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Intramuscular but not nebulized administration of a mRNA vaccine against *Rhodococcus equi* stimulated humoral immune responses in neonatal foals

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OBJECTIVE
Design and evaluate immune responses of neonatal foals to a mRNA vaccine expressing the virulence-associated protein A (VapA) of *Rhodococcus equi*.

ANIMALS
Cultured primary equine respiratory tract cells; Serum, bronchoalveolar lavage fluid (BALF), and peripheral blood mononuclear cells (PBMCs) from 30 healthy Quarter Horse foals.

METHODS
VapA expression was evaluated by western immunoblot in cultured equine bronchial cells transfected with 4 mRNA constructs encoding VapA. The mRNA construct with greatest expression was used to immunize foals at ages 2 and 21 days in 5 groups: (1) 300 µg nebulized mRNA (n = 6); (2) 600 µg nebulized mRNA (n = 4); (3) 300 µg mRNA administered intramuscularly (IM) (n = 5); (4) 300 µg VapA IM (positive controls; n = 6); or (5) nebulized water (negative controls; n = 6). Serum, BALF, and PBMCs were collected at ages 3, 22, and 35 days and tested for relative anti-VapA IgG_1, $IgG_{4/7}$, and IgA activities using ELISA and cell-mediated immunity by ELISpot.

RESULTS
As formulated, nebulized mRNA was not immunogenic. However, a significant increase in anti-VapA $IgG_{4/7}$ activity ($P < .05$) was noted exclusively in foals immunized IM with VapA mRNA by age 35 days. The proportion of foals with anti-VapA IgG_1 activity > 30% of positive control differed significantly ($P = .0441$) between negative controls (50%; 3/6), IM mRNA foals (100%; 5/5), and IM VapA (100%; 6/6) groups. Natural exposure to virulent *R equi* was immunogenic in some negative control foals.

CLINICAL RELEVANCE
Further evaluation of the immunogenicity and efficacy of IM mRNA encoding VapA in foals is warranted.

Keywords: mRNA, vaccine, *Rhodococcus equi*, foal, immunity

Rhodococcus equi (*RE*) is a facultative intracellular pathogen that is widely distributed in the soil and environment of horses worldwide.[1] Many foals at horse-breeding farms are infected, causing severe pneumonia in a proportion of those infected foals, whereas many other foals resolve infection without developing clinical signs.[2,3] Rhodococcal pneumonia is an important health problem because the disease is recurrent at many breeding farms, annual incidence

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may be high, and recovered foals may have reduced athletic performance.[1,4]

To date, a vaccine to prevent *RE* pneumonia has not been licensed in the United States (US). A vaccine remains elusive largely because foals appear to be infected very soon after birth[5] when they are highly susceptible to infection[6] presumably because of naïve and immature immune responses of foals[7–11] and interference of maternal antibodies (Abs).[12,13] Despite their naïve and immature immunity, neonatal foals can mount effective immune responses against intracellular bacteria.[14–19]

mRNA vaccines have gained attention for their success against COVID-19[20] because they can achieve superior cell-mediated and humoral immune responses to those from traditional vaccines.[21,22] Efficacy of mRNA vaccines has been demonstrated in preclinical models of bacterial infection.[23–26] Furthermore, vaccination of neonatal mice against influenza with in vitro transcribed (IVT) mRNA resulted in humoral and cell-mediated immune (CMI) responses in the face of maternal Ab that protected against influenza infection.[27] These findings prompted us to investigate a mRNA vaccine to protect foals against *RE* pneumonia.

Virulent strains of *RE* carry a plasmid that encodes the virulence-associated protein A (VapA) that is necessary for *RE* to cause disease in foals.[28] VapA is highly immunogenic,[29–32] because it is surface-expressed and secreted and consequently accessible to immune effector cells and antibodies. Foals gavaged with live, virulent *RE* develop antibody titers to virulent *RE* and VapA during the first weeks after birth[16–19] that correlate with protection against pneumonia following challenge with virulent *RE.*[18–19] Additionally, passive immunization with anti-VapA antibodies protects foals against *RE* pneumonia,[6,33–35] further indicating a protective role for anti-VapA antibodies.

Thus, our goal is to develop a mRNA vaccine encoding VapA to immunize foals for protection against *RE* pneumonia. Because *RE* infection is considered to occur via inhalation,[1,5,6] we hypothesized that immunizing foals via nebulization could be a more effective method than intramuscular (IM) immunization by inducing local immune responses. Here, we report in silico design of 4 IVT mRNA constructs expressing VapA, in vitro selection of a VapA mRNA construct as a vaccine candidate, and in vivo immunogenicity in foals receiving a selected mRNA construct administered either via nebulization or IM.

Methods

Ethics statement

All methods were performed in accordance with relevant guidelines and regulations for animal use and for laboratory practices (Texas A&M University Infectious Biohazard Committee IBC# 2017-105; IACUC AUP# 2020-0306).

Design of mRNA constructs

Four alternative mRNA constructs encoding versions of the VapA protein were designed **(Figure 1)** based on the *vapa* gene (NCBI accession, #BAA04768.1; **Supplementary Material S1**). These 4 constructs were in vitro transcribed with a mammalian Kozak consensus sequence, base substitution with 5-methoxyuridine (5-moU), poly-adenylation, and poly-A tail by TriLink BioTechnologies.

In vitro evaluation of mRNA constructs

Equine bronchial epithelial cells (EBECs) and bronchial fibroblasts (EBFs) were harvested postmortem from 4 adult horses with no history of respiratory disease. Primary cultures were established as previously described (summarized in Supplementary Material S1).[36–38] Each of the 4 mRNA constructs was formulated with a commercial transfection reagent (Lipofectamine MessengerMAX, ThermoFisher Scientific) according to the manufacturer's instructions, delivered to cell media in cultures, and incubated for 24 hours. Each condition was repeated in duplicate within the experiment, and the experiment was replicated 3 times. Supernatants and cell lysates of cultured EBECs and EBFs were harvested to assess secretion and intracellular expression of VapA by western immunoblotting. Western immunoblotting was performed using a monoclonal mouse anti-VapA antibody (Santa Cruz Biotechnologies; Cat #sc-390576; detailed in Supplementary Material S1) to confirm and compare VapA expression to select a vaccine candidate.

