

Title: SARS-CoV2 variant-specific replicating RNA vaccines protect from disease and pathology and reduce viral shedding following challenge with heterologous SARS-CoV2 variants of concern.

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Abstract. Despite mass public health efforts, the SARS-CoV2 pandemic continues as of late-2021 with resurgent case numbers in many parts of the world. The emergence of SARS-CoV2 variants of concern (VoC) and evidence that existing vaccines that were designed to protect from the original strains of SARS-CoV-2 may have reduced potency for protection from infection against these VoC is driving continued development of second generation vaccines that can protect against multiple VoC. In this report, we evaluated an alphavirus-based replicating RNA vaccine expressing Spike proteins from the original SARS-CoV-2 Alpha strain and recent VoCs delivered *in vivo* via a lipid inorganic nanoparticle.

Vaccination of both mice and Syrian Golden hamsters showed that vaccination induced potent neutralizing titers against each homologous VoC but reduced neutralization against heterologous challenges. Vaccinated hamsters challenged with homologous SARS-CoV2 variants exhibited complete protection from infection. In addition, vaccinated hamsters challenged with heterologous SARS-CoV-2 variants exhibited significantly reduced shedding of infectious virus. Our data demonstrate that this vaccine platform elicits significant protective immunity against SARS-CoV2 variants and supports continued development of this platform.

Introduction

Since the emergence of SARS-CoV2 in China in late 2019, over 200 million confirmed cases have been reported. The global public health burden caused by SARS-CoV2 and its resulting disease, Coronavirus Disease 2019 (COVID-19), has driven the rapid development of several promising vaccine candidates with a few currently being used for large scale vaccination efforts (Subbarao, 2021). However, the continued emergence of novel SARS-CoV2 variants of concern (VoC) and the diminished efficacy of current vaccines against infection by these VoC has raised concerns that novel SARS-CoV2 variants could evade current vaccine-mediated immunity (Subbarao, 2021). While leading mRNA vaccines provide protection from severe COVID-19 disease and hospitalization in individuals infected with VoCs (Lopez Bernal et al., 2021) , breakthrough infections, particularly in the upper airways of vaccinated individuals, are increasing and can lead to transmission events , even within clusters of vaccinated individuals (Brown CM et al., 2021). This highlights the need for continued vaccine development activities to improve protection from infection as well as disease to reduce transmission in the population. In addition, although current vaccine candidates are widely available in developed countries, access to vaccines in less developed countries is still lacking (Fontanet et al., 2021). Thus, development of new vaccine candidates capable of inducing broad and protective immunity and amenable to worldwide

distribution is still necessary to increase the availability of vaccines to address the pandemic and reduce the likelihood of vaccine-resistant strains of SARS-CoV2 emerging.

Previously, we reported the development of an Venezuelan Equine Encephalitis Virus (VEEV)-based replicating RNA (repRNA) vaccine encoding the spike protein of the original A.1 lineage of SARS-CoV2, delivered by a Lipid InOrganic Nanoparticle (LION) (Erasmus et al., 2020a) . We have since transitioned this technology through current good manufacturing practices (cGMP)-compliant production of both the repRNA and LION components and demonstrated safety and tolerability in a preclinical toxicology study leading to our open investigational new drug (IND) status under the drug product name, HDT-301, a B.1.351-specific vaccine that is pending phase I trials in US. Additionally, this technology is currently being evaluated in a Phase I clinical trial in India under the drug product designation HGC019 with pending filings in Brazil, South Korea, and China.

In this report we evaluated repRNA vaccine expressing the SARS-CoV2 spike protein of the SARS-CoV2 variants, B.1.1.7 and B.1.351 in mice and hamsters, using LION to mediate delivery via intramuscular injection. Vaccination of mice or hamsters with LION/repRNA vaccines expressing spikes of distinct VoCs induced potent neutralizing antibody responses against each virus after a prime-boost regimen.

Vaccinated hamsters challenged with homologous SARS-CoV2 strains were completely protected from infection. In addition, vaccinated hamsters challenged with heterologous viruses were significantly protected against viral shedding, viral replication, and exhibited little-to-no viral-induced pathology in the lung tissue. Cumulatively, our data provide direct evidence that a repRNA/LION vaccine can provide complete protection from exposure to homologous viruses and substantial protection from disease via accelerated clearance of virus when exposed to a new heterologous VoC. These data support further development of the LION/repRNA vaccine.

Materials and Methods

Biosafety and Ethics. All procedures with infectious SARS-CoV2 were conducted under high biocontainment conditions in accordance with established operating procedures approved by the Rocky Mountain Laboratories (RML) institutional biosafety committee (IBC). Sample inactivation followed IBC approved protocols (Haddock et al., 2021). Animal experiments were approved by the corresponding institutional animal care and use committee and performed by experienced personnel under veterinary oversight. Mice were group-housed, maintained in specific pathogen-free conditions, and entered experiments at 6-8 weeks of age. Hamsters were group-housed in HEPA-filtered cage systems and acclimatized to high containment conditions prior to start of SARS-CoV2 challenge. They were provided with nesting material and food and water ad libitum.

Viruses and cells. Viruses used for hamster challenge were as described previously (Hansen et al., 2021). For *in vitro* assays: Vero USAMRIID (a gift from Ralph Baric, UNC-Chapel Hill), VeroE6-TMPRSS2 (JCRB1819, JCRB Cell Bank, NIBIOHN), and Vero-hACE2-TMPRSS2 (a gift from Michael Diamond, Washington Univ) cells were cultured at 37C in DMEM supplemented with 10% FBS, and 100U/ml of penicillin-streptomycin. In addition, VeroE6-TMPRSS2 and Vero-hACE2-TMPRSS2 cells were cultured in the presence of 1mg/ml G418 and 10ug/ml puromycin, respectively. The SARS-CoV-2 Isolate hCoV-19/Germany/BavPat1/2020 (B.1)(NR-52370, BEI Resources), hCoV-19/England/204820464/2020 (B.1.1.7)(NR-54000, BEI Resources), and hCoV-19/USA/PHC658/2021 (B.1.617.2) (NR-55612, BEI Resources) were obtained from BEI Resources. Virus stocks were generated by expanding the virus in Vero-USAMRIID cells. Isolate 501Y.V2.HV001 (B.1.351) was obtained from Alex Sigal, AHRI (African Health Research Institute) and amplified in Vero-hACE2-TMPRSS2 cells upon reception. Virus stocks generated were tittered on VeroE6-TMPRSS2 cells. Viral RNAs were purified from the stock virus by using Quick-RNA viral kit (Zymo Research) and sent for RNA-seq for verification (University of Washington).

Vaccine constructs. Spike variants were constructed on the background of either the KV995PP substitution to stabilize the pre-fusion conformation of spike, or the native spike without prefusion stabilizing substitutions. The full-length spike open reading frame derived from the original Washington isolate was used as the reference A.1 lineage spike and the various deletions and substitutions that define the B.1, B.1.1.7, and B.1.351 lineages are depicted in Figure 1. All constructs were cloned by Gibson assembly of three overlapping fragments synthesized on a bioxp (Codex DNA) and codon optimized for human codon usage. Plasmids were then Sanger sequenced to confirm nucleotide identity and then linearized by NotI digestion prior to transcription and capping as described (Erasmus et al., 2020b).

Mouse studies. For mouse studies, 6-8 week old female C57BL/6 mice (Jackson laboratory) received 1μg of each vaccine, as outlined in Table 1, via intramuscular injections on days 0 and 28. Animals were then bled 2 weeks after the booster immunization and sera was evaluated for neutralizing antibody responses by plaque reduction neutralization test against A.1, B.1, B.1.1.7, and B.1.351 viruses.

Hamster studies. For hamster studies, 7 to 8-week-old male Syrian Golden hamsters were purchased from Envigo. Hamsters were randomly assigned to study groups and acclimatized for several days prior to vaccination. Hamsters were vaccinated with 20μg of indicated repRNA complexed to LION. RNA was diluted in water and LION diluted in 40% sucrose and 100mM sodium citrate to achieve a theoretical nitrogen:phosphate (N:P) ratio of 15. RNA and LION were allowed to complex for 30 minutes at 4°C. Hamsters were primed with a single 50μL intramuscular (IM) injection to the hind limb on day 0 and boosted four weeks later. Mock vaccinated hamsters received identical IM immunizations with saline. To monitor antibody responses to vaccination, blood was collected via retroorbital bleeds 25 days after prime vaccination and 14 and 21 days after boost vaccination. Hamsters were monitored daily for appetite, activity and weight loss and no adverse events were observed among the LION/repRNA vaccinated groups. Following the first vaccination, one mock vaccinated hamster developed a testicular

abscess and per veterinarian recommendation was euthanized. Data from this hamster was excluded from all analyses. For SARS-CoV2 challenge, hamsters were inoculated with 1000 TCID₅₀ indicated SARS-CoV2 variant via 50µL intranasal instillation. Following challenge, hamsters were weighed and monitored daily. Hamsters were orally swabbed on days 2 and 4 post-infection (PI). Swabs were placed in 1mL DMEM without additives. A scheduled necropsy at day 4 PI was performed on all animals to harvest blood and lung tissue. Studies were performed once.

Viral RNA quantification. Viral RNA from swabs was isolated using Qiaamp RNA mini kit (Qiagen) and viral RNA was isolated from tissues using RNEasy mini kit (Qiagen) according to provided protocols. Viral RNA was quantified by one-step qRT-PCR using QuantiFast Probe PCR reagents (Qiagen) and primers and probes specific for the SARS-CoV2 sub-genomic E RNA as previously described (Corman et al., 2020). For both assays, cycling conditions were as follows: initial hold of 50°C for 10min, initial denaturation of 95°C for 5min, and 40 cycles of 95°C for 15sec followed by 60°C 30sec. SARS-CoV2 RNA standards with known copy number were prepared in house, diluted, and run alongside samples for quantification. The limit of detection was based on the standard curve and defined as the quantity of RNA that would give a Ct value of 40.

