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Supplementary Information for

Swine Acute Diarrhea Syndrome Coronavirus Replication in Primary Human Cells Reveals Potential Susceptibility to Infection

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Supplementary Text
Materials and Methods.

Virus and cells

The recombinant SADS-CoV and its red fluorescent protein expressing derivative (SADS-CoV tRFP) were propagated in Vero CCL-81 and LLC-PK1 cells. All cells were maintained prior to infection in complete DMEM media (Gibco) supplemented with 10% fetal clone II (HyClone), non-essential amino acids, sodium pyruvate, and 1% Pen-Strep antibiotic (Gibco). To generate virus stocks, cells were washed twice with PBS and cultured in serum-free DMEM-H media (Gibco) supplemented with 8% tryptose phosphate broth and 2.5-10ug/mL trypsin (Gibco) and grown at 37°C in a humidified CO_2 incubator. Samples were then titered by plaque assay on Vero CCL-81 cells. Cells were plated at 800,000 cells per well. To enhance viral growth and spread, trypsin was added at 10ug/mL to the overlay for plaque assay. Plaques were visualized using neutral red staining at 4-5 days post infection. Growth curves were performed in Huh7.5 and LLC-PK1 cells, and supernatants were titered on Vero CCL-81 cells as previously described (1, 2). Huh7.5 cells did not require additional trypsin for viral growth and spread, though it is likely that the inoculum used to perform the growth curves contained minimal levels of residual trypsin. For growth curves of SADS-CoV tRFP, fluorescent foci were counted to determine titer. Primary human airway epithelial cells, lung fibroblast, and lung microvascular endothelial cells prepared as described previously (3) were purchased from the Marsico Lung Institute, Tissue Procurement and Cell Culture Core, University of North Carolina at Chapel Hill, and used to evaluate SADS-CoV growth using previously described methods (1, 2). The cells obtained from human lungs were approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #03-1396). 2D primary human intestinal cells were derived from human ileal crypts that form a continuous polarized epithelium with proliferative and non-proliferative zones (4). All primary cells (lung and intestinal) were infected with 200ul of SADS-CoV-tRFP diluted at 1:10 in PBS. Inoculated cultures were incubated at 37°C in a humidified CO_2 incubator for 2 hours before washing 3 times with 500ul PBS. Viral titers were determined using apical washes of airway ALI cultures, or apical media of intestinal cultures at appropriate time points. All recombinant SADS-CoVs are maintained in a BSL3 laboratory to enhance biosafety and all waste material is disinfected and autoclaved prior to removal from the facility. All personnel were equipped with fully protective Tyvek suits, double gloves, and a PAPR as previously described by our group (5).

Systematic assembly of a full length SADS-CoV cDNA

The SADS-CoV clone was designed using the previously published sequence (6). Six contiguous cDNAs (A-F) flanked by unique restriction endonuclease sites (BsmBI/SapI/BglI) were purchased from BioBasic (**Fig. 1**). Breakpoints were designed to maximize the stability of cloned fragments

in bacteria as previously described by our group (7-9). The fragments were digested, separated through 0.8% agarose gels, visualized, excised, and purified using a QIAquick Gel Extraction Kit (Qiagen). The SADS-CoV cDNAs were ligated overnight at 4°C, phenol/chloroform extracted, and precipitated. Full-length T7 RNA transcripts were generated *in vitro* as described by the manufacturer (Ambion mMessage mMachine) with modifications (10). RNA transcripts (wild-type or tRFP) were added to 800ul of Vero CCL-81 cell suspension ($8x10^6$) in an electroporation cuvette, and 4 electrical pulses of 450V at 50uF were distributed with a Gene Pulser II electroporator (Bio-Rad). As previously published, N gene transcripts were included during electroporation due to the increased infectivity of CoV transcripts when incorporated (10, 11). Transfected cells were allowed to recover for 10 minutes at room temperature before incubating at 37°C for 4 days in a $75cm^2$ tissue culture flask. Viral progeny were passaged once in either Vero CCL-81 or LLC-PK1 cells for 2-4 days and used to generate a virus stock for future use (GenBank, accession number MT039231).