Study population

Thirty Quarter Horse foals that were healthy at birth, had transfer of passive immunity assessed by semiquantitative immunoassay (SNAP Foal IgG Test, IDEXX), and had CBC results within reference ranges at age 1 day were included in the study. The sample size was calculated using a statistical power of 80%, significance level of 0.05, and expected proportions of foals having IgG OD ratio values > 0.3 of the positive control among 25% of control foals (natural exposure, based on unpublished data from our laboratory among foals born and housed at our facility) and 100% in mRNA vaccinates. The *mod-VapA* mRNA construct was investigated as a vaccine candidate (Figure 1) by comparing the following groups: (1) 300 µg of mRNA delivered by nebulization (n = 6, low-dose); (2) 600 µg of mRNA delivered by nebulization (n = 4, high-dose); (3) 300 µg of mRNA administered IM (n = 5); (4) negative control foals nebulized with water (n = 6); and (5) positive controls immunized IM with purified recombinant VapA (rVapA) protein with an adjuvant (300 µg VapA; n = 6). We planned to have 6 foals in each group; however, because of supply-chain disruptions and COVID-19 illness, we were unable to formulate mRNA in lipid nanoparticles (LNPs) for 3 foals (2 in the high-dose group and 1 in the IM mRNA group). All foals were immunized at ages 2 and 21 days. Mare-foal pairs were maintained in stalls for 4 days after foaling, then housed with 2 to 3 other mare-foal pairs in paddocks at a research facility that had housed both *RE*-infected and uninfected foals and their dams in previous years. Our study was weighted toward more foals

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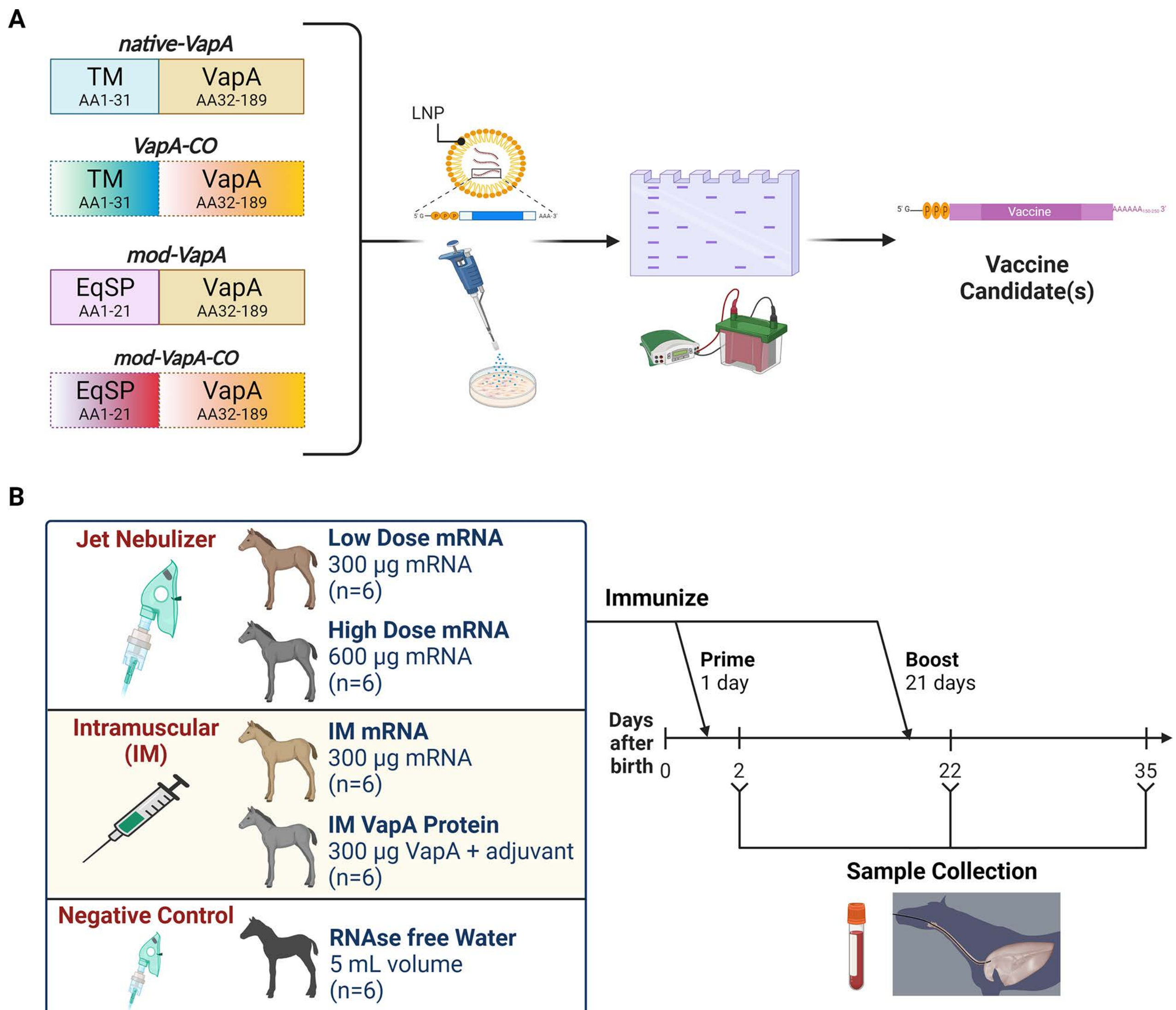

Figure 1—Study design of IVT mRNA vaccine candidate selection and assessment of in neonatal foals. Four mRNA constructs expressing VapA were designed (A) as either expressing native VapA (*native-VapA*); codon-optimized VapA (*VapA-CO*); modified sequence (*mod-VapA*) with the transmembrane domain (TM) of VapA removed and replaced with an equine-specific signal peptide (EqSP); and finally, this modified construct was codon-optimized (*mod-VapA-CO*). Constructs were delivered to primary cultures of equine bronchial epithelial cells and bronchial fibroblasts, and evaluated for VapA protein expression by western immunoblot to select a vaccine candidate. The selected mRNA construct was then evaluated (B) by immunizing foals at ages 2 and 21 days in 5 groups: (1) 300 µg nebulized mRNA (n = 6); (2) 600 µg nebulized mRNA (n = 6); (3) 300 µg mRNA administered intramuscularly (IM) (n = 6); (4) 300 µg VapA IM (positive controls; n = 6); or, (5) nebulized water (negative controls; n = 6). Serum, bronchoalveolar lavage fluid (BALF), and PBMCs were collected from foals at ages 3, 22, and 35 days to test for relative anti-VapA IgG_1, $IgG_{4/7}$, and IgA activities by ELISA and cell-mediated immunity by ELISpot.

receiving nebulized mRNA than IM mRNA because we hypothesized that intrapulmonary VapA expression might induce more effective immune responses in the lungs.

