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Evaluation of luciferase and prefusion-stabilized F protein from respiratory syncytial virus mRNA/LNPs in pre-clinical models using jet delivery compared to needle and syringe

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ABSTRACT

Described here is the evaluation of a luciferase (luc) and respiratory syncytial virus (RSV) messenger RNA / lipid nanoparticle (mRNA/LNP) vaccine using a Needle-free Injection System, Tropis®, from PharmaJet® (Golden, Colorado USA). Needle-free jet delivery offers an alternative to needle/syringe. To perform this assessment, compatibility studies with Tropis were first performed with a luc mRNA/LNP and compared to needle/syringe. Although minor changes in particle size and encapsulation efficiency were observed when using Tropis on the benchtop, *in vitro* luciferase activity remained the same. Next, the luc mRNA/LNP was administered to rats intramuscularly using Tropis or needle/syringe and tracking of the injection and distribution was performed. Lastly, an mRNA encoding a prefusion-stabilized F protein from RSV was delivered intramuscularly using both Tropis and needle/syringe at 1 and 5 mcg mRNA. An equivalent IgG response was observed using both Tropis and needle/syringe. The cell mediated immune (CMI) response was also evaluated, and responses to RSV-F were detected from animals immunized with needle/syringe at all dose levels, and from the animals immunized with Tropis in the 5 and 25 ug groups. These results indicated that delivery of mRNA/LNPs with Tropis is a potential means of administration and an alternative to needle/syringe.

Introduction

The use of messenger RNA / lipid nanoparticle (mRNA/LNP) vaccines have come to the forefront during the COVID-19 pandemic (2019 to the present) [1,2]. These vaccines offer the ability to respond quickly to new outbreaks. Rapid synthesis of mRNAs encoding key antigens (such as the spike glycoprotein for SARS-CoV-2) followed by packaging into LNPs allows this rapid deployment [1,2]. At least two of the mRNA/LNP COVID vaccines are shipped as frozen liquids in glass vials, thawed, and administered with needle/syringe into the deltoid muscle [2]. Although the needle/syringe is being used for the majority of immunizations against COVID-19, other delivery methods for administering mRNA/LNPs have been developed, one of which is with needle-free jet injection [3].

Jet delivery with vaccines may be performed either intradermally (ID), subcutaneously, or intramuscularly (IM). One example is Tropis, which is commercially available and has been used to vaccinate over seven million people (mostly children) around the world ([5–9], PharmaJet personal communication). In general, there have been a number of vaccines evaluated using jet delivery, targeting protection from COVID, polio, rabies, hantaviruses, human papillomavirus (HPV), zika, HIV, chikungunya, dengue, influenza, and measles-mumps-rubella (MMR) [3,4,6,10–28]. These vaccines have been composed of mRNA or DNA that encode viral antigens, or attenuated or inactivated viruses [3–24]. A protamine-complexed mRNA vaccine for rabies was evaluated in a Phase I trial using jet delivery (Tropis (ID) and Stratis (IM)) and was found to be safe and effective when delivered via jet injection but not effective when needle/syringe was used [10].

In this report, an mRNA/LNP vaccine encoding for a prefusion-stabilized F protein from RSV was evaluated in pre-clinical models using intramuscular jet delivery with Tropis and compared to needle/syringe [29]. In humans, Tropis is utilized for intradermal administration, whereas intramuscular injections can be accomplished in pre-clinical rodent models [4]. RSV is a virus that causes upper and lower

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respiratory illness, especially in infants and immunocompromised and older adults. A licensed vaccine for individuals 60 years of age and older was recently approved by the FDA.

Materials and methods

Messenger RNA (mRNA) / lipid nanoparticles (LNPs)

Messenger RNA (mRNA) encoding firefly luciferase and the prefusion-stabilized F protein from respiratory syncytial virus (RSV A2 strain) was manufactured by TriLink Biotechnologies (San Diego, CA). The construct utilized is shown in Fig. 6 (see Results). mRNA/LNP formulations were generated using rapid nanoprecipitation as described previously [30]. LNPs contained an asymmetric ionizable amino lipid, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol and poly(ethylene glycol) 2000-dimyristoylglycerol (PEG2000-DMG) with their composition similar to those described previously [29]. Prior to administration, formulations were evaluated for particle size, lipid concentration, mRNA concentration and mRNA encapsulation.