Infectious virus titration. Infectious virus in swabs or tissues was quantified by tissue-culture infectious dose 50 assay (TCID₅₀) on Vero cells. Tissues were weighed and homogenized in 1mL DMEM supplemented with 2% FBS and penicillin and streptomycin. Homogenate was clarified of large debris by centrifugation. Samples were then serially 10-fold diluted in DMEM 2% FBS and applied to wells beginning with the 1:10 dilution in triplicate. Cells were incubated for six days before cytopathic effect (CPE) was read. TCID₅₀ was determined by the Reed and Muench method (Reed and Muench, 1938). The limit of detection was defined as at least two wells positive in the 1:10 dilution. To distinguish samples with no detectable infectious virus from those with single positive wells (<1 median TCID), Table 1 reports the fraction of samples from each group with any wells positive for CPE.

Plaque reduction neutralization tests (PRNTs). Two-fold serial dilutions of heat inactivated serum and 600 plaque-forming units (PFU)/ml solution of A.1, B.1, B.1.1.7, B.1.351, or B.1.617.2 viruses were mixed 1:1 in DMEM and incubated for 30 min at 37C. Serum/virus mixtures were added, along with virus only and mock controls, to Vero E6-TMPRSS2 cells (ATCC) in 12-well plates and incubated for 30 min at 37C. Following adsorption, plates were overlaid with a 0.2% agarose DMEM solution supplemented with Penicillin/Streptomycin (Fisher Scientific). Plates were then incubated for 2 days at 37C. Following incubation, 10% formaldehyde (Sigma-Aldrich) in DPBS was added to cells and incubated for 30 minutes at room temperature. Plates were then stained with 1% crystal violet (Sigma-Aldrich) in 20% EtOH (Fisher Scientific). Plaques were enumerated and percent neutralization was calculated relative to the virus-only control.

Histology and Immunohistochemistry. At time of necropsy, lungs were dissected and insufflated with 10% neutral buffered formalin and then submerged in 10% neutral buffered formalin for a minimum of 7 days with 2 changes. Tissues were placed in cassettes and processed with a Sakura VIP-6 Tissue Tek, on a 12-hour automated schedule, using a graded series of ethanol, xylene, and ParaPlast Extra. Prior to staining, embedded tissues were sectioned at 5 µm and dried overnight at 42°C. Using GenScript U864YFA140-4/CB2093 NP-1 (1:1000) specific anti-CoV immunoreactivity was detected using the Vector Laboratories ImPress VR anti-rabbit IgG polymer (# MP-6401) as secondary antibody. The tissues were then processed using the Discovery Ultra automated processor (Ventana Medical Systems) with a ChromoMap DAB kit Roche Tissue Diagnostics (#760-159). Sections were scored by certified pathologists who were blinded to study groups.

Statistical Analyses. Statistical analyses as described in the figure legends were performed using Prism 8.4.3 (GraphPad).

Results.

Variant-specific immunization provides distinct cross-neutralization profiles. We first evaluated whether the vaccination with constructs expressing the native, pre-fusion stabilized or furin cleavage site mutated version of the SARS-CoV-2 spike protein would elicit distinct humoral immune responses as it has been found that stabilization of the pre-fusion conformation may improve vaccine responses to coronavirus vaccines (Pallesen et al., 2017). We found that mice vaccinated with repRNA constructs expressing any of the spike protein versions generated similar ELISA and neutralization titers against the A.1 SARS-CoV-2 strain (Supplemental Figure 1). These results suggest that particularly in the context of genetic vaccines, modification of the spike protein may be unnecessary. Nevertheless, given the existing body of data on the clinical safety of vaccines expressing the pre-fusion stabilized spike protein, we chose to further evaluate repRNA constructs expressing pre-fusion stabilized spike proteins. Given the continued emergence of SARS-CoV2 VoC, effective vaccines against COVID-19 and/or SARS-CoV2 infection will need to provide broadly protective immune responses to existing and emergent SARS-CoV2 VoCs. In response to the ever-changing landscape of such variants, we constructed 3 repRNA vaccine candidates (**Figure 1**) based on the A.1, B.1, B.1.1.7 or B.1.351 lineage spike, all with the KV995PP pre-fusion stabilizing mutation. To evaluate homologous and heterologous neutralization following prime and boost vaccination, we immunized mice with LION/repRNA expressing the SARS-CoV2 spike from several VoC, including the native spikes of A.1 or B.1 lineages as well as the pre-fusion spikes of B.1, B.1.1.7, or B.1.351 lineage viruses. Mice were vaccinated with 1 μ g of RNA and boosted with the identical immunogens 28 days later. At fourteen days post-boost, serum samples were assayed

by 80% plaque reduction neutralization test (PRNT₈₀) against A.1, B.1, B.1.1.7, or B.1.351 viruses.

Native	600-A.1	sp	S1	S2	TM	CD
	675-B.1	sp	*D614G		TM	CD
Pre-fusion	604-A.1	sp	S1	S2 **KV995PP	TM	CD
	676-B.1	sp	*D614G		TM	CD
	673-B.1.1.7	sp	Δ69-70 ΔY144	*A570D *N501Y *D614G *P681H *T716I *S982A **KV995PP *D1118H	TM	CD
	671-B.1.351	sp	Δ242-244 *I18F *D80A *D215G *R246I *K417N *F484K *N501Y *D614G *A701V	**KV995PP	TM	CD

Figure 1: repRNA constructs evaluated in this study. Schematic representations of the SARS-CoV2 spike are shown with the signal peptide (sp), S1 and S2 domains, transmembrane domain (TM), and cytoplasmic domain (CD) indicated. The repRNAs are identified as 600 expressing the A.1 spike, 675 (B.1 spike), 604 (A.1. pre-fusion stabilized spike), 676 (B.1. pre-fusion stabilized spike), 673 (B.1.1.7 pre-fusion stabilized spike) and 671 (B.1.351 pre-fusion stabilized spike) with the mutations relative to the A.1 strain shown.

In A.1 spike vaccinated mice, we observed similar neutralization activity against B.1 challenge virus compared to homologous A.1 challenge, while we found a significant 4.4-fold and 14.7 fold drop in neutralization activity against B.1.1.7 and B.1.351 challenge virus, respectively (**Figure 2A**). In B.1.1.7 vaccinated mice we found a significant 11.3-fold drop in neutralization activity against the B.1.351 VoC compared to homologous B.1.1.7 challenge virus (**Figure 2B**). In B.1.351 vaccinated mice, we found a significant 12.3 fold and 4-fold drop in neutralization activity against the B.1.1.7 and A.1 challenge virus, respectively (**Figure 2C**). Cumulatively, our data show that the B.1.351 VoC was the most resistant to

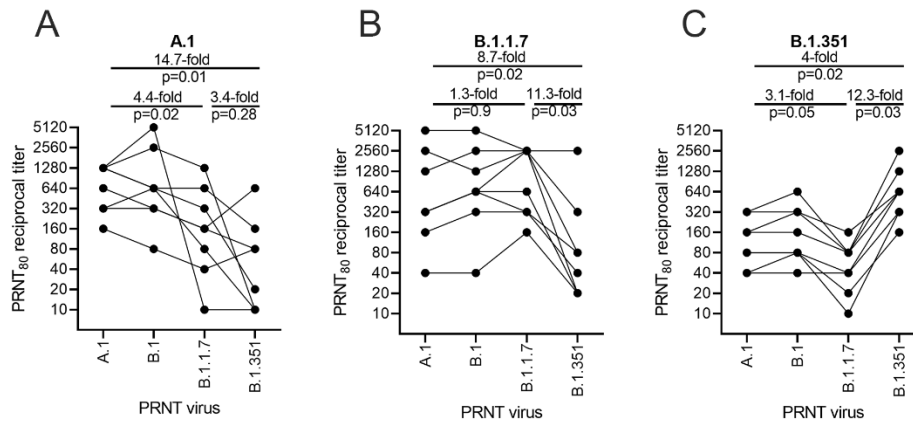


Figure 2: Relative neutralizing antibody responses induced by each vaccine candidate as measured against 4 variants of concern. C57BL/6 mice (n=8/group) received a 1ug intramuscular injection on days 0 and 28 of LION/repRNA encoding either the native conformation of spike derived from A.1, or B.1 viruses, or the prefusion-stabilized conformation of spike derived from B.1, B.1.1.7, or B.1.351 viruses. Mice were bled 14 days after the boost immunization and neutralizing antibody responses measured by 80% plaque reduction neutralization tests (PRNT₈₀) against A.1 (A), B.1.1.7 (B), or B.1.351 (C) viruses. Data in A-C are presented as geometric mean and geometric standard deviation with each individual sample data point overlaid and were analyzed by one-way ANOVA. P < 0.05 were considered statistically significant.

Vaccination induces homologous and heterologous neutralizing antibodies in hamsters. Given the

distinct nAb profiles of the B.1.1.7 and B.1.351, relative to each other, we next evaluated these two candidates, as well as a reference A.1 lineage spike, on the same pre-fusion-stabilized spike backbone, in the Syrian Golden hamster model of SARS-CoV2 infection. Hamsters were vaccinated with 20μg of LION/repRNA via IM injections and boosted with identical immunizations four weeks later (**Figure 3A**). To evaluate immunogenicity of LION/repRNA in hamsters, at day 14 post-boost, serum samples were assayed by 80% plaque reduction neutralization test (PRNT₈₀) against A.1, B.1.351, or B.1.1.7 viruses as well as the more recently described B.1.617.2 virus (Delta) variant. Against homologous virus, each vaccine candidate elicited robust neutralizing antibody (nAb) titers ranging from 1:320 to 1:10,240 with 2- to 14-fold reductions when measured against heterologous virus (**Figure 3B**). As observed in mice, the most “vaccine-resistant” VoC was the B.1.351 strain, where 13- to 14-fold reductions in nAb titers were observed in hamsters immunized with A.1- or B.1.1.7-specific vaccines, respectively (**Figure 3B**). The A.1

and B.1.1.7 strains of SARS-CoV2 were similarly neutralized by A.1 or B.1.1.7 vaccination exhibiting a roughly 2-fold reduction in neutralizing activity when comparing homologous to heterologous challenge, however, a ~4-5-fold reduction in nAb titer was observed against these viruses in hamsters receiving the B.1.351-specific vaccine (**Figure 3**), recapitulating observations in mice (Figure 2). Against the B.1.617.2 VoC, the A.1 and B.1.1.7 spike vaccinated hamsters had a 4- to 6-fold reduction in serum neutralization titer compared to homologous virus challenge while hamsters vaccinated with the B.1.351 spike had a

10-fold reduction in neutralization titer (**Figure 3B**).