Vaccine preparation and delivery

The IVT mRNA was formulated in LNPs (Onpattro formulation; detailed in Supplementary Material S1) and stored at 4 °C until use. Immediately before nebulization, the mRNA-LNP solution was diluted to a total volume of 5 mL in RNAse-free sterile water (Invitrogen, Cat #10977-015). Negative control foals were nebulized with 5 mL RNAse-free water (Invitrogen). mRNA (or water) was delivered via a jet nebulizer (Supplementary Material S1).[39] All IM immunizations were delivered to the triceps muscle with a 1.5" X 22 gauge needle. The IM mRNA vaccine was delivered as 1 mL of LNP-mRNA solution. The IM VapA protein vaccine was prepared using 300 µg rVapA and 10% Montanide Gel 01 adjuvant (Seppic), diluted to 1 mL volume with sterile 0.9% NaCl. No IM placebo injection group was incorporated in this study. Foals were examined twice daily by a veterinarian for adverse effects of vaccination.

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Sample collection

Blood and bronchoalveolar lavage (BAL) fluid were collected at ages 2, 22, and 35 days for ELISA and ELISpot. BAL was performed at age 2 days under general anesthesia, and at ages 22 and 35 days with standing sedation (detailed in Supplementary Material S1). Recovered BAL fluid (BALF) was processed immediately by filtering through sterile gauze and centrifuging at 400 X *g* for 10 minutes. Fluid was harvested and stored at −80 °C for subsequent analysis.

Anti-VapA antibody testing via ELISA

Anti-VapA IgG_1, $IgG_{4/7}$, and IgA antibody activities in serum and BALF were determined by indirect ELISA (detailed in Supplementary Material S1). An OD ratio was calculated by dividing the OD value of each sample by that of the positive control on the same plate. A sample OD ratio of > 30% of the positive control (> 0.3) was defined as a positive result; this cut-point was selected because it represented values greater than the mean plus 3 SDs of OD ratios for all study foals at age 3 days. The proportion of foals achieving this positive result at age 35 days was compared among groups for both IgG subisotypes. The ratio of IgA activity from BALF relative to the IgA activity in serum was calculated as a marker of increased local anti-VapA IgA activity within the respiratory tract.

ELISpot testing

As a marker of CMI response to vaccination, interferon gamma (IFN-γ) production from isolated foal peripheral blood mononuclear cells (PBMCs) stimulated with a lysate of virulent *RE* (strain ATCC 33701) was measured using a commercial ELISpot kit (Cat #3117-2A; Mabtech) according to manufacturer's directions (summarized in Supplementary Material S1).

Data analysis

All analysis was performed using R statistical software (Version 4.3.1; R Foundation for Statistical Computing). Correlation between variables was quantified using the cor.test method with the Spearman option. Proportions were compared between variables using Fisher's exact tests using the fisher.test method in R. Effects of age, treatment group, and their interaction with activity levels of IgG_1, $IgG_{4/7}$, or IgA in serum or BALF were assessed using linear mixed-effects models using the nlme package in R with an exchangeable correlation structure. Effects of mRNA vaccination, time, and vaccination-by-time interaction on ELISpot counts were assessed using linear mixed-effects modeling using R (nlme package) with pair-wise comparisons between groups made using the method of Tukey. Significance was set at $P < .05$.

Results

In vitro mRNA construct selection

VapA expression in supernatants and cells of cultured EBECs and EBFs was detected by immunoblot for the constructs lacking the TM domain (ie, *mod-VapA* and *mod-VapA-CO*) but not those with the TM domain **(Figure 2)**. Furthermore, the *mod-VapA* construct was deemed better expressed than the *mod-VapA-CO* based on band strength relative to the loading control.

Immunogenicity of nebulized VapA mRNA

Activities of IgG_1, $IgG_{4/7}$, and IgA in serum and BALF and cell-mediated immune (CMI) responses (ie, IFNγ activity after VapA stimulation of PBMCs determined by ELISpot) were compared between control foals and foals nebulized with either low- or high-dose of mRNA. No significant ($P < .05$) differences were observed in IgG_1 activities between groups within age (ie, ages 3, 22, or 35 days; **Figure 3**). Serum IgG_1 activities were significantly higher for controls at age 35 days than at either age 3 ($P = .0207$) or 22 days ($P = .0156$). Unlike control foals, serum IgG_1 activities were not significantly higher at age 35 days for mRNA nebulized foals. Serum $IgG_{4/7}$ activities appeared to increase with age (Figure 3) but no significant ($P < .05$) pairwise differences between groups or times were detected. Serum IgA activities decreased significantly from age 3 days to 22 days in all groups ($P = .0004$, .0483, and .0263 for controls, low-dose, and high-dose groups, respectively; Supplementary Material S1). Although values at age 35 days appeared lower than at age 3 (Supplementary Material S1), a significant difference was only noted for the low-dose mRNA group ($P = .0090$) but not for the controls ($P = .2668$) or the high-dose group ($P = .0899$). Within ages (ie, 3, 22, or 35 days), however, no significant differences between groups were detected. In summary, serum IgA decreased at age 22 days for foals but did not rise consistently and tended to remain low for the low-dose nebulized group.

Similarly, although activity levels appeared to increase with age, no significant effects of age, group, or age-by-group interaction were detected for IgG_1 or $IgG_{4/7}$ activities in BALF (Figure 3). IgA activity in BALF increased significantly between ages 3 and 22 days (Supplementary Material S1) for the control group ($P = .0090$) and for the high-dose group ($P = .0050$) but not the low-dose group ($P = .9474$). Similarly, IgA activities were significantly higher at age 35 days than 3 days for control foals ($P = .0005$) and the high-dose foals ($P = .0009$) but not the low-dose foals ($P = .0533$; Supplementary Material S1). The ratio of anti-VapA IgA activity in BALF to serum (Figure 3) increased significantly in all groups at age 22 days ($P = .0090$, .0293, .0090 for control, low-dose, and high-dose groups, respectively) and at age 35 days ($P < .0010$ for all groups). No significant differences were detected between IgA activities at ages 22 and 35 days within groups. Within age categories, no significant differences between groups were detected.