Tropis preparation

For all uses of Tropis, the vaccine is first loaded into a needle-free syringe which is then inserted into the device. The syringe opening is the location where the stream of vaccine is dispensed into the muscle, or alternatively for compatibility studies, into an Eppendorf tube.

Compatibility studies of luciferase mRNA with Tropis and Needle/Syringe

Luciferase mRNA/LNPs at volumes of 0.15 mL were loaded either into the Tropis Needle-free Syringe or into a standard needle/syringe image, then dispensed at a volume of 0.1 mL into Eppendorf tubes. The material was characterized using particle size analysis, encapsulation efficiency, and *in vitro* activity assays.

Particle size analysis

Particle Size Analysis was performed on a WYATT DynaPro PlateReader II. Samples were loaded into the cell and particle size measured in diameter (nm). The polydispersity values (nm) were also obtained.

Encapsulation efficiency (EE)

Encapsulation efficiency (EE) was performed using a Quanti-iT™ Ribogreen™ RNA assay kit (Invitrogen). Samples were analyzed on a SpectraMax Gemini XPS plate reader *in vitro*.

Luciferase activity assay

An *In vitro* luciferase activity assay was performed using HepG2 cells (ATCC HB-8065) that were grown in a monolayer. The cells were plated at 50,000 cells per well in ½ area 96-well tissue culture treated (TC) plates (white, clear bottom, Perkin Elmer). Following plating, the cells were grown for 24 h followed by the transfection of of 2.4 Âµg RNA (in LNPs) per well. The luciferase mRNA/LNPs were dispensed through either Tropis or needle/syringe. The cells remained transfected for 8 h which was followed by luciferase reagent (Promega Luciferase assay system kit [E1500]. Luciferase activity was measured on a Perkin Elmer VICTOR™ X4 2030 luminometer.

Intramuscular delivery with Tropis

Intramuscular injections using Tropis were performed as described by Brocato et al [4]. Rats were shaved on the hindlimbs followed by pressing Tropis against the skin, which is referred to as the 'over-the-muscle' technique [4]. The Vastus lateralis muscle was injected using Tropis. Some of the rats received injections in both hind limbs while others received injections only on the left hind limb. In the latter case, measurements were made at 1 day (24 h) only while in the former case, measurements were taken at both 1 day and 2 days (24 and 48 h).

In vivo ultrasound studies

Ultrasound imaging was performed in rats (n = 3) to verify injection placement using a high frequency preclinical ultrasound (VisualSonics Vevo 2100) paired with a MS550 linear array transducer (center frequency of 40 MHz). To facilitate non-invasive visualization of the depot in skin, animals were anesthetized using Isoflurane (Zoetis Inc. Kalamazoo, MI) at an approximate 1.5 % concentration supplied by medical air through a vaporizer. They were positioned supine on a heated imaging platform (VSI, Toronto, Canada) equipped with integrated temperature sensor and ECG electrodes for monitoring heart and respiratory rate. Prior to any injections or imaging, the skin surface was cleared of hair using a #50 A5 clipper blade. Immediately after the injection, acoustic gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ) was applied to the skin between the transducer surface to facilitate ultrasound transmission. The transducer was positioned free-hand over the injection site to acquire various B-mode images of the region of interest.

In vivo imaging studies

Imaging studies were performed with 350 g Sprague-Dawley male rats and analyzed on an IVIS system. Rats were anaesthetized using isoflurane. Administration of luciferase mRNA/LNPs was performed on the hind limb in the Vastus lateralis with either Tropis or needle/syringe. Following administration, rats were injected subcutaneously with D-luciferin reconstituted at 30 mg/mL at 90 mg/kg. Ten minutes post-D-luciferin administration, animals were placed into the imaging chamber and images were acquired using medium bidding on FOV D. Acquisition times varied across groups to ensure capture of at least 3000 photons. Animals were first imaged in the dorsal position and then ventral position. Analysis of the injection site was done on dorsal images. Prism software was used to graph the data and Minitab® software (ver. 21.1) was used to perform the statistical analysis.