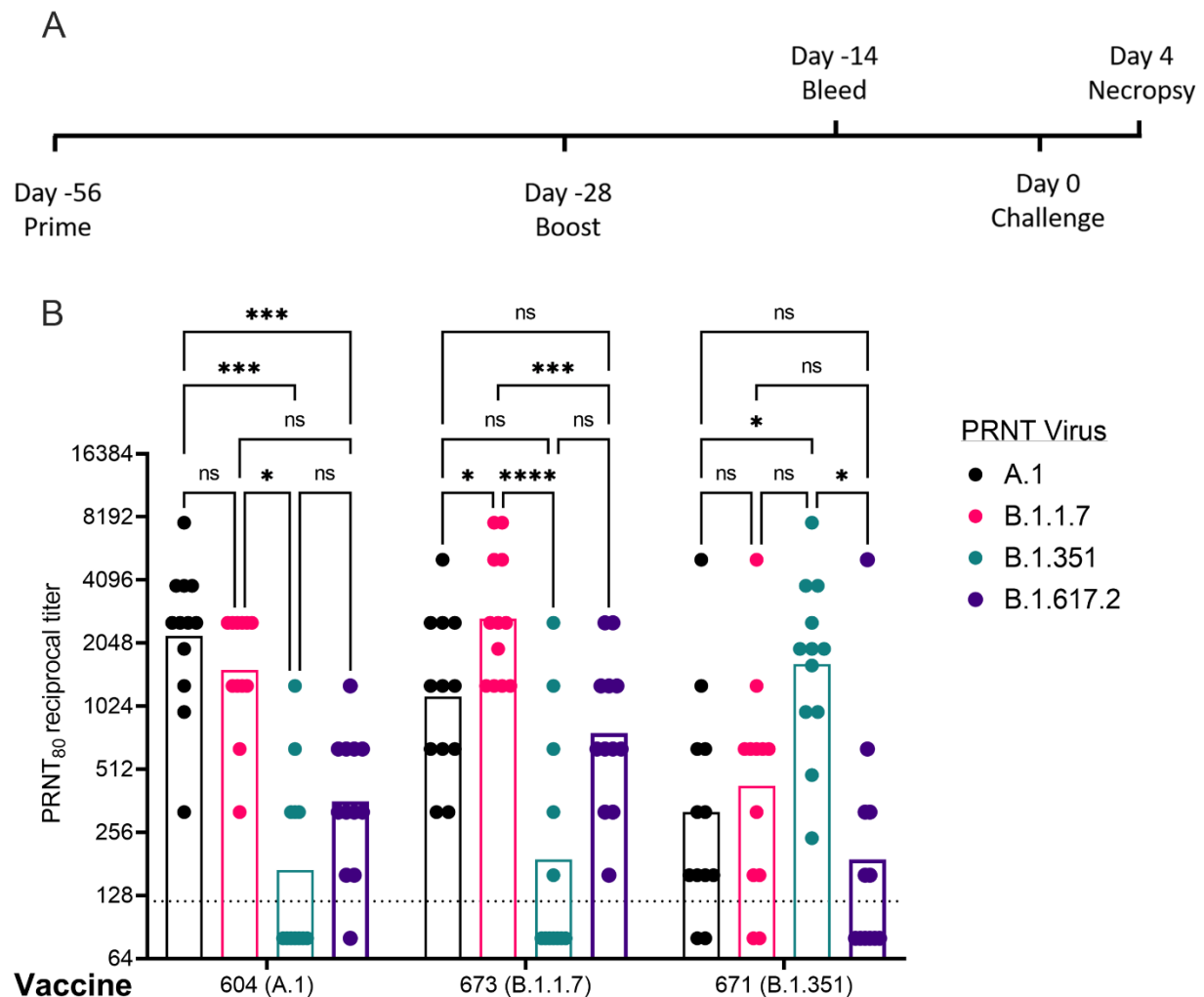


Figure 3: Post-boost neutralizing antibody responses in Syrian golden hamsters. Sera from animals immunized with LION complexed with repRNA vaccine variants A.1, B.1.1.7, or B.1.351 were incubated with live virus of variant A.1 (black), B.1.1.7 (pink), B.1.351 (green), or B.1.617.2 (purple) as indicated. Plaque-reduction neutralizing titers are indicated by individual symbols with geometric mean titers represented by the height of the bars. Indicated statistical comparisons performed using a two-way ANOVA with Tukey's multiple comparisons test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Vaccination significantly reduced viral shedding. To determine the protective benefit of the candidate vaccines, hamsters were challenged with 1000 TCID₅₀ of the A.1 strain or the B.1.351 or B.1.1.7 VoC four weeks after boosting (**Figure 3A**). Thus, all RNA vaccines would be evaluated against homologous and heterologous SARS-CoV2 challenge. For reference, the median infectious dose (ID₅₀) in hamsters for the A.1 strain is 5 TCID₅₀ (Rosenke et al., 2020). A significant criterion of an effective SARS-CoV2 vaccine is an

ability to reduce transmission of the virus from vaccinated individuals to susceptible persons by protecting the upper airway from infection. To evaluate whether vaccination could reduce viral shedding in the upper airway of SARS-CoV2 challenged hamsters oral swabs were collected on days 2 and 4 PI and viral loads quantified by qRT-PCR to measure sub-genomic RNA (Sg) indicative of active viral replication and infectious virus by TCID₅₀ assay. In addition, we also reported samples with any detectable infectious virus, even when <1 TCID₅₀ to distinguish animals with no infectious virus from those with low levels of infectious virus (Table 1).

Against the A.1 strain, only vaccination with the B.1.1.7 repRNA significantly reduced Sg RNA levels (**Figure 4A**). However, all three repRNAs significantly reduced shedding of infectious virus in the oral cavity at day 2 PI and both the A.1 and B.1.1.7 repRNAs significantly reduced infectious virus at day 4 PI (**Figure 4B**). Notably, no infectious virus could be isolated from the swabs of B.1.17 repRNA-vaccinated animals at day 4 PI (Table 1), indicating rapid control of viral shedding. These data indicate that against the SARS-CoV2 A.1 strain, vaccination with any of the three repRNAs can significantly reduce viral shedding.

Against the B.1.351 variant, compared to mock vaccinated hamsters at day 2 PI, hamsters vaccinated with the homologous B.1.351 repRNA had significantly reduced Sg RNA (**Figure 4C**) and by day 4 PI, significantly reduced viral RNA was seen in all vaccinated hamsters (**Figure 4C**). Similar to the A.1 challenge, against the B.1.351 variant challenge, vaccination with any of the repRNAs significantly reduced infectious virus at both day 2 and 4 PI (**Figure 4D**). Notably, at day 2 PI, infectious virus could only be isolated from the oral cavity of 2 of 6 hamsters vaccinated with the B.1.351 repRNA, while virus was isolated from 6 of 6 vaccinated with the A.1 or 3 of 6 vaccinated with the B.1.1.7 vaccine (Table 1), suggesting more rapid clearance of virus in B.1.351-infected hamsters vaccinated with B.1.351 repRNA.

Finally, against the B.1.1.7 variant, the B.1.351 and B.1.1.7 vaccine repRNAs significantly reduced Sg RNA at day 2 PI, and at day 4 PI both the A.1 and B.1.351 repRNAs significantly reduced Sg RNA (**Figure 4E**). Similar to A.1 or B.1.351 variant challenges, vaccination with any of the repRNAs lead to significantly reduced titers of infectious virus in oral swabs at both day 2 and 4 PI (**Figure 4F**), and more rapid clearance of virus was observed in vaccine-matched B.1.1.7 vaccinated/challenged animals, with live virus isolated in oral cavities only 1 of 6 B.1.1.7-vaccinated hamsters. In contrast, live virus could be isolated in 6 of 6 A.1- or 2 of 6 B.1.351-vaccinated hamsters challenged with the B.1.1.7 VoC at day 2 PI (Table 1). Overall, across all three SARS-CoV2 challenges, 19 of 54 and 32 of 54 vaccinated hamsters had no detectable infectious virus in their swabs at day 2 and 4 PI, respectively (Table 1). Cumulatively these data demonstrate that vaccination with any of the three repRNAs significantly reduced viral shedding against homologous and heterologous SARS-CoV2 challenge with more rapid clearance observed in

vaccine-matched VoC challenges.