Within group, CMI responses (ie, IFNγ activity after VapA stimulation of PBMCs) were significantly ($P < .05$) increased for all treatment groups at ages

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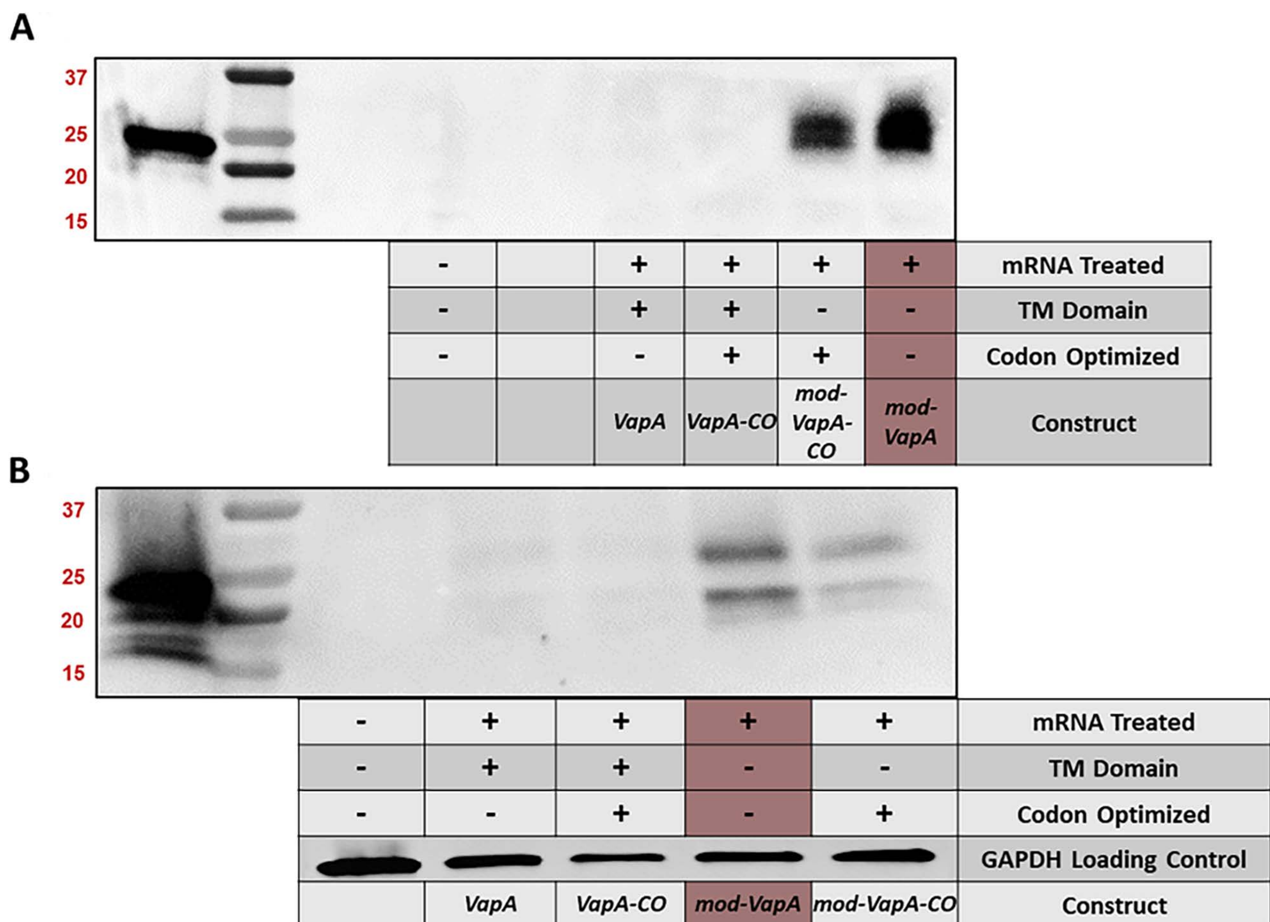

Figure 2—In vitro evaluation of VapA protein expression from the 4 IVT mRNA vaccine candidates. Representative image of western immunoblot demonstrating VapA expression in cell culture supernatants (A) and cell lysates (B) of equine bronchial fibroblasts (EBFBs) transfected with mRNA constructs lacking the TM domain (*mod-VapA* and *mod-VapA-CO*), using monoclonal anti-VapA antibody, recombinant VapA protein as positive control (2 different sources), and nontransfected cells as negative control. Greater VapA expression was seen without codon optimization (*mod-VapA*). Immunoblotting results were similar for cell lysates and supernatants of EBECs.

22 and 35 days relative to age 3 days (**Figure 3**). No significant differences were detected between ages 35 and 22 days for any of the groups, nor between groups at ages 35 or 22 days. Collectively, these results indicated no significant effects of nebulized mRNA on either serum or BALF anti-VapA antibody activity or CMI responses to *RE*.

Immunogenicity of IM VapA mRNA

Activities of IgG_1, $IgG_{4/7}$, and IgA in serum and BALF and CMI responses were compared between foals administered VapA mRNA IM, positive control foals administered VapA protein IM, and negative controls (nebulized with RNase-free water; N.B., the same control foals described above). No IM placebo group was used in this study.