Immunogenicity studies

Rats were immunized with RSV Pre-F mRNA/LNPs at doses of 1, 5, or 25 μg mRNA or placebo (1 X phosphate buffered saline) at Day 0 and Day 21. Following administration, blood samples for serum were collected at Day 14 (2 weeks post-dose 1), and Day 35 (2 weeks post-dose 2) to evaluate anti-RSV F antibodies using an IgG-specific ELISA.

Spleens were collected at Day 42 (3 weeks post-dose 2) and processed to isolate splenocytes. Red blood cells were lysed with ACK lysis buffer (Invitrogen multispecies buffer 00-4300-54). The enzyme-linked immunospot (ELISPOT) assay was used to enumerate antigen-specific gamma interferon (IFN-γ)-secreting cells from rat spleens using mabTech catalog 332-4APW-10. ELISPOT plates were washed and blocked for 30 min with 200 mcl/well of complete R10 medium. To each well, 2×10^5 cells were added with 2 μg/ml of RSV F specific peptide pools (15mer overlapping by 11, JPT Peptide Solutions, Germany) or equal quantity of DMSO as a negative control for each sample. Cells were incubated for 20 h at 37 °F. Following incubation, plates were washed and developed according to the ELISPOT manufacturer's instructions. Spots were enumerated with an imager (AID, Germany), and the data were normalized to an input of 10^6 cells. Each sample was run in duplicate, and the result are the average of the two wells.

Statistical evaluation

Throughout the manuscript, figures and plots show the raw data obtained, and tables describe the statistical evaluation performed. In the

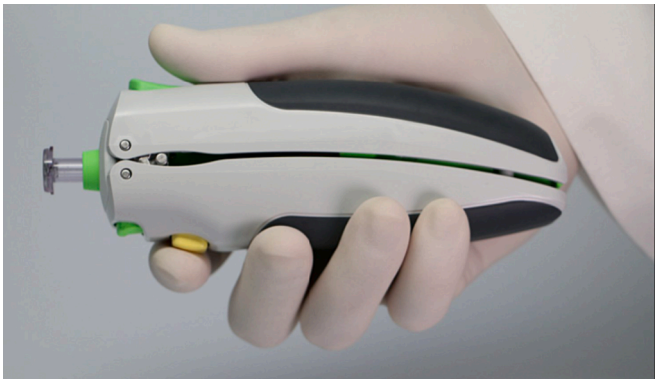

Fig. 1. The Tropis Needle-free Injector from Pharmajet.

Tables, a term of the form "X*Y" represents the interaction between factor X and factor Y. An interaction is the additional effect on the response above and beyond the effect due to the individual factors. In any experiment, some of the variation observed in the response data is due to random effects and some is due to systematic effects. A factor is an explanatory variable that is studied as part of an experimental design. It is varied in a systematic manner. Changes in the response mean are caused by factor effects. An experimental unit (EU) is the material that is being evaluated in the study. Generally, each factor is randomly assigned to one or more experimental units. There can be different sizes of EUs in the same experiment. Variation in the response data is caused by variation among and within the EUs. For the *in vivo* imaging studies, data were collected on a total of 20 rats of which four were injected with PBS using the Tropis device and served as a negative control group. The data on these animals were not included in the statistical analysis.

The totality of data obtained from the *in vivo* imaging studies represent a four-factor treatment structure in a split-split-plot design structure. There are three sizes of experimental units (EUs): The entire rat, a hind limb of a rat, and the time interval between measurements made on the same limb. The primary factors of interest are the injection **Device** (tropis or needle/syringe) and the injection **Dose** of luciferase mRNA/LNP (1 ug or 5 ug). Both of these factors are applied to the entire rat. The injection **Position** (left hind leg or right hind leg) factor is applied to the hind limb of a rat. The **Time** of measurement (1 day or 2 days) factor is applied to the time interval between the measurements made at 1 day and 2 days. Note that measurements made between the two hind limbs or between the two time points within the same hind limb represent repeated measures. A repeated measures analysis of variance (RMANOVA) is utilized to analyze the overall data. The primary hypothesis of interest focused on comparing the two injection devices at an injection dose of 1 ug. The data on the response variable, Total Flux (p/s), were log-transformed prior to analysis to better satisfy statistical assumptions of normality and homoscedasticity.