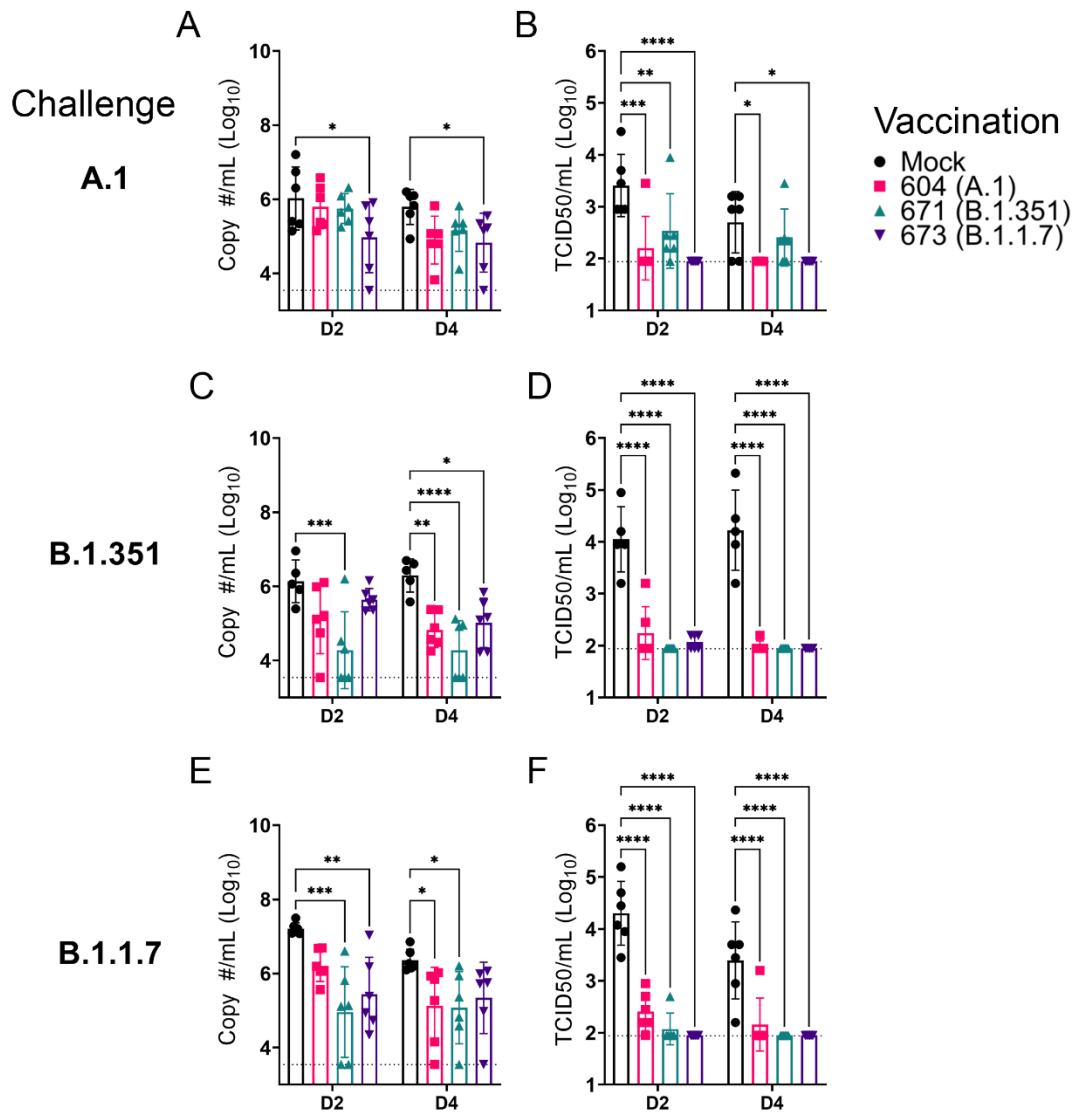


Figure 4: repRNA vaccination significantly reduces viral shedding. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 2 or 4 PI, oral swabs were collected. SARS-CoV2 in the swabs was quantified by qRT-PCR specific for the SgE RNA (A, C, E) or infectious virus by TCID₅₀ assay (B, D, F). N = 5 (mock-vaccinated, B.1.351 challenge) or 6 (all other groups). A two-way ANOVA with Dunnett's multiple comparison test against mock-vaccinated hamsters was performed. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Comparisons without indicated P-values were considered non-significant (P > 0.05).

Vaccination significantly reduced viral burden in lung tissue. As the hamster model of SARS-CoV2

infection is not lethal, we performed a timed necropsy on day 4 PI to evaluate viral burdens in the lungs

of infected hamsters via qRT-PCR and measured infectious virus in the lungs by TCID₅₀. All three vaccine

repRNAs significantly reduced Sg viral RNA in the lungs of hamsters challenged with any of the three variants of SARS-CoV2 (**Figure 5A, C, E**). Notably, the majority of vaccinated animals across the three SARS-CoV2 variant challenges had no detectable Sg RNA, suggestive of substantial immunity in the lungs of these animals. We also quantified the amount of infectious virus in the lungs and found that all three RNA vaccines significantly reduced infectious virus in animals challenged with any of the three SARS-CoV2 variants (**Figure 5C, F, I**). Against infection with the B.1.351 and B.1.1.7 variants, no infectious virus was detected in the lungs of any vaccinated hamster (**Figure 5C, F, I and Table 1**). Against the A.1 strain, infectious virus was only found in the lungs of 1 of 6 animals in the A.1 and B.1.351 repRNA vaccinated animals and 0 of 6 animals in the B.1.1.7 repRNA vaccinated animals (Table 1). Cumulatively, by highly sensitive qRT-PCR for Sg RNA and by measurement of infectious virus, vaccination with any of the repRNAs resulted in significant reduction of SARS-CoV2 burden in the lungs indicating that the

LION/repRNA vaccine platform is highly protective against SARS-CoV2 infection in the lung.

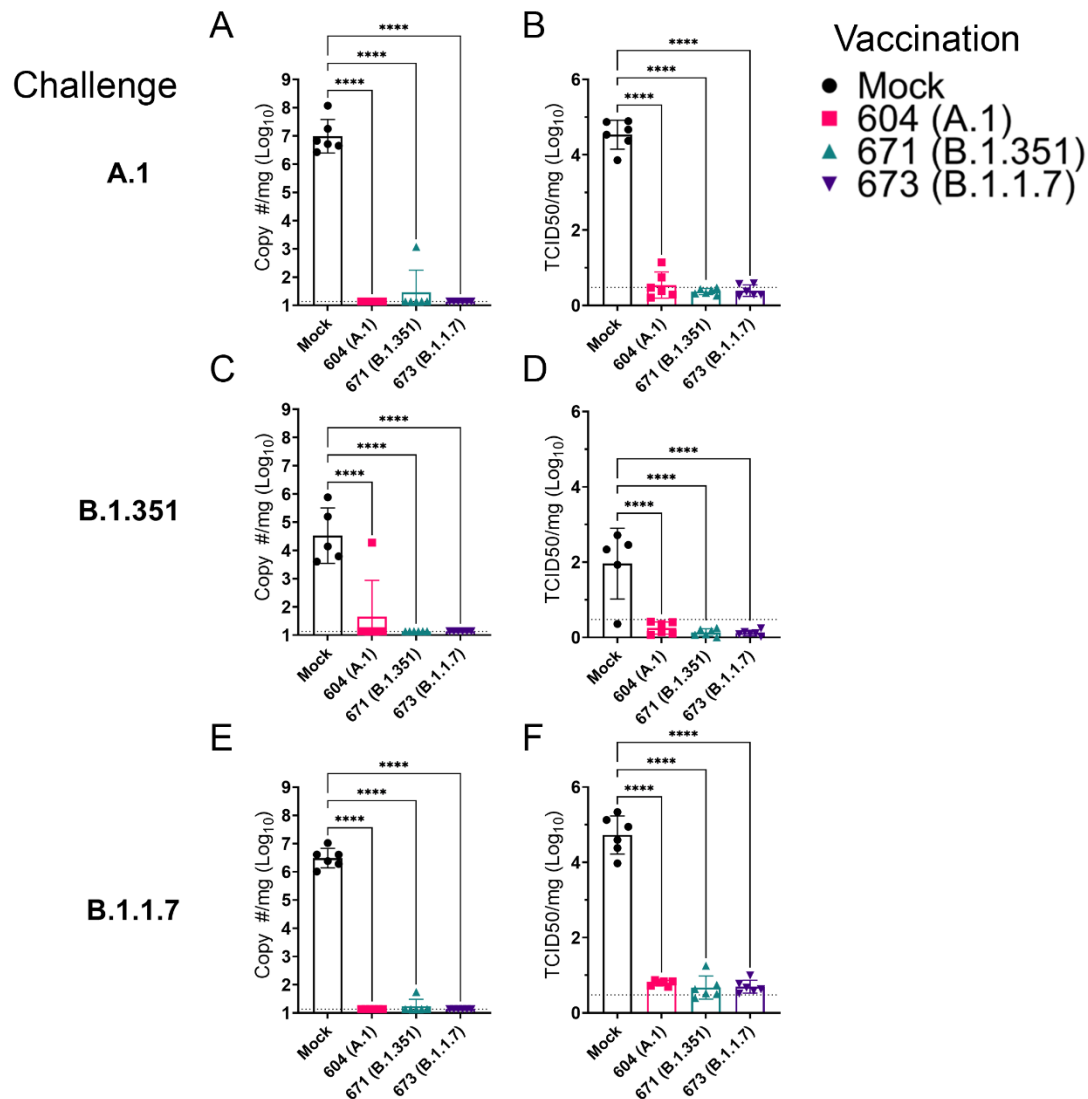


Figure 5: repRNA vaccination significantly reduces viral burden in the lungs. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized, and lung tissue collected. SARS-CoV2 burden in the lung was quantified by qRT-PCR specific for the SgE RNA (A, C, E). Infectious virus in the lungs was quantified by TCID₅₀ assay (B, D, F). Indicated statistical comparisons performed using a One-way ANOVA with Dunnett's multiple comparison test against mock-vaccinated hamsters. * P < 0.05, **** P < 0.0001. Comparisons without indicated P-values were non-significant (P > 0.05)

Vaccination protected against lung pathology.