For all 3 groups, serum IgG_1 activity was significantly ($P < .0001$) increased at age 35 days relative to ages 3 and 22 days **(Figure 4)**. No significant differences in IgG_1 activity were detected between groups at age 35 days; however, IgG_1 activity was more consistent for foals in the IM groups (mRNA or rVapA protein) than controls, suggesting a more uniform response to IM immunization compared to responses attributed to natural exposure in control foals. At age 35 days, the proportion of foals with anti-VapA IgG_1 activity > 30% of the positive control was 50% for controls (3/6) compared with 100% each for the IM mRNA (5/5) and the IM VapA (6/6) groups; this difference was significant ($P = .0441$). Serum $IgG_{4/7}$ activities (Figure 4) appeared more variable than those for serum IgG_1 activities. The only significant differences detected within groups were that values were significantly higher for the IM mRNA group at age 35 days relative to ages 3 days ($P = .0012$) and 22 days ($P < .0001$), and for the control foals at age 35 days relative to age 22 days ($P = .0148$); however, controls did not differ significantly between ages 3 and 35 days ($P = .2726$) and between ages 3 and 22 days ($P = .9747$). Collectively, these data indicate that while all foals demonstrated increased serum activity of IgG_1 and $IgG_{4/7}$ at age 35 days, results for the IM groups were stronger and more consistent. Serum IgA activities decreased significantly from age 3 days to 22 days in control and IM VapA protein groups ($P = .0010$ and $.0028$, respectively) but not

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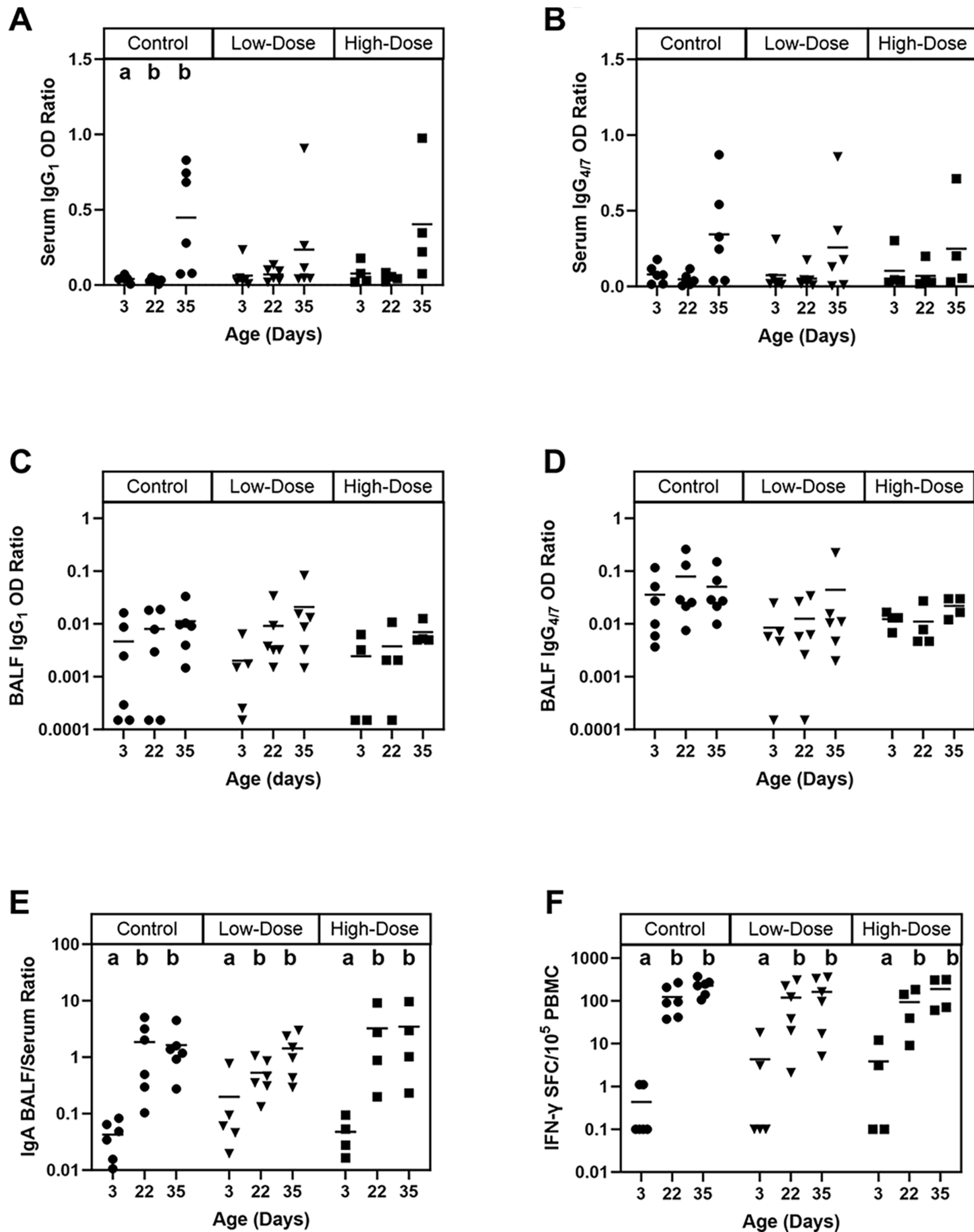

Figure 3—Humoral and cell mediated immune (CMI) responses to nebulized mRNA vaccine in neonatal foals. Ratio of serum activities of anti-VapA IgG_1 (A) and $IgG_{4/7}$ (B) relative to positive control (serum of an *Rhodococcus equi* hyper-immunized horse) was compared between the 2 nebulized mRNA groups (high-dose and low-dose) and the negative controls (nebulized with water), and compared by age. While IgG_1 activities increased over time in control foals, no significant differences were seen in foals that received nebulized mRNA in either IgG_1 or $IgG_{4/7}$ serum activities. BALF anti-VapA IgG_1 (C) and $IgG_{4/7}$ (D) activities also did not have any significant differences between age, group, or age by group interaction. Ratio of BALF anti-VapA IgA activity relative to serum anti-VapA IgA activity (E) was also compared between the 2 nebulized mRNA groups (high-dose and low-dose) and the negative controls (nebulized with water), and compared by age. The ratio of anti-VapA IgA in BAL fluid relative to serum increased significantly with age within all 3 groups from age 3 days to age 22 days (P = .0090 for controls, P = .0293 for low-dose group, and P = .0090 for high-dose group) and age 35 days (P < .0010 for all 3 groups). Within groups, no significant differences were detected between IgA ratio at ages 22 and 35 days. Within age, no significant differences between groups were detected. ELISpot results (*RE*-stimulated minus negative control) of interferon gamma secretion were compared between the 2 nebulized mRNA groups (high-dose and low-dose) and the negative controls (nebulized with water) and by age (F). There were significant increases in all foals over time (P < .05), but no significant differences were detected between nebulized groups within age. Different letters denote significant differences between groups (P < .05) within specific ages, and there were no pairwise differences between groups within specific ages. Black bars are means.