For the immunogenicity studies, data were collected on a total of 42 rats of which six were injected with PBS using a needle/syringe device and served as the placebo group. The data on those animals were not included in the statistical analysis. Titer measurements were obtained on blood samples drawn at two-weeks post-dose 1 and two-weeks post-dose 2. Measured titers less than the limit of detection of the assay were assigned a value of 25 for the purpose of analysis. The totality of data describes a three-factor treatment structure in a split-plot design structure. The primary factors of interest are the injection **Device** (Tropis or Needle/Syringe) and the injection **Dose** of RSV Pre-F mRNA/LNPs (1 ug, 5 ug or 25 ug mRNA). Both of these factors are applied to the entire animal. The **Time** of measurement (two-weeks post-dose 1 or two-weeks post-dose 2) factor is applied to the time interval between the measurements made at these time points. Note that titers obtained between the two time points within the same animal represent repeated measures. A repeated measures analysis of variance (RMANOVA) is utilized to analyze the overall data. The primary hypothesis of interest focused on comparing the two injection devices at two-weeks post-dose 2. The titer data were log-transformed prior to analysis to better satisfy statistical assumptions of normality and homoscedasticity.

Table 1
Results from biophysical analysis of luciferase mRNA/LNPs dispensed through Tropis or needle/syringe.[+]

Sample Name	Concentration	%EE	Average Diameter	Average PDI
Tropis	0.151	71	131	0.241
Needle/Syringe	0.159	86	99	0.212

[+] Studies performed with n = 3.

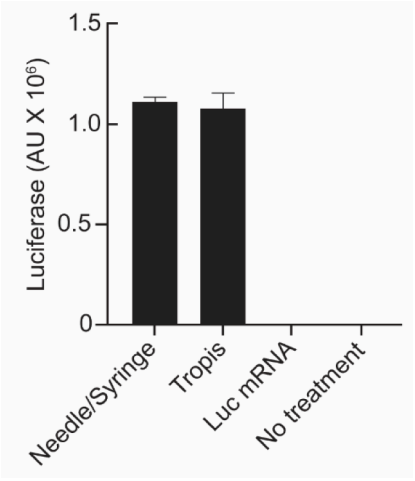

Fig. 2. *In vitro* expression analysis of luciferase mRNA/LNP in HepG2 cells following compatibility studies with Tropis and needle/syringe.n = 3. Error bars are the standard deviation.

Results

Compatibility studies with luciferase mRNA/LNPs

The Tropis device used for this study is shown in Fig. 1. Tropis is a spring-activated Needle-free injection system. To determine if mRNA/LNPs were compatible with Tropis, luciferase mRNA/LNPs were loaded in both the device as well as a needle/syringe. The contents were dispensed into Eppendorf tubes and the concentration evaluated and biophysical analysis was performed. These assays included measuring concentration by UV/VIS, encapsulation efficiency (EE), and particle size, and are summarized in Table 1. The results indicated that mRNA concentrations were similar between Tropis and needle/syringe, and a lower encapsulation efficiency and larger particle size was observed with Tropis, as compared to needle/syringe (Table 1).

To determine if the differences in encapsulation efficiency or particle size had an impact on the activity of the luciferase mRNAs/LNPs, *in vitro* activity in HepG2 cells was evaluated. The luciferase mRNA/LNPs were introduced to the HepG2 cell culture, followed by analysis. It was determined that activity was similar between samples dispensed using Tropis or needle/syringe, which are summarized in Fig. 2. These results indicated despite some differences in biophysical attributes, the activity of the luciferase mRNA remained unchanged when evaluated *in vitro*.