We further evaluated vaccine efficacy by evaluating lung pathology by H&E staining, IHC for viral antigen, and by lung-to-body weight ratio (**Figure 6**). H&E staining of lung sections demonstrated that

among mock vaccinated hamsters challenged with A.1 or B.1.1.7 strains of SARS-CoV2 developed lesions typical of SARS-CoV2 with the A.1 and B.1.1.7 variant infected hamsters developing more, and more severe, lesions than those infected with the B.1.351 variant. The lesions were multifocal and consisted of inflammatory cells, mostly viable and degenerate neutrophils, filling alveoli and alveolar septa were thickened by edema, fibrin, alveolar and septal macrophages and variable amounts of type II pneumocyte hyperplasia. Bronchiolitis was present in most hamsters as was vasculitis in, or adjacent to, affected areas (**Figure 6**). In contrast, hamsters vaccinated with any of the repRNAs were protected from lung pathology and had diminished viral antigen (**Figure 6**). The majority of hamsters had no evident lesions and no detectable viral antigen and in the few that had lesions or IHC reactivity, they were minor

(Figure 6). The complete histological and IHC findings are provided in supplemental table 1.

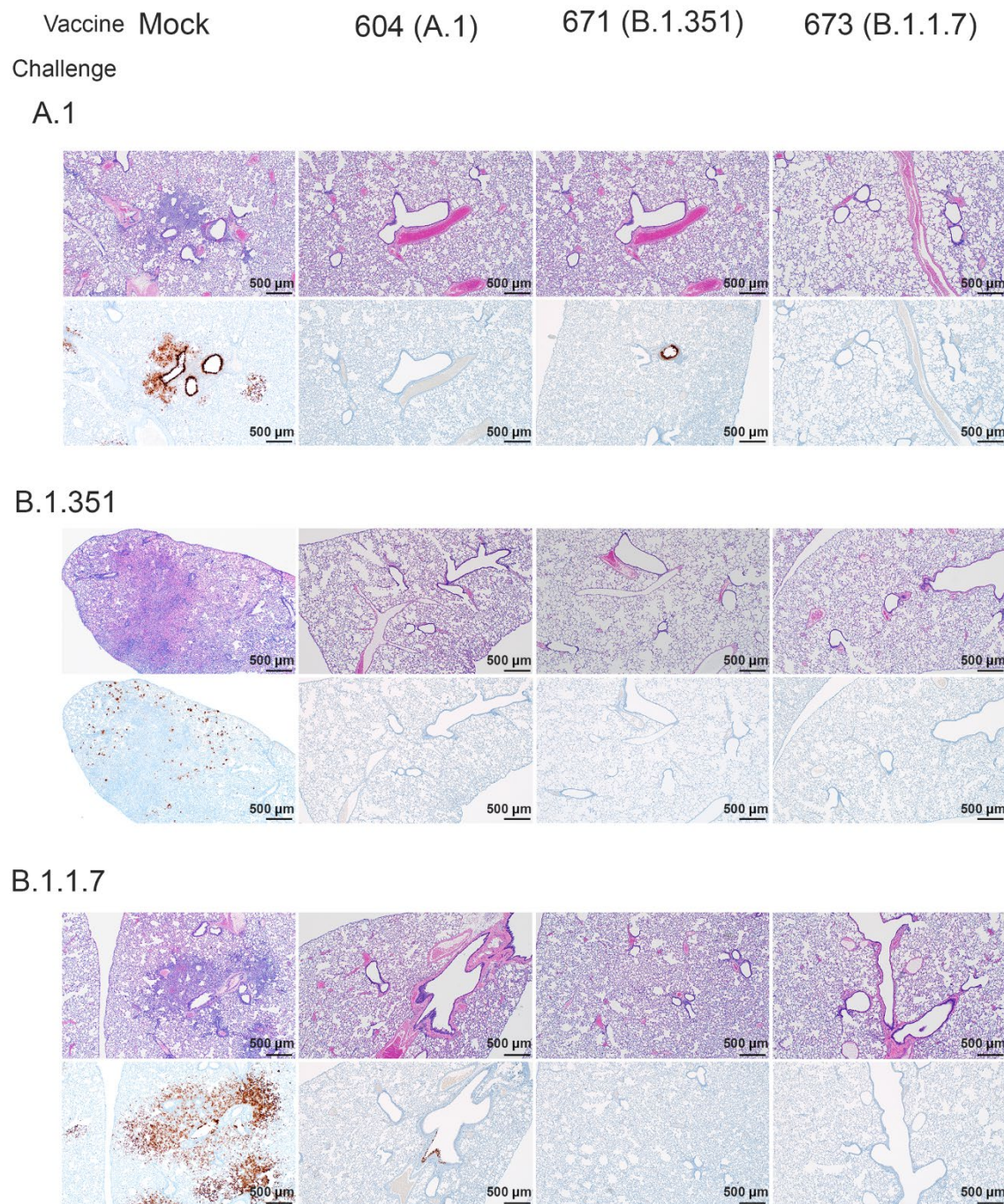


Figure 6: repRNA vaccination reduces lung pathology and SARS-CoV2 antigen burden in lung tissue. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized and lungs formalin-fixed and paraffin-embedded. Sections were stained with H&E (top-row of each challenge) or for SARS-CoV2 viral antigen (bottom-row of each challenge). Representative images of each group are shown.

To quantify lung pathology, we measured lung-to-body weight ratio, expressed as % of body weight and also had the H&E and IHC sections scored by a pathologist blinded to study groups. Hamsters vaccinated with any of the three repRNAs and challenged with either the A.1 or B.1.1.7 variant had significantly reduced lung-to-body weight ratios compared to similarly challenged mock-vaccinated hamsters (**Figure 7A and C**). Against the B.1.351 variant, only hamsters vaccinated with the A.1 repRNA had significantly reduced lung-to-body weight ratio compared to mock-vaccinated hamsters (**Figure 7B**). Cumulative pathology scores of H&E stained (**Figure 7D – F**) or lung sections stained for viral antigen (**Figure 7G – I**) showed that all vaccines conferred significant protection against lung pathology and significantly reduced viral antigen induced by any of the SARS-CoV2 challenge strains. The table summarizing the complete histological and IHC findings are provided in supplemental table 1. Overall mock-vaccinated B.1.351 challenged hamsters had reduced viral loads (**Figure 5**) and reduced evidence of virally induced pathology (**Figure 7B, E, H**) than mock-vaccinated hamsters challenged with either the A.1 or B.1.1.7 VoC

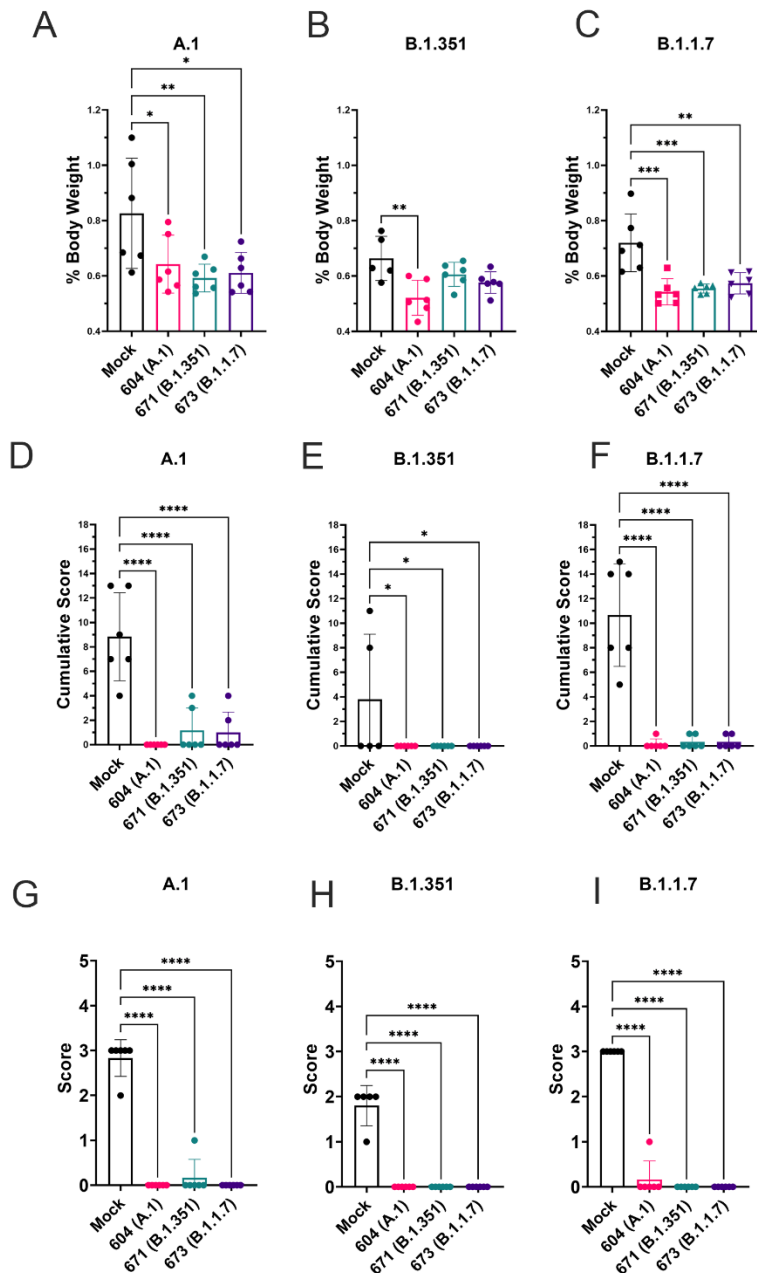


Figure 7: repRNA vaccination significantly protects against lung pathology in hamsters. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized, lung weighed and lungs formalin-fixed and paraffin-embedded. Lung weights as percentage of body weight are reported (A – C). Lung sections were stained with H&E (D – F) or for SARS-CoV2 viral antigen (G – I). Sections were scored by a pathologist blind to study groups and assigned a score for percent area affected by SARS-CoV2 lesions and cumulative score presented (A – C) or presence of viral antigen (D – F). Indicated statistical comparisons performed using a one-way ANOVA with Dunnett's multiple comparison test against mock-vaccinated hamsters. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Discussion.

