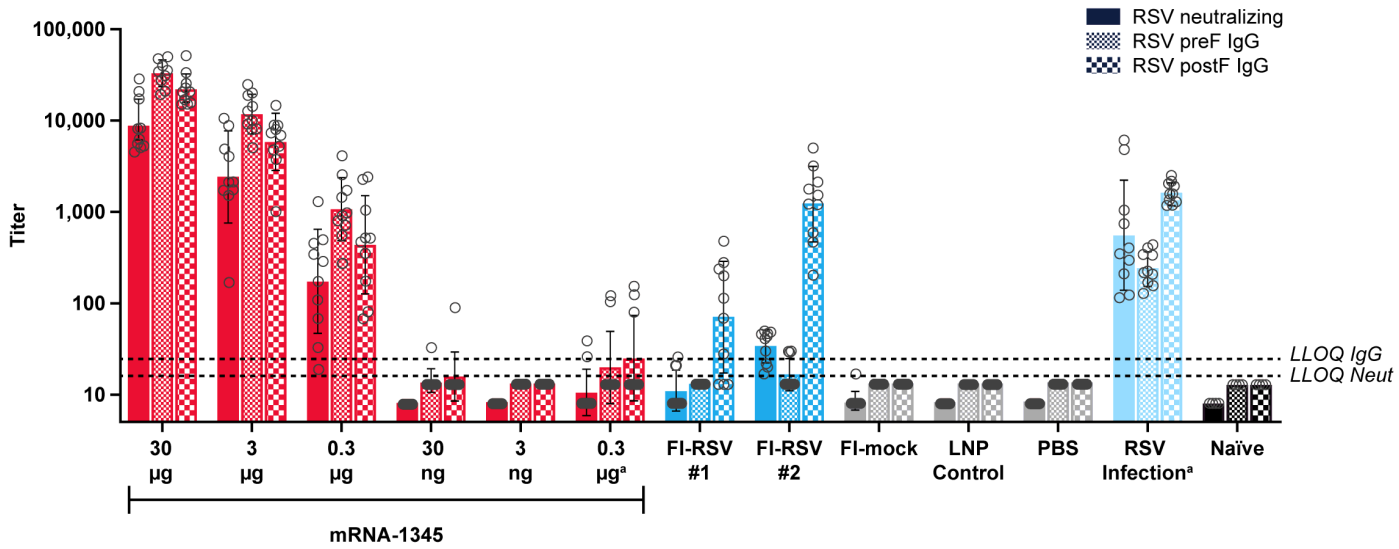


Figure 3

Characterization of $CD4^+$ and $CD8^+$ T-cell responses to RSV F vaccines in BALB/c mice



	mRNA-1345 30 µg	mRNA-1345 3 µg	mRNA-1345 0.3 µg	mRNA-1345 30 ng	mRNA-1345 3 ng	mRNA-1345 0.3 µg[a]	FI-RSV #1	FI-RSV #2	FI-mock	LNP Control	PBS	RSV Infection[a]	Naive
preF IgG : RSV nAb	3.8	4.9	6.2	–	–	–	–	0.48	–	–	–	0.43	–
postF IgG : RSV nAb	2.5	2.4	2.5	–	–	–	6.4	36.0	–	–	–	2.9	–
preF IgG : postF IgG	1.5	2.0	2.5	–	–	–	0.19	0.01	–	–	–	0.15	–

Figure 4

RSV-neutralizing and binding antibody responses following mRNA-1345 vaccination in cotton rats

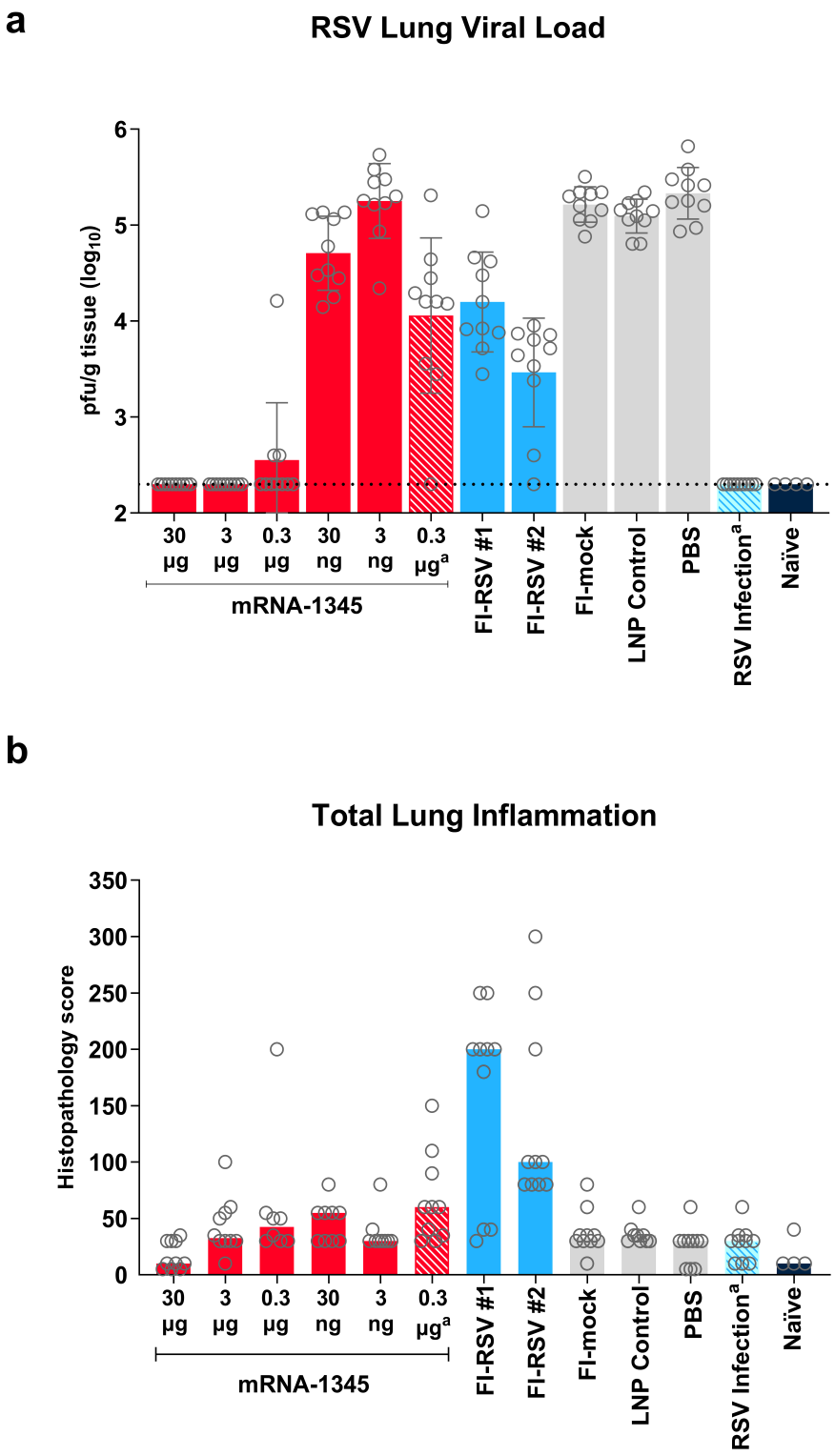


Figure 5

mRNA-1345 mediated protection assessed by lung viral load 5 days after RSV challenge and post-infection lung inflammation in cotton rats

Supplementary Files

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