Following the determination that the luciferase mRNA remained active *in vitro*, an *in vivo* evaluation was performed. A previous publication had demonstrated that Tropis, which is utilized for intradermal delivery in humans, could be used for intramuscular (IM) delivery in small animals such as rodents [4]. Following the procedure as described by Brocato et. al. with Tropis, intramuscular injections were

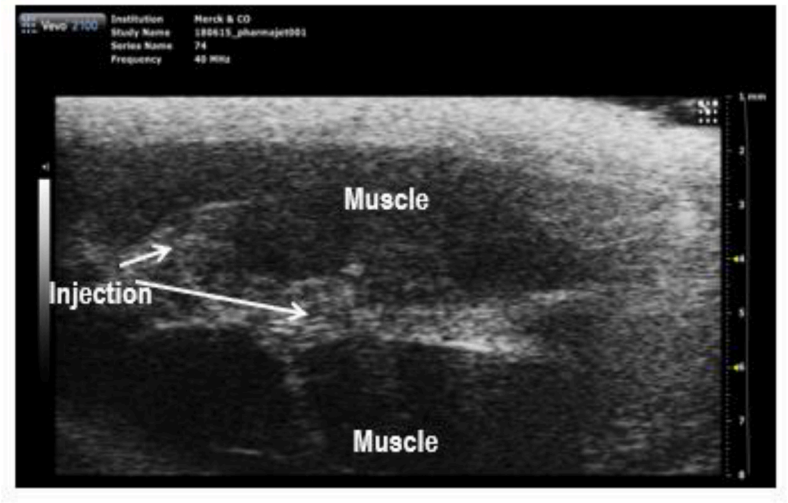

Fig. 3. Representative ultrasound imagee (n = 3) of IM injection performed with Tropis.

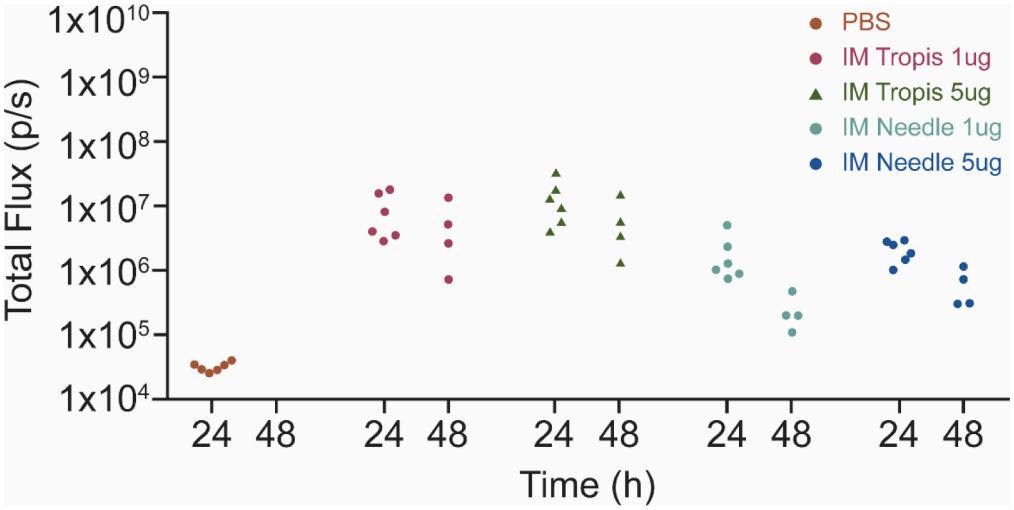

Fig. 4. *In vivo* expression of luciferase mRNA/LNPs in rats. Luciferase doses at 1 and 5 μg mRNA were administered intramuscularly (IM) in the quadriceps with a 0.1 mL injection volume using Tropis or needle/syringe. Points shown on the plot are the raw data obtained. The 24-hour time points had an n = 6 and the 48-hour points had an n = 4.

Table 2
Tests of Fixed Effects from the RMANOVA.[+]

Term	DF Num	DF Den	F-Value	P-Value
Device	1.00	16.47	36.20	0.000
Dose (ug)	1.00	16.47	1.75	0.204
Time (h)	1.00	13.18	76.78	0.000
Position	1.00	16.71	2.47	0.134
Device*Dose (ug)	1.00	16.82	0.03	0.873
Device*Time (h)	1.00	13.23	4.55	0.052
Device*Position	1.00	16.47	0.00	0.996
Dose (ug)*Time (h)	1.00	13.23	0.08	0.784
Dose (ug)*Position	1.00	16.47	0.05	0.826
Time (h)*Position	1.00	13.18	1.93	0.188

[+] Statistical analysis of data obtained from Fig. 4.