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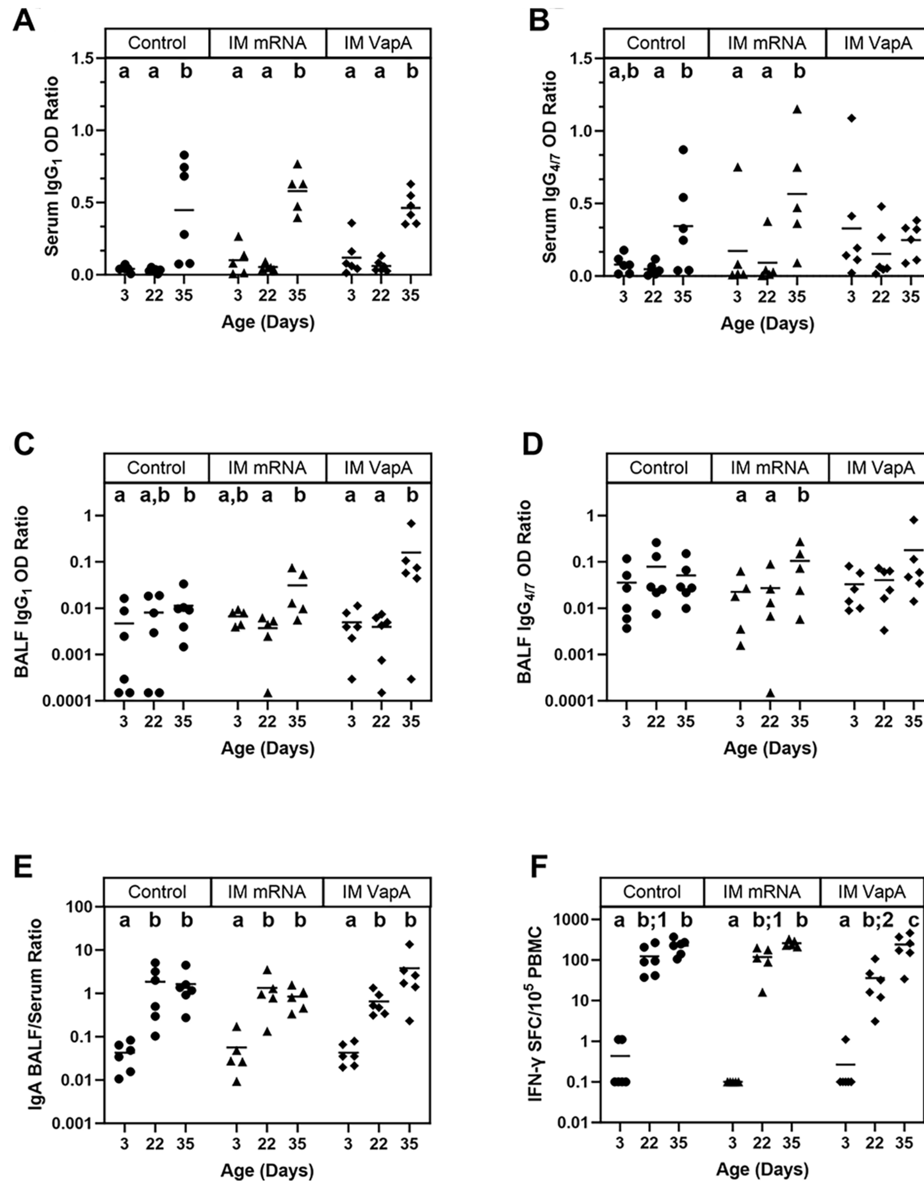

Figure 4—Humoral and cell mediated immune (CMI) responses to intramuscular VapA vaccine in neonatal foals. Ratio of serum activities of anti-VapA IgG_1 (A) and $IgG_{4/7}$ (B) relative to positive control (serum of an *Rhodococcus equi* [*RE*] hyperimmunized horse) compared between the 2 intramuscular VapA groups (VapA mRNA and VapA protein) and the negative controls (nebulized with water), and compared by age. There were significant increases in all foals over time ($P < .05$). At age 35 days, the proportion of foals with anti-VapA IgG_1 activity > 30% of the positive control was 50% for controls (3/6) compared with 100% each for the IM mRNA (5/5) and the IM VapA (6/6) groups. Serum $IgG_{4/7}$ activities were significantly higher for the IM mRNA group at age 35 days relative to ages 3 days ($P = .0012$) and 22 days ($P < .0001$), and for the control foals at age 35 days relative to age 22 days ($P = .0148$). BALF anti-VapA IgG_1 (C) and $IgG_{4/7}$ (D) activities were similar to serum activities. At age 35 days, values of IgG_1 activity in BALF were significantly higher for foals in the IM mRNA group ($P = .0155$) and the VapA protein group ($P = .0010$). Activities of $IgG4_{/7}$ in BALF only differed significantly for the foals in the IM mRNA group: values at age 35 days were significantly higher than those at ages 3 ($P = .0326$) and 22 days ($P = .0028$). Effects of vaccine group and age on the ratios of BALF anti-VapA IgA activity relative to serum anti-VapA IgA activity (E) were compared between foals in the 2 intramuscular VapA groups (VapA mRNA and VapA protein) and the negative controls (nebulized with water). Within group, the ratio of BALF to serum IgA activity was significantly higher at ages 22 ($P < .0001$) and 35 ($P < .0001$) days; however, values at age 35 days were not significantly higher than those at age 22 days ($P = .1600$). Within age, no significant differences between groups were detected. ELISpot results (*RE*-stimulated minus negative control) of interferon gamma secretion were compared between the 2 intramuscular VapA groups (VapA mRNA and VapA protein) and the negative controls (nebulized with water), and compared by age (F). There were significant increases in all foals over time ($P < .05$), but no significant differences were detected between nebulized groups within age. No significant differences in CMI response were detected between ages 35 and 22 days for either the controls or the IM mRNA group, but values for the IM VapA protein group were significantly lower at both ages 35 and 22 days ($P < .0001$). Different letters denote significant differences between groups ($P < .05$) within specific ages, different numbers denote numerals indicate any significant ($P < .05$) differences between groups within age, and black bars are means.