Despite the availability of several vaccines for SARS-CoV2 in the majority of the developed world, as of mid-2021, the COVID-19 pandemic continues with resurgent case numbers in multiple countries despite widespread vaccine deployment (WHO, 2021). The emergence of the delta VoC and emerging evidence that existing vaccines have diminished efficacy against the delta VoC compared to previous VoC (Lopez Bernal et al., 2021; Sheikh et al., 2021) along with breakthrough infections among fully vaccinated individuals resulting in similar viral loads to unvaccinated individuals (Brown CM et al., 2021) together indicate that continued development of vaccines for COVID-19 is warranted.

Several COVID-19 alphavirus-based replicon vaccines have been evaluated in animal models. In mice vaccinated with a lipid nanoparticle delivered VEEV repRNA expressing the pre-fusion stabilized form of the original Wuhan SARS-CoV2 isolate, mice developed potent neutralizing antibody responses against infectious SARS-CoV2 (McKay et al., 2020). Similarly, a DNA-launched Semliki-forest virus replicon expressing the Wuhan SARS-CoV2 isolate spike induced humoral and cellular immunity against SARS-CoV2 in mice (Szurgot et al., 2021). We have previously evaluated the LION/repRNA vaccine expressing the native A.1 spike in mice and pigtail macaques and found that vaccination induced potent neutralizing antibody activity (Erasmus et al., 2020a). Challenge studies showed vaccinated pigtail macaques were protected against SARS-CoV2 challenge [manuscript in preparation]. However, these studies did not evaluate the ability of these vaccines to protect against emerging VoCs and we therefore sought to determine the ability of LION/repRNA to confer broad protection against heterologous SARS-CoV2 challenge.

In both mice and hamsters, we found distinct neutralizing responses when comparing homologous to heterologous infectious virus. Relative to homologous vaccination and challenge virus, the greatest decrease in neutralization activity was seen when mice or hamsters were vaccinated with A.1 or B.1.1.7

repRNAs and nAbs measured against B.1.351 virus or when animals were vaccinated with B.1.351 repRNA and nAbs measured against B.1.1.7 virus. Overall, however, these changes were not significant, supporting the conclusion that while pre-fusion stabilized spike confers advantages in the context of recombinant proteins, those advantages are not particularly obvious within the context of gene-encoded approaches, as demonstrated by others (Kalnin et al., 2021; Mercado et al., 2020; Yu et al., 2020). Similar observations were made in mice receiving native, pre-fusion-stabilized, furin cleavage site-deleted, or a combination of pre-fusion- and furin cleavage site-mutants expressed from repRNA, where no significant differences in neutralizing antibody titers were observed between groups. In hamsters vaccinated with A.1 or B.1.1.7 repRNA, a >8-fold decrease in neutralization activity against the B.1.351 strain was found compared to hamsters vaccinated with the B.1.351 repRNA. These findings are consistent with previous studies showing reduced neutralization of convalescent and A.1 vaccinated sera against the B.1.351 and B.1.1.7 variants (Shen et al., 2021a; Shen et al., 2021b; Wang et al., 2021; Zhou et al., 2021) and provides new insights into the design of VoC-specific vaccines and their ability to provide cross-neutralizing immunity. Nevertheless, although we observed reduced neutralization activity against these heterologous variants, hamsters vaccinated with any of the three repRNAs were still significantly protected against viral replication in the lungs and lung pathology following heterologous SARS-CoV2 challenge. We also found significant reduction of viral shedding in the upper airway of even heterologous challenged hamsters, indicating that all vaccines induced immunity above the threshold likely necessary for protection from disease in hamsters.

Given the recent and alarmingly rapid spread of the B.1.617.2 (Delta) VoC, we also evaluated the ability of sera from these vaccinated hamsters to cross-neutralize B.1.617.2 and compare vaccine resistance to other VoCs. Similar to recent reports (Edara et al., 2021; Planas et al., 2021), the B.1.617.2 VoC exhibited a 6-fold reduction in nAb titers in A.1 vaccinated hamsters, and B.1.351 VoC still remained the most resistant to A.1 vaccination. Interestingly, B.1.1.7 vaccination induced robust cross-neutralization of

B.1.617.2 with only a 3.5-fold reduction in nAb titers relative to homologous neutralization. Vaccination with B.1.351, however, did not provide very robust cross-neutralization of B.1.617.2, with a 10-fold reduction in nAb titers relative to homologous neutralization, suggesting that those previously infected with B.1.351 may remain susceptible to re-infection with the B.1.617.2 VoC.

While protection from SARS-CoV2 infection in the lower respiratory tract is likely important for prevention of severe disease and death, reduction of shedding of virus from the upper respiratory tract is likely key for interruption of SARS-CoV2 transmission. Indeed, a recent study demonstrated that despite sterilizing protection in the lower airway of vaccinated hamsters challenged with homologous virus, transmission was still observed upon co-housing with naïve animals (Wu et al., 2021). And while a significant reduction in upper airway viral load, as measured by Sg RNA qPCR, was observed in these vaccinated animals, an attempt to isolate infectious virus was not reported. Our data showed that VoC-matched vaccination (i.e. B.1.1.7- or B.1.351-repRNA) induced more rapid clearance of homologous virus with sterilizing protection in the oral cavity observed for majority of animals at the day 2 PI oral swab. And while we and others have demonstrated sterilizing protection in the lower airway of animals, protection from upper respiratory infection appears to be a higher bar as a measure of vaccine efficacy as several vaccine studies in animal models have shown reduced control of SARS-CoV2 replication in the upper respiratory tract. In ChAdOx1 nCoV-19 vaccinated hamsters, vaccination reduced shedding of SARS-CoV2 in B.1.1.7 challenged but not B.1.351 challenged hamsters, despite sterile protection in the lungs (Fischer et al., 2021). In ChAdOx1 nCov-19 vaccinated rhesus macaques, no differences in SARS-CoV2 RNA loads in nasal swabs between vaccinated or unvaccinated animals were observed despite significantly reduced viral loads in the lungs (van Doremalen et al., 2020). Intranasal delivery of the ChAd nCoV-19 vaccine may improve control of viral shedding as intranasal but not IM vaccination resulted in significantly reduced viral shedding (Hassan et al., 2021; van Doremalen et al., 2021). In contrast, rhesus macaques vaccinated with mRNA-1273 showed significantly reduced viral RNA in both bronchoalveolar-

lavage (BAL) and nasal swabs (Corbett et al., 2020). Similarly, rhesus macaques vaccinated with Ad26 had significantly reduced upper and lower respiratory titers although across vaccine candidates evaluated, more robust protection was observed in the BAL than nasal swabs (Mercado et al., 2020). Of the vaccine efficacy studies have been conducted in hamsters (Brocato et al., 2021; Fischer et al., 2021; Hörner et al., 2020; Meyer et al., 2021; Mohandas et al., 2021; Rauch et al., 2021; van der Lubbe et al., 2021; Wu et al., 2021; Yinda et al., 2021; Zhang et al., 2021), those that evaluated infectious virus in upper airways, including unmodified mRNA (Rauch et al., 2021), measles-vectored (Hörner et al., 2020), and Ad26-vectored (van der Lubbe et al., 2021) approaches, were unable to induce sterilizing protection in the upper airway of a majority of animals while an inactivated rabies virus-vectored approach was able to do so (Kurup et al., 2021). An important consideration of the above referenced studies is that these were within the context of A.1 vaccination and matched A.1 viral challenge therefore, continued evaluation of deployed vaccines and those under development against emergent VoCs is needed.

Together our data demonstrate that LION/repRNA vaccines can induce broadly protective immunity against multiple SARS-CoV2 strains. Vaccination significantly reduced SARS-CoV2 viral burden in both the upper and lower respiratory tract and prevented development of SARS-CoV2 lung pathology even when challenged with heterologous SARS-CoV2 strains. Additionally, given the distinct heterologous neutralizing antibody responses elicited by B.1.1.7- or B.1.351-specific repRNA, coupled with the observation that matched vaccination provides superior protection in the upper airway against homologous VoC challenge, a bivalent mixture consisting of these two VoC-specific vaccines may provide superior cross-neutralizing capacity than each monovalent vaccine alone and decrease the frequency of vaccine breakthrough-mediated transmission events. Our data cumulatively support continued development of the LION/repRNA vaccine platform for COVID-19.

Table 1: Fraction of hamster samples with any detectable infectious virus

Challenge	RNA	Oral swabs		Lung tissue
		D2	D4	D4
A.1	Mock	6/6	6/6	6/6
	A.1 (604)	6/6	2/6	1/6
	B.1.351 (671)	6/6	6/6	1/6
	B.1.17 (673)	3/6	0/6	0/6
B.1.351	Mock	5/5	5/5	5/5
	A.1 (604)	6/6	2/6	0/6
	B.1.351 (671)	2/6	2/6	0/6
	B.1.17 (673)	3/6	2/6	0/6
B.1.1.7	Mock	6/6	6/6	6/6
	A.1 (604)	6/6	2/6	0/6
	B.1.351 (671)	2/6	3/6	0/6
	B.1.17 (673)	1/6	3/6	0/6

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Conflicts of Interest J.H.E., A.P.K., J.A., P.B., D.H.F, and M.G. have equity interest in HDT Bio. J.H.E. is a consultant for InBios. P.B. is a consultant for Arcturus, Sensei, and Next Phase. D.C. is on the scientific advisory board of Genemod and Compliment Corp. D.H.F. is a consultant for Gerson Lehrman Group, Orance, Abacus Bioscience, Neoleukin Therapeutics. J.H.E., A.P.K., and D.C. are co-inventors on U.S. patent application no. 62/993,307 “Compositions and methods for delivery of RNA” pertaining to the LION formulation. All other authors declare that they have no competing interests.