Table 3
Tukey simultaneous tests for differences of means.[+]

Difference of Device Levels	Differenceof Means	SE ofDifference	95 % CI
IM Tropis - IM Needle	0.860	0.143	(0.557, 1.162)

Difference of Device*Dose (ug) Levels	Differenceof Means	SE ofDifference	Simultaneous 95% CI	AdjustedP-Value
(IM Tropis 1) - (IM Needle 1)	0.882	0.197	(0.321, 1.442)	0.002

Statistical analysis of data obtained from Fig. 4[+].

demonstrated in rats [4]. Verification that the injected material was delivered to the muscle was confirmed using ultrasound (Fig. 3). Following this demonstration, *in vivo* imaging with a luciferase mRNA/LNP and immunogenicity studies using RSV F mRNA/LNPs delivered IM were performed.

Imaging studies were performed *in vivo* in adult Sprague-Dawley rats to assess distribution and uptake of luciferase mRNA/LNPs. Rats were anaesthetized and then injected intramuscularly with the luciferase mRNA/LNPs using either Tropis or with needle/syringe. The substrate D-luciferin was injected subcutaneously. Luciferase signal generated from the luciferase mRNA was monitored in real time. The results of this

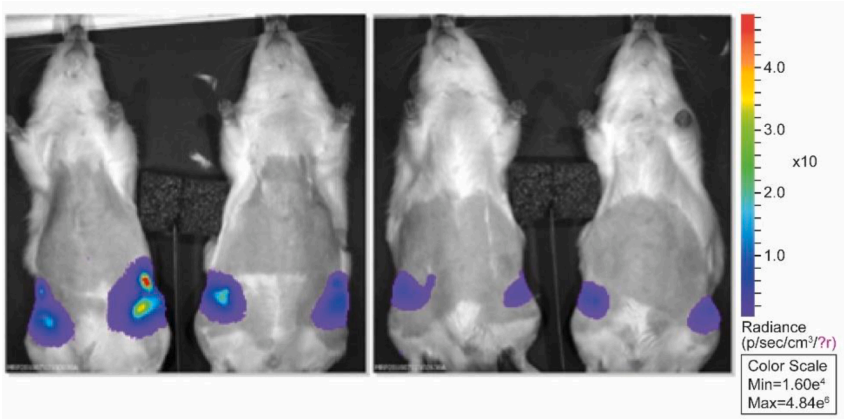

Fig. 5. *In vivo* imaging analysis of luciferase following IM administration of 5 mcg mRNA in the Vastus medialis of rats followed by imaging after 24 h in the presence of luciferin substrate. n = 6, representative image shown.

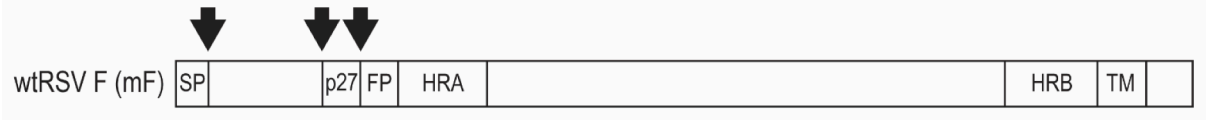

Fig. 6. wtRSV (mf) (rsv a2 strain) protein expressed by the wtRSV (mf) mrna construct incorporated into lnps and utilized in immunogenicity studies. sp, signal peptide; p27, p27 peptide; fp, fusion peptide; hra, heptad repeat a;HRB, heptad repeat B; TM, transmembrane peptide. Arrows indicate the proteolytic cleavage sites in the expressed protein [29].

study are summarized in Fig. 4 and Tables 2 and 3.