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in the IM mRNA group (P = .0601; Supplementary Material S1). At age 35 days, serum IgA was not significantly lower than at age 3 days for control foals (P = .2337) or IM mRNA foals (P = .0899); however, activities of IgA for the IM VapA protein foals were significantly (P = .0021) lower at age 35 days than at age 3 days. Serum IgA concentrations at days 22 and 35 did not differ within groups. Within age, there were no significant differences between groups. In summary, serum IgA decreased at age 22 days for foals but did not rise consistently and tended to remain low for the IM VapA protein group.

Results for IgG subisotype activities in BALF had a pattern similar to those for serum (Figure 4). For the controls and VapA protein groups, IgG_1 activities at age 35 days were significantly higher than at ages 3 days (P = .0278 and P = .0008, respectively); although values for the IM VapA mRNA group appeared higher at age 35 days than 3 days, this difference was not significant (P = .6617). At age 35 days, IgG_1 activity in BALF was significantly higher than at age 22 days for foals in the IM mRNA group (P = .0155) and the VapA protein group (P = .0010) but not the controls (P = .5112). No significant differences were detected in BALF IgG_1 activity at age 35 days between groups, and although the proportion of foals with BALF IgG_1 activity > 1% of the positive control value varied among control (17%; 1/6), IM mRNA (60%; 3/5), and VapA protein (83%; 5/6), this difference was not significant (P = .0586). $IgG_{4/7}$ activities in BALF differed significantly only for the foals in the IM mRNA group: values at age 35 days were significantly higher than those at ages 3 (P = .0326) and 22 days (P = .0028) (Figure 4); although values appeared to increase with age for foals in other groups, no other differences between ages or groups were significant. Values of IgG_1 in serum and BALF and $IgG_{4/7}$ in BALF were significantly correlated (Pearson's ρ = 0.5813, P < .0001 and Pearson's ρ = .6684, P < .0001, respectively), likely explaining the similar results observed for serum and BALF. For anti-VapA IgA activity in BALF, no significant effects of group or group-by-age interaction were detected (Supplementary Material S1). Accounting for the effects of group, anti-VapA IgA activity was significantly higher at ages 22 (P < .0001) and 35 (P < .0001) days, and values on day 35 were significantly (P = .0003) higher than those on day 22. Similarly, no significant effects of group or group-by-age interaction were detected for the ratio of anti-VapA IgA activity in BAL fluid to that in serum (Figure 4). Accounting for the effects of the group, the ratio of BALF to serum IgA activity was significantly higher at ages 22 (P < .0001) and 35 (P < .0001) days; however, values at age 35 days were not significantly higher than those at age 22 days (P = .1600).

Within group, activity of IFNγ after VapA stimulation of PBMCs were significantly (P < .0001) increased for all treatment groups at ages 22 and 35 days relative to age 3 days (Figure 4). No significant differences in this CMI response were detected between ages 35 and 22 days for either the controls or the IM mRNA group, but values for the IM VapA protein group were significantly lower (P < .0001) at age 22 than age 35 days. At age 22 days, values of CMI for the IM VapA protein group were significantly lower than those in either the controls (P = .0107) or the IM mRNA group (P = .0295). No other significant differences were noted. Collectively, these data indicated that CMI responses to *RE* characterized by IFN-γ expression increased with age in all foals, and these responses appeared graphically most consistent for the IM mRNA group at age 35 days.

Safety data

None of the foals in any of the groups developed pneumonia or coughing during the study. None of the foals developed lameness after their initial immunization but 3 foals in the IM VapA group developed lameness after their boost lasting 2 days duration. Six foals developed fever (rectal temperature ≥ 39.4 °C [≥ 103.0 °F]) of 1 day duration (maximum temperatures among the foals are summarized in Supplementary Material S1). Of these, fever occurred in 2 foals in the low-dose nebulized group after the primary immunization, 3 foals in the IM VapA protein group, and 1 foal in the IM mRNA group after the second immunization. Localized swelling at the injection site was seen in 1 of 5 foals in the IM mRNA group after the primary immunization and none after the second immunization. For the IM VapA protein group, 3 foals developed localized swellings after the primary immunization and 5 developed swelling after the second immunization. The frequency of localized swellings was significantly (P = .0115) higher in the IM VapA protein group than in the IM mRNA group. The swelling lasted 1 day in the foal in the IM mRNA group and ranged from 1 to 4 days (median, 2 days) in the IM VapA protein group.

Discussion

The primary objective of our study was to determine whether nebulizing mRNA encoding VapA in foals could stimulate increased activity of antibodies recognizing VapA systemically (serum) and locally in the lungs (BALF) and increased systemic CMI to VapA. Our first step was to select a mRNA construct encoding VapA based on the results of in vitro expression in EBECs and EBFs. Our results indicated that a VapA mRNA construct with a modification of the TM domain was best expressed in cells and secreted into the cell culture supernatants. Removing the TM domain of the bacterial VapA and replacing it with a host species-specific signal sequence was intended to limit the anchoring of expressed protein to the surface of host cells and to enhance secretion. This has been a successful strategy for several mRNA vaccine constructs, including a candidate for *Yersinia pestis*,[26] a facultative intracellular pathogen like *RE*. Unexpectedly, the noncodon-optimized construct appeared to be better expressed than the construct optimized for enhanced expression in equine cells. Codon optimization may be both species- and tissue-specific.[40,41] To the authors' knowledge, the tissue of origin for equine codon optimization is

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unspecified,[42] and codon optimization for equine respiratory cells has not been investigated. Moreover, noncodon-optimization can sometimes prolong protein translation, resulting in improved expression and enhanced protein folding.[40,41] Results of our in vitro studies underscore the importance of empirically testing mRNA constructs in host cells to select those most effectively translated.

As formulated, nebulized mRNA encoding VapA failed to stimulate greater activity of IgG_1, $IgG_{4/7}$, or IgA recognizing VapA in either the serum or BALF, or greater CMI to VapA, in nebulized foals relative to controls. Intrapulmonary mRNA has induced immune responses in rodents; however, mRNA was delivered either intranasally (that largely enters the lungs in rodents) or intratracheally via aerosol spray.[43] Intrapulmonary mRNA vaccines can increase local IgA activity,[43] and bronchial-associated lymphoid tissue (BALT) can also generate antigen-specific IgG within the respiratory tract.[43–45] It is unclear why our construct failed to be immunogenic given our evidence of in vitro expression. The nebulizer may have caused destruction of the LNPs and degradation of the mRNA.[46] It is also possible that the nebulizer failed to deliver particles to the lungs. Particle size data for the nebulizer were generated with a different liposomal drug,[40] thus the droplet size profile might differ for the mRNA-LNP solution we used. Furthermore, intrapulmonary particle deposition from nebulizers in human infants and pediatric patients can differ markedly from that in adults.[47] LNPs may be inappropriate carriers for nebulizing horses: formulating mRNA in polymers rather than LNPs has been demonstrated to be more effective for nebulizing mRNA in multiple animal species.[48] Moreover, different mRNA vaccine constructs can provide superior mucosal immune responses.[49] Thus, the construct and/or the formulation of mRNA could have impacted our results.