Figure Legends

Figure 1: repRNA constructs evaluated in this study. Schematic representations of the SARS-CoV2 spike are shown with the signal peptide (sp), S1 and S2 domains, transmembrane domain (TM), and cytoplasmic domain (CD) indicated. The repRNAs are identified as 600 expressing the A.1 spike, 675 (B.1 spike), 604 (A.1. pre-fusion stabilized spike), 676 (B.1. pre-fusion stabilized spike), 673 (B.1.1.7 pre-fusion stabilized spike) and 671 (B.1.351 pre-fusion stabilized spike) with the mutations relative to the A.1 strain shown.

Figure 2: Relative neutralizing antibody responses induced by each vaccine candidate as measured against 4 variants of concern. C57BL/6 mice (n=8/group) received a 1ug intramuscular injection on days 0 and 28 of LION/repRNA encoding either the native conformation of spike derived from A.1, or B.1 viruses, or the prefusion-stabilized conformation of spike derived from B.1, B.1.1.7, or B.1.351 viruses. Mice were bled 14 days after the boost immunization and neutralizing antibody responses measured by 80% plaque reduction neutralization tests (PRNT₈₀) against A.1 (A), B.1.1.7 (B), or B.1.351 (C) viruses. Data in A-C are presented as geometric mean and geometric standard deviation with each individual sample data point overlaid and were analyzed by one-way ANOVA. $P < 0.05$ were considered statistically significant.

Figure 3: Post-boost neutralizing antibody responses in Syrian golden hamsters. Sera from animals immunized with LION complexed with repRNA vaccine variants A.1, B.1.1.7, or B.1.351 were incubated with live virus of variant A.1 (black), B.1.1.7 (pink), B.1.351 (green), or B.1.617.2 (purple) as indicated. Plaque-reduction neutralizing titers are indicated by individual symbols with geometric mean titers represented by the height of the bars. Indicated statistical comparisons performed using a two-way ANOVA with Tukey's multiple comparisons test. ns $P > 0.05$, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4: repRNA vaccination significantly reduces viral shedding. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 2 or 4 PI, oral swabs were collected. SARS-CoV2 in the swabs was quantified by qRT-PCR specific for the SgE RNA (A, C, E) or infectious virus by TCID₅₀ assay (B, D, F). N = 5 (mock-vaccinated, B.1.351 challenge) or 6 (all other groups). A two-way ANOVA with Dunnett's multiple comparison test against mock-vaccinated hamsters was performed. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Comparisons without indicated P-values were considered non-significant (P > 0.05).

Figure 5: repRNA vaccination significantly reduces viral burden in the lungs. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized, and lung tissue collected. SARS-CoV2 burden in the lung was quantified by qRT-PCR specific for the SgE RNA (A, C, E). Infectious virus in the lungs was quantified by TCID₅₀ assay (B, D, F). Indicated statistical comparisons performed using a One-way ANOVA with Dunnett's multiple comparison test against mock-vaccinated hamsters. * P < 0.05, **** P < 0.0001. Comparisons without indicated P-values were non-significant (P > 0.05)

Figure 6: repRNA vaccination reduces lung pathology and SARS-CoV2 antigen burden in lung tissue. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized and lungs formalin-fixed and paraffin-embedded. Sections were stained with H&E (top-row of each challenge) or for SARS-CoV2 viral antigen (bottom-row of each challenge). Representative images of each group are shown.

Figure 7: repRNA vaccination significantly protects against lung pathology in hamsters. Mock or repRNA vaccinated hamsters were challenged with 1000 TCID₅₀ of the indicated SARS-CoV2 strains via the IN route. At day 4 PI, hamsters were euthanized, lung weighed and lungs formalin-fixed and paraffin-embedded. Lung weights as percentage of body weight are reported (A – C). Lung sections were stained

with H&E (D – F) or for SARS-CoV2 viral antigen (G – I). Sections were scored by a pathologist blind to study groups and assigned a score for percent area affected by SARS-CoV2 lesions and cumulative score presented (A – C) or presence of viral antigen (D – F). Indicated statistical comparisons performed using a one-way ANOVA with Dunnett’s multiple comparison test against mock-vaccinated hamsters. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Comparisons without indicated P-values were considered non-significant ($P > 0.05$).

Supplemental Figure 1. Effect of spike modifications on binding and neutralizing antibody responses in mice. C57BL/6 mice (n=5/group) were immunized with LION-formulated repRNA encoding the wild-type (WT), the prefusion-stabilized (PreF), the furin cleavage site-deleted (Fur_{mut}), or a combination of the PreF and Fur_{mut} modifications of the full-length spike of A.1 lineage SARS-CoV2. Vaccinations were administered on days 0 and 28, and serum collected on days 14, 28, and 38 assayed for (A) anti-spike binding IgG by enzyme linked immunosorbent assay and serum collected on days 28 and 38 assayed for (B) neutralizing antibody responses by 80% plaque reduction neutralization assay against A.1 SARS-CoV2 (WA-1 isolate).

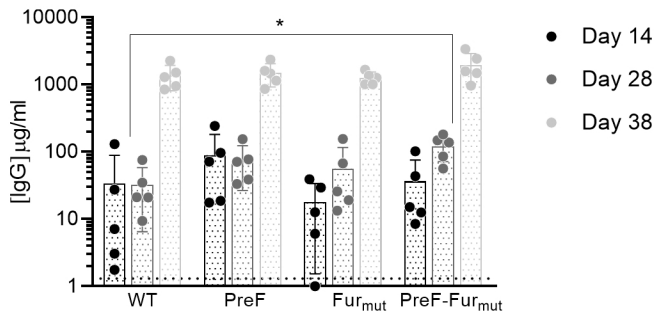
References

- Brocato, R.L., Kwilas, S.A., Kim, R.K., Zeng, X., Principe, L.M., Smith, J.M., and Hooper, J.W. (2021). Protective efficacy of a SARS-CoV-2 DNA vaccine in wild-type and immunosuppressed Syrian hamsters. *npj Vaccines* 6, 16.
- Brown CM, J, V., and H, J. (2021). Outbreak of SARS-CoV-2 Infections, Including COVID-19 Vaccine Breakthrough Infections, Associated with Large Public Gatherings — Barnstable County, Massachusetts, July 2021. *MMWR Morb Mortal Wkly Rep* 2021 0:1059-1062.
- Corbett, K.S., Flynn, B., Foulds, K.E., Francica, J.R., Boyoglu-Barnum, S., Werner, A.P., Flach, B., O'Connell, S., Bock, K.W., Minai, M., *et al.* (2020). Evaluation of the mRNA-1273 Vaccine against SARS-CoV-2 in Nonhuman Primates. *New England Journal of Medicine* 383, 1544-1555.
- Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., *et al.* (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* 25, 2000045.
- Edara, V.-V., Pinsky, B.A., Suthar, M.S., Lai, L., Davis-Gardner, M.E., Floyd, K., Flowers, M.W., Wrammert, J., Hussaini, L., Ciric, C.R., *et al.* (2021). Infection and Vaccine-Induced Neutralizing-Antibody Responses to the SARS-CoV-2 B.1.617 Variants. *New England Journal of Medicine* 385, 664-666.
- Erasmus, J.H., Khandhar, A.P., O'Connor, M.A., Walls, A.C., Hemann, E.A., Murapa, P., Archer, J., Leventhal, S., Fuller, J.T., Lewis, T.B., *et al.* (2020a). An Alphavirus-derived replicon RNA vaccine induces SARS-CoV-2 neutralizing antibody and T cell responses in mice and nonhuman primates. *Science translational medicine* 12, eabc9396.
- Erasmus, J.H., Khandhar, A.P., Walls, A.C., Hemann, E.A., O'Connor, M.A., Murapa, P., Archer, J., Leventhal, S., Fuller, J., Lewis, T., *et al.* (2020b). Single-dose replicating RNA vaccine induces neutralizing antibodies against SARS-CoV-2 in nonhuman primates. *bioRxiv : the preprint server for biology*.
- Fischer, R.J., van Doremalen, N., Adney, D.R., Yinda, C.K., Port, J.R., Holbrook, M.G., Schulz, J.E., Williamson, B.N., Thomas, T., Barbican, K., *et al.* (2021). ChAdOx1 nCoV-19 (AZD1222) protects hamsters against SARS-CoV-2 B.1.351 and B.1.1.7 disease. *bioRxiv : the preprint server for biology*, 2021.2003.2011.435000.
- Fontanet, A., Autran, B., Lina, B., Kieny, M.P., Karim, S.S.A., and Sridhar, D. (2021). SARS-CoV-2 variants and ending the COVID-19 pandemic. *Lancet (London, England)* 397, 952-954.
- Hansen, F., Meade-White, K., Clancy, C., Okumura, A., Hawman, D., Friederike, F., Kaza, B., Jarvis, M., Rosenke, K., and Feldmann, H. (2021). Prior SARS-CoV-2 infection prevents acute disease and lung pathology in reinfecting Syrian hamsters but not virus replication in the upper respiratory tract. *Cell Press Sneak Peak*.
- Hassan, A.O., Feldmann, F., Zhao, H., Curiel, D.T., Okumura, A., Tang-Huau, T.-L., Case, J.B., Meade-White, K., Callison, J., Chen, R.E., *et al.* (2021). A single intranasal dose of chimpanzee adenovirus-vectored vaccine protects against SARS-CoV-2 infection in rhesus macaques. *Cell Rep Med* 2, 100230-100230.
- Hörner, C., Schürmann, C., Auste, A., Ebenig, A., Muraleedharan, S., Dinnon, K.H., Scholz, T., Herrmann, M., Schnierle, B.S., Baric, R.S., *et al.* (2020). A highly immunogenic and effective measles virus-based Th1-biased COVID-19 vaccine. *Proceedings of the National Academy of Sciences* 117, 32657-32666.
- Kalnin, K.V., Plitnik, T., Kishko, M., Zhang, J., Zhang, D., Beauvais, A., Anosova, N.G., Tibbitts, T., DiNapoli, J., Ulinski, G., *et al.* (2021). Immunogenicity and efficacy of mRNA COVID-19 vaccine MRT5500 in preclinical animal models. *npj Vaccines* 6, 61.
- Kurup, D., Malherbe, D.C., Wirblich, C., Lambert, R., Ronk, A.J., Zabihi Diba, L., Bukreyev, A., and Schnell, M.J. (2021). Inactivated rabies virus vectored SARS-CoV-2 vaccine prevents disease in a Syrian hamster model. *PLOS Pathogens* 17, e1009383.

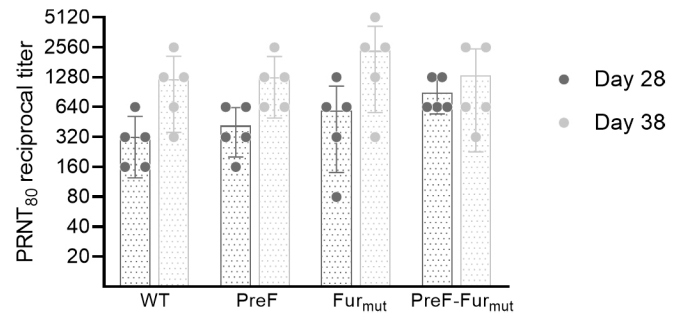
- Lopez Bernal, J., Andrews, N., Gower, C., Gallagher, E., Simmons, R., Thelwall, S., Stowe, J., Tessier, E., Groves, N., Dabrera, G., *et al.* (2021). Effectiveness of Covid-19 Vaccines against the B.1.617.2 (Delta) Variant. *New England Journal of Medicine*.
- McKay, P.F., Hu, K., Blakney, A.K., Samnuan, K., Brown, J.C., Penn, R., Zhou, J., Bouton, C.R., Rogers, P., Polra, K., *et al.* (2020). Self-amplifying RNA SARS-CoV-2 lipid nanoparticle vaccine candidate induces high neutralizing antibody titers in mice. *Nature Communications* 11, 3523.
- Mercado, N.B., Zahn, R., Wegmann, F., Loos, C., Chandrashekar, A., Yu, J., Liu, J., Peter, L., McMahan, K., Tostanoski, L.H., *et al.* (2020). Single-shot Ad26 vaccine protects against SARS-CoV-2 in rhesus macaques. *Nature* 586, 583-588.
- Meyer, M., Wang, Y., Edwards, D., Smith, G.R., Rubenstein, A.B., Ramanathan, P., Mire, C.E., Pietzsch, C., Chen, X., Ge, Y., *et al.* (2021). mRNA-1273 efficacy in a severe COVID-19 model: attenuated activation of pulmonary immune cells after challenge. *bioRxiv : the preprint server for biology*.
- Mohandas, S., Yadav, P.D., Shete-Aich, A., Abraham, P., Vadrevu, K.M., Sapkal, G., Mote, C., Nyayanit, D., Gupta, N., Srinivas, V.K., *et al.* (2021). Immunogenicity and protective efficacy of BBV152, whole virion inactivated SARS- CoV-2 vaccine candidates in the Syrian hamster model. *iScience* 24, 102054.
- Pallesen, J., Wang, N., Corbett, K.S., Wrapp, D., Kirchdoerfer, R.N., Turner, H.L., Cottrell, C.A., Becker, M.M., Wang, L., Shi, W., *et al.* (2017). Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. *Proceedings of the National Academy of Sciences* 114, E7348-E7357.
- Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M.M., Planchais, C., Porrot, F., Robillard, N., Puech, J., *et al.* (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature* 596, 276-280.
- Rauch, S., Roth, N., Schwendt, K., Fotin-Mleczek, M., Mueller, S.O., and Petsch, B. (2021). mRNA-based SARS-CoV-2 vaccine candidate CVnCoV induces high levels of virus-neutralising antibodies and mediates protection in rodents. *npj Vaccines* 6, 57.
- Reed, L.J., and Muench, H. (1938). A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS¹². *American Journal of Epidemiology* 27, 493-497.
- Rosenke, K., Meade-White, K., Letko, M., Clancy, C., Hansen, F., Liu, Y., Okumura, A., Tang-Huau, T.-L., Li, R., Saturday, G., *et al.* (2020). Defining the Syrian hamster as a highly susceptible preclinical model for SARS-CoV-2 infection. *Emerg Microbes Infect* 9, 2673-2684.
- Sheikh, A., McMenamin, J., Taylor, B., and Robertson, C. (2021). SARS-CoV-2 Delta VOC in Scotland: demographics, risk of hospital admission, and vaccine effectiveness. *The Lancet* 397, 2461-2462.
- Shen, X., Tang, H., McDanal, C., Wagh, K., Fischer, W., Theiler, J., Yoon, H., Li, D., Haynes, B.F., Sanders, K.O., *et al.* (2021a). SARS-CoV-2 variant B.1.1.7 is susceptible to neutralizing antibodies elicited by ancestral spike vaccines. *Cell Host & Microbe* 29, 529-539.e523.
- Shen, X., Tang, H., Pajon, R., Smith, G., Glenn, G.M., Shi, W., Korber, B., and Montefiori, D.C. (2021b). Neutralization of SARS-CoV-2 Variants B.1.429 and B.1.351. *New England Journal of Medicine* 384, 2352-2354.
- Subbarao, K. (2021). The success of SARS-CoV-2 vaccines and challenges ahead. *Cell Host & Microbe* 29, 1111-1123.
- Szurgot, I., Hanke, L., Sheward, D.J., Vidakovics, L.P., Murrell, B., McInerney, G.M., and Liljeström, P. (2021). DNA-launched RNA replicon vaccines induce potent anti-SARS-CoV-2 immune responses in mice. *Scientific Reports* 11, 3125.
- van der Lubbe, J.E.M., Rosendahl Huber, S.K., Vijayan, A., Dekking, L., van Huizen, E., Vreugdenhil, J., Choi, Y., Baert, M.R.M., Feddes-de Boer, K., Izquierdo Gil, A., *et al.* (2021). Ad26.COVS.2.S protects Syrian hamsters against G614 spike variant SARS-CoV-2 and does not enhance respiratory disease. *NPJ Vaccines* 6, 39.

- van Doremalen, N., Lambe, T., Spencer, A., Belij-Rammerstorfer, S., Purushotham, J.N., Port, J.R., Avanzato, V.A., Bushmaker, T., Flaxman, A., Ulaszewska, M., *et al.* (2020). ChAdOx1 nCoV-19 vaccine prevents SARS-CoV-2 pneumonia in rhesus macaques. *Nature* **586**, 578-582.
- van Doremalen, N., Purushotham, J.N., Schulz, J.E., Holbrook, M.G., Bushmaker, T., Carmody, A., Port, J.R., Yinda, C.K., Okumura, A., Saturday, G., *et al.* (2021). Intranasal ChAdOx1 nCoV-19/AZD1222 vaccination reduces shedding of SARS-CoV-2 D614G in rhesus macaques. *bioRxiv : the preprint server for biology*, 2021.2001.2009.426058.
- Wang, P., Nair, M.S., Liu, L., Iketani, S., Luo, Y., Guo, Y., Wang, M., Yu, J., Zhang, B., Kwong, P.D., *et al.* (2021). Antibody resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7. *Nature* **593**, 130-135.
- WHO (2021). Weekly epidemiological update on COVID-19 - 27 July 2021.
- Wu, Y., Huang, X., Yuan, L., Wang, S., Zhang, Y., Xiong, H., Chen, R., Ma, J., Qi, R., Nie, M., *et al.* (2021). A recombinant spike protein subunit vaccine confers protective immunity against SARS-CoV-2 infection and transmission in hamsters. *Science translational medicine* **13**, eabg1143.
- Yinda, C.K., Port, J.R., Bushmaker, T., Fischer, R.J., Schulz, J.E., Holbrook, M.G., Shaia, C., de Wit, E., van Doremalen, N., and Munster, V.J. (2021). Prior aerosol infection with lineage A SARS-CoV-2 variant protects hamsters from disease, but not reinfection with B.1.351 SARS-CoV-2 variant. *Emerg Microbes Infect* **10**, 1284-1292.
- Yu, J., Tostanoski, L.H., Peter, L., Mercado, N.B., McMahan, K., Mahrokhian, S.H., Nkolola, J.P., Liu, J., Li, Z., Chandrashekar, A., *et al.* (2020). DNA vaccine protection against SARS-CoV-2 in rhesus macaques. *Science*, eabc6284.
- Zhang, B.Z., Wang, X., Yuan, S., Li, W., Dou, Y., Poon, V.K., Chan, C.C., Cai, J.P., Chik, K.K., Tang, K., *et al.* (2021). A novel linker-immunodominant site (LIS) vaccine targeting the SARS-CoV-2 spike protein protects against severe COVID-19 in Syrian hamsters. *Emerg Microbes Infect* **10**, 874-884.
- Zhou, D., Dejnirattisai, W., Supasa, P., Liu, C., Mentzer, A.J., Ginn, H.M., Zhao, Y., Duyvesteyn, H.M.E., Tuekprakhon, A., Nutalai, R., *et al.* (2021). Evidence of escape of SARS-CoV-2 variant B.1.351 from natural and vaccine-induced sera. *Cell* **184**, 2348-2361.e2346.

A



B



Supplemental Figure 1. Effect of spike modifications on binding and neutralizing antibody responses in mice. C57BL/6 mice (n=5/group) were immunized with LION-formulated repRNA encoding the wild-type (WT), the prefusion-stabilized (PreF), the furin cleavage site-deleted (Furmut), or a combination of the PreF and Furmut modifications of the full-length spike of A.1 lineage SARS-CoV2. Vaccinations were administered on days 0 and 28, and serum collected on days 14, 28, and 38 assayed for (A) anti-spike binding IgG by enzyme linked immunosorbent assay and serum collected on days 28 and 38 assayed for (B) neutralizing antibody responses by 80% plaque reduction neutralization assay against A.1 SARS-CoV2 (WA-1 isolate).