A statistically significant difference between Tropis and Needlel/Syringe was detected (p = 0.000) as well as a statistically significant difference between 24 and 48 h (p = 0.000). The marginally statistically significant interaction between **Device** and **Time** (p = 0.052) suggests the difference in log(Flux) between Tropis and Needle/Syringe was not entirely consistent between 24 and 48 h. This can be seen in Fig. 4 where the differences between 24 and 48 h for the Neede/Syringe device appear to be slightly larger than the differences between 24 and 48 h for the Tropis device. No other statistically significant effects were detected.

Overall, the mean difference in log (Total Flux) between Tropis and Needle/Syringe (95 % confidence interval) was estimated to be 0.860 (0.557, 1.162). In the original scale this implies that the geometric mean Total Flux associated with Tropis was $10^{0.860}$ = 7.24-fold greater (95 % CI: $10^{0.557}$ = 3.61-fold, $10^{1.162}$ = 14.5-fold) than that for the Needle/Syringe device (top part of Table 3). For the specific comparison of Tropis to Needle/Syringe at the 1 ug injection dose, the difference was statistically significant (p = 0.002, Tukey's simultaneous test for differences in means). The geometric mean Total Flux associated with Tropis was $10^{0.882}$ = 7.62-fold greater (95 % CI: $10^{0.321}$ = 2.09-fold, $10^{1.442}$ = 27.7-fold) than that for the Needle/Syringe device (bottom part of Table 3).

Imaging analysis of rats injected with luciferase mRNA/LNPs indicated higher expression in the lymph nodes when using Tropis (Fig. 5). These data suggest that the mRNA may have been transported to the lymph node by immune cells originating near the muscle where the mRNA/LNPs were administered. Alternatively, there may have been more uptake and expression of the Tropis-administered material.

In the next study, an evaluation of immunogenicity with mRNA encoding the prefusion-stabilized F protein from RSV were utilized. A schematic of the mRNA utilized is shown in Fig. 6. The RSV mRNA encoding the prefusion-stabilized F protein from RSV combined with LNPs was administered with either Tropis or needle/syringe to the muscle. The study design is shown in Fig. 7A. The results of these studies are depicted in Fig. 7B. As displayed in Table 4, the titers after 5 weeks of immunization indicated a statistically similar result when comparing Tropis to needle/syringe (p = 0.974 associated with the Device term in Table 4), which was consistent among the three doses studied (p = 0.974 associated with the Device*Dose(ug) term in Table 4). The CMI response was also evaluated using an IFN-γ ELISPOT assay. T-cell responses to RSV-F were detected from animals immunized with needle/syringe at all dose levels, and from the animals immunized with Tropis delivery in the 5 and 25 ug groups. (Fig. 7C). Data from two animals in Group 1 were excluded from the data summary for failing quality control criteria (high levels of spontaneous IFN-γ release as observed in the unstimulated [DMSO] control well).

Discussion and conclusions

The goal of this study was to investigate the ability of modern jet delivery technology to deliver mRNA/LNPs intramuscularly in a rodent model. Luciferase mRNA was used as a model to evaluate compatibility and expression using *in vivo* imaging analysis. An mRNA encoding the prefusion-stabilized F protein from RSV was delivered intramuscularly using Tropis in rodent models and the immune response measured. This was the first time, to our knowledge, that an RSV mRNA/LNP was delivered using Tropis and immunogenicity evaluated in a rodent model.

The data indicated changes in encapsulation efficiency following transfer of luc mRNA/LNP through Tropis, however the luciferase mRNA demonstrated the same expression *in vitro* as material transferred through a needle/syringe. At the administration site, the Tropis-delivered material resulted in a higher local signal and increased expression at the local lymph nodes relative to the standard of care (needle/syringe). Although this was found to be a statistically significant finding, the practical impact is unknown. The quantitative data suggested Tropis-administered material trended higher than the standard needle/syringe. These studies will need to be repeated to confirm the significance of this trend. For the RSV mRNA/LNP, the humoral response was equivalent between Tropis and needle/syringe when administered intramuscularly. CMIs were induced in rodents using either method of delivery. These CMI responses (group mean) were higher in the rodents immunized with needle/syringe compared to those immunized with Tropis. The reasons for this observation are unknown