Anti-VapA IgA activity in foal serum appeared to decrease by age 22 days, presumably attributable to maternal antibody degradation, irrespective of treatment group, and generally remained low at age 35 days relative to age 3 days. In contrast, BALF anti-VapA IgA activity and the ratio of activity in BALF to serum increased with age irrespective of group. Presumably, the increase in anti-VapA IgA activity in BALF is attributable to natural *RE* exposure because it was observed for all treatment groups. The ratio of BALF to serum IgA is considered to reflect the production of IgA within the lower respiratory tract presumably by resident B cells.[45] It is unclear whether the failure of nebulized mRNA foals to respond is attributable to a lack of effective VapA expression within the respiratory tract or whether foals were unable to mount effective immune responses to the nebulized mRNA. As noted above, formulations of mRNA in carriers other than LNPs or different nebulizers might be more effective. The role of this increased antirhodococcal IgA activity in the lungs in protecting foals against *RE* infection merits further investigation.

We included the VapA protein IM group as a positive control for systemic responses relative to control foals and included the IM VapA mRNA to compare to responses to IM protein administration. Both these immunization methods appeared to generate more consistent increases in anti-VapA antibody activity in serum and BALF than those observed for the control group by age 35 days. The correlation between serum and BALF is expected because IgG is the predominant isotype in BALF and the majority of these antibodies are serum-derived.[44] Although antibody responses to the primary immunization were not detected at age 22 days, the initial immunization at age 3 days may have stimulated other innate and adaptive immune responses that contributed to more consistent responses to VapA. The proportion of foals with anti-VapA IgG_1 activity > 30% of the positive control at age 35 days was significantly higher in the 2 IM groups than in the control foals, and the only group for which anti-VapA $IgG_{4/7}$ activity was increased at age 35 days was the IM mRNA group. While this was a small study, results are promising for the potential of active immunization using mRNA vaccines in neonatal foals, similar to findings in neonatal mice.[27] Based on these results, we will study the immunogenicity and efficacy of this VapA mRNA construct administered intramuscularly in a larger study powered to detect differences in immunogenicity based on the results of this study.

Foals in our control group developed anti-VapA activity in serum and BALF. This finding was attributed to natural exposure to virulent *RE*, which is ubiquitous in the environment of foals.[1] At affected farms, more foals presumed to be infected with *RE* recover spontaneously than require treatment.[2,3] Thus, it is possible that the foals with anti-VapA antibody activity represent foals that have developed protective immune responses after natural exposure. Gavage with and natural exposure to live, virulent *RE* induce increased serum anti-VapA IgG_1 activity that correlates with protection against intrabronchial infection.[16–19] Thus, it is possible that our results demonstrate that IM immunization with VapA mRNA can induce immune responses in those foals that do not develop immunity via natural exposure and are more susceptible to infection. Without a IM placebo group, we cannot exclude the possibility that the difference between the IM immunized groups and control foals was not due to chance differences in natural exposure between groups; housing practices, however, make this explanation unlikely.

Foals in all groups had significant increases in VapA-specific CMI by 22 days. This likely reflects both natural exposure to *RE* in the environment of foals and maturation of adaptive immune responses: newborn foals are known to express less IFNγ than older foals[8,11,50], and respond less effectively to intracellular bacteria soon after birth than when older.[6] The period of reduced CMI responses corresponds with a period during which foals are highly susceptible to infection.[6]

This study had important limitations. The sample size was relatively small, primarily because of the costs of maintaining mares and foals and space limitations for housing. We originally planned to conduct a 2-year study; however, after observing that nebulizing VapA mRNA failed to induce immune

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responses, we elected to stop the project and refocus on studying the immunogenicity and efficacy of IM mRNA administration. We evaluated only the activity of 2 IgG subisotypes, IgA, and IFNγ responses of PBMCs against VapA. It is possible that other innate or adaptive immune responses were stimulated by nebulizing with mRNA-LNPs, which has been documented in rodents but is dependent on LNP composition.[51] While nebulized LNP-mRNA solutions can be translated to proteins in lungs,[43,52] evidence exists that LNPs are not optimal as a carrier for nebulized mRNA.[48] Because of the high cost of LNPs, we used nebulized water rather than nebulized empty LNPs as a negative control. For nebulized mRNA, this concern is moot because there was no difference between nebulized mRNA foals and control foals. For our IM controls, however, it would have been useful to have had an IM placebo group, particularly an IM LNP control: we cannot exclude the possibility that the VapA-specific responses in both the IM VapA protein and IM VapA mRNA groups were attributable to nonspecific (ie, adjuvanting/innate immune) effects of the vaccines. An IM placebo group was not included in this study because of funding, limited numbers of research foals, and our original intention to prioritize immunization via nebulization; we prioritized nebulized controls because nebulizing saline with 2.8% glycerol may enhance innate immune responses in the lungs of foals that might contribute to protection against *RE*.[53] This lack of IM control foals will be addressed in our future studies of IM VapA mRNA. We did not have a group immunized only at age 22 days; thus, we cannot rule out that immune responses at 35 days were attributable to immunization at age 22 days alone; that is, it is possible that immunization at age 3 days had no effect. Further experimentation will be needed to address this concern.

Despite these limitations, we identified a mRNA construct that is expressed by equine cells and documented the importance of evaluating expression of mRNA constructs in live host cells. As formulated, IM—but not nebulized—delivery of mRNA might generate immune responses in foals. Further evaluation of the immunogenicity and efficacy of this construct by the IM route is planned. Further evaluation of mucosal immunization with alternative carriers and formulations of mRNA merits consideration.

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Disclosures

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Supplementary Materials

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