Recombinant SADS-CoV tRFP expression construct

To generate a SADS-CoV tRFP recombinant virus, the nonstructural ORF, NS3a, was replaced with tomato red fluorescent protein (tRFP) via generation of three PCR amplicons and ligation into the SADS F fragment. One PCR amplicon was generated using primers SADS tRFP #1+ (5'-gtgcattgttgctaaggacgg-3') and SADS tRFP #2 rev (5'-nnnnnngctcttcttggacgtggacctttcaatctc-3'). A second PCR amplicon was generated using primers SADS tRFP #3+ (5'-nnnnnngctcttctccaataatggtgagcaagggcgaggag-3') and SADS tRFP #4 rev (5'-nnnnnngctcttcattacttgtacagctcgtccatg-3'). A third PCR amplicon was generated using primers SADS tRFP #5+ (5'-nnnnnngctcttcgtaatactaacacacctttttgttggtatc-3') and SADS tRFP #6 rev (5'-ggcgcaaagagtgacaatgg-3'). The three amplicons were digested with SapI, as indicated in the primer sequences above, and ligated. The ligation product was then digested with Bpu10I and EciI prior to insertion into the SADS F plasmid. Recovery of recombinant viruses encoding tRFP are as described previously (GenBank, accession number MT039232) (10, 11).

VRP expressing SADS nucleocapsid and spike genes

The SADS nucleocapsid and spike genes were cloned separately into pVR21 3526 to generate virus replicon particles (VRP), as previously described (12, 13). Briefly, pVR21 is an expression vector carrying the VEE genome in which the VEE structural genes are replaced by the SADS nucleocapsid or spike gene following the 26S promoter. The SADS nucleocapsid and spike gene, with the deletion of 7 amino acids from the C terminus, was PCR amplified off the full SADS cDNA before insertion into the pVR21 backbone. The SADS-pVR21 construct, a plasmid containing the VEE 3526 envelope glycoproteins, and a plasmid containing the VEE capsid protein were used to generate T7 RNA transcripts. The RNA transcripts were then electroporated

into BHK cells. VRP were harvested 48 hours later and purified via high speed ultra-centrifugation. Groups of 5 to 7-week-old BALB/c mice (Jackson Labs) were then inoculated via footpad injection with VRP expressing the SADS nucleocapsid protein. Mice were boosted at 21 days, euthanized at 14 days post boost, and serum was collected for antibody against the nucleocapsid protein.

Western blot analysis

To produce protein lysates for analysis by western blot, infected cells were washed with 1X PBS and lysed in buffer containing 20mM Tris-HCl (pH 7.6), 150mM NaCl, 0.5% deoxycholine, 1% nonidet P-40, 0.1% SDS. After initial lysis and removal of nuclei, supernatants were added to an equal volume of 10mM EDTA and 0.9% SDS for a final concentration of SDS of 0.5%. Following lysis, samples were incubated for 15 minutes at room temperature prior to use in western blot. Proteins were separated using a 7.5% polyacrylamide gel. For each sample, 30ul of the protein lysate was mixed with 4X loading dye and heated to 95°C for 10 min. Samples were loaded into the gel and separated by electrophoresis in 1X running buffer (3.03g Tris-HCl, 14.4g glycine, 1g SDS, 1L dH2O) at 110V for 5 min, followed by 90V for 65 mins. Proteins were transferred onto an Immuno-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 115mA for 30 min using 1X dry transfer buffer (5.82g Tris base, 2.92g glycine, 100ml methanol, 1L dH2O). The membranes were blocked in 1X phosphate-buffered saline–Tween 20 (PBST)–5% milk at 4°C overnight with shaking. Membranes were then treated with primary antibody using mouse anti-SADS nucleocapsid sera (1:250)–1X PBST-5% milk and incubated at 37°C for 2 hours with shaking. Membranes were washed three times with 1X PBST (10 min per wash) before treatment with secondary antibody. Secondary antibody of goat anti-mouse IgG horseradish peroxidase (HRP) (GE Healthcare) (1:5,000)–1X PBST–5% milk and were incubated at room temperature for 1 h with shaking. Membranes were washed six times with 1X PBST (5 min per wash) and developed using LI-COR WesternSure PREMIUM Chemiluminescent Substrate (LI-COR). Membranes were incubated at room temperature for 5 min before imaging was performed using the LI-COR C-DiGit blot scanner (LI-COR Biosciences, Lincoln, NE, USA).

Northern Blot Analysis

Total RNA was extracted from icSADS or icSADS-tRFP infected cells approx. 20hr post infection using TRIzol Reagent (ThermoFisher). Messenger (poly A) RNA was isolated from the total RNA using an Oligotex mRNA Mini Kit (Qiagen). Messenger RNA (0.6-0.7µg) was separated on an agarose gel and transferred to BrightStar-Plus membrane using a NorthernMax-Gly Kit (Invitrogen). Blots were hybridized with a biotin labeled oligomer (5'-BiodT/CTTTGATTACTCCACCACACCAGACA/BiodT-3'), then detected using a Chemiluminescent Nucleic Acid Detection Module (ThermoFisher).

RT-PCR of leader containing transcripts

RNA, isolated using TRIzol Reagent, was reverse transcribed using a SuperScript II kit (Invitrogen) and random primers. PCR was performed using a SADS leader primer (5'-GACTTTCCAGTCTACTCTTCTC-3') and a reverse primer in the N gene (5'-CGAGACTGTGAACGTGAAGC-3').

***In vivo* infection of Balb/c and IFNR mice**

10-week-old Balb/c or IFN Type I/II knock-out mice (IFNR) were infected with $1x10^5$ PFU of SADS-CoV. IFNR mice were infected either intraperitoneally (IP) or by oral gavage, and Balb/c mice were infected intranasally or by oral gavage. Mice infected by oral gavage were given 1 x 10^5 virus in a 100ul volume. Mice infected IP were given 1 x 10^5 SADS-CoV in a 200ul volume. Mice were also infected intranasally with a 50ul volume containing 1 x 10^5 SADS-CoV. Mice were weighed daily, and samples were harvested at 2 dpi, including the liver, spleen, and intestines. Intestinal samples were harvested by section including duodenum, jejunum, ileum, and colon. RNA was extracted from each tissue sample using a Directzol RNA kit (Zymo) and qRT-PCR was run (45 cycles) to determine the amount of viral RNA present in each mouse and tissue.

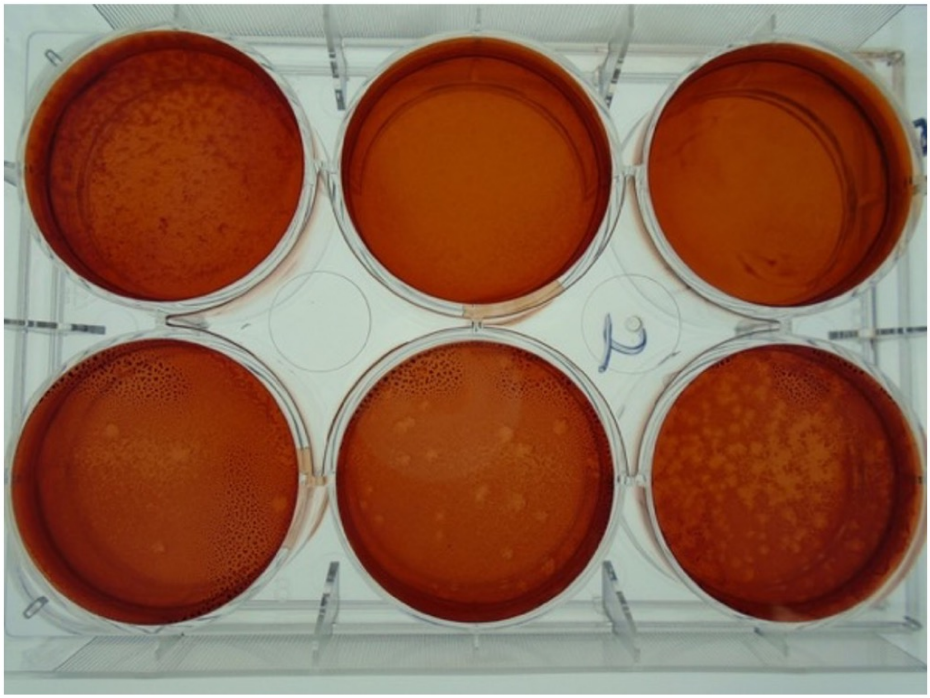
Neutralization Assays with hCoV Sera

Neutralization assays were performed using known hCoV sera against SADS-CoV tRFP and NL63 GFP, using methods previously described (14). NL63 GFP was used as a human coronavirus expected to be neutralized by human donor sera. SADS-CoV tRFP and NL63 GFP diluted to 10^5 PFU were incubated with sera from 4 donors at 1:2 serial dilutions for 1 hr at 37°C. All dilutions were performed in serum-free media containing 1% antibiotic. Poly-L lysine treated 96-well plates seeded with Huh7.5 cells were infected with each condition of virus and sera, as well as with PBS and sera free virus as controls, for 1 hr at 37°C for SADS-CoV tRFP and 32°C for NL63 GFP. Following infection, virus and sera was removed and cells were washed twice with 1X PBS before overlaid with serum-free media. Cells were imaged for fluorescence and percent neutralization was calculated using the area of fluorescence.

Inhibition of SADS-CoV by Remdesivir