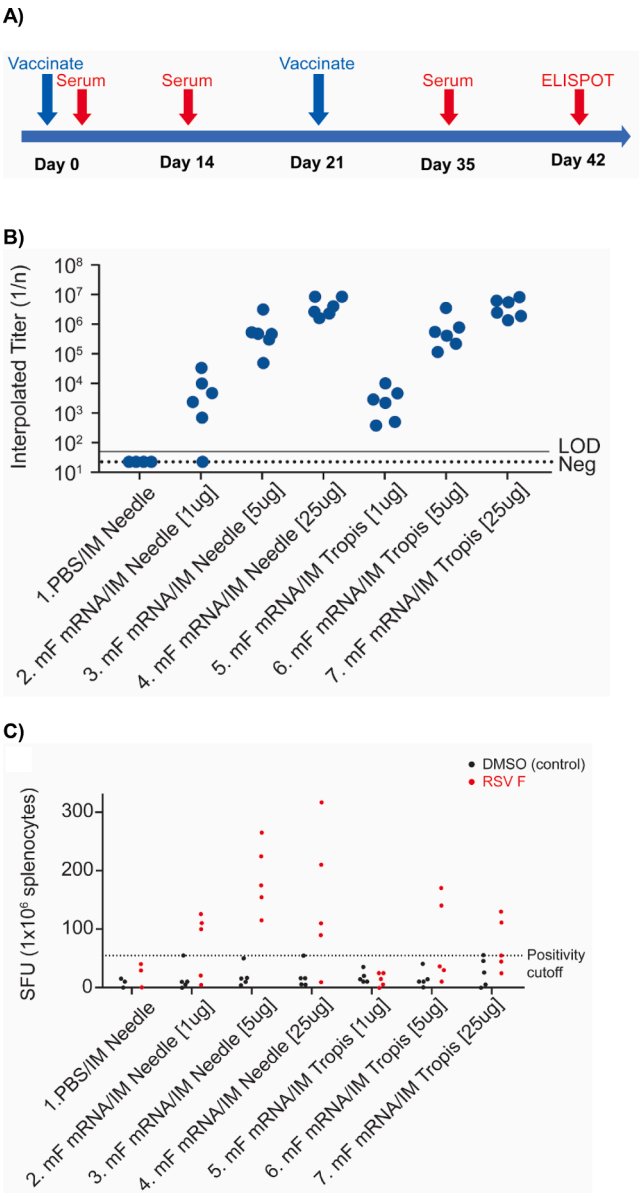

Fig. 7. Immunogenicity results of the RSV mRNA with Tropis vs. needle/syringe. Study design (A), as measured 2 weeks post-dose 2 in RSV-F specific ELISA (B) and at 3 weeks post-dose 2 in an RSV-F specific IFN-γ ELISPOT, n = 6 (C) ELISPOT analysis. n = 5 for RSV F and n = 5 for DMSO (control) Two animals in group 1 (PBS injected control, initial n = 5) of the ELISPOT assay were excluded for quality control due tohigh levels of IFN- γ background (n = 3).

Table 4
Analysis of Variance for Log (Titer).[+]

Source	DF	SS	MS	F	P
Device	1	0.0004	0.0004	0.00	0.974
Dose (ug)	2	69.0812	34.5406	90.63	0.000
Device*Dose (ug)	2	0.0201	0.0100	0.03	0.974
Error	30	11.4332	0.3811		
Total	35	80.5349			

[+] Statistical analysis of data obtained from Fig. 7B.

and studies in additional species such as non-human primates will be needed to fully evaluate the induction of CMI using jet delivery methods.

The implementation of an RSV vaccine is believed to have the potential to reduce disease burden. mRNA/LNP-based vaccines are now considered an essential tool for combating disease as demonstrated with the COVID-19 vaccines [31]. Additionally, the ability to rapidly administer vaccines with a needle-free device would have advantages in many parts of the world. The use of a needle-free device may improve global access to mRNA vaccines as this type of vaccine is now becoming more prevalent. In conclusion, studies of mRNA/LNP-based vaccines combined with jet delivery technology is an expanding area and has the potential for future growth.

Animal procedures

All animal procedures were performed according to IACUC guidelines and approved animal procedure statements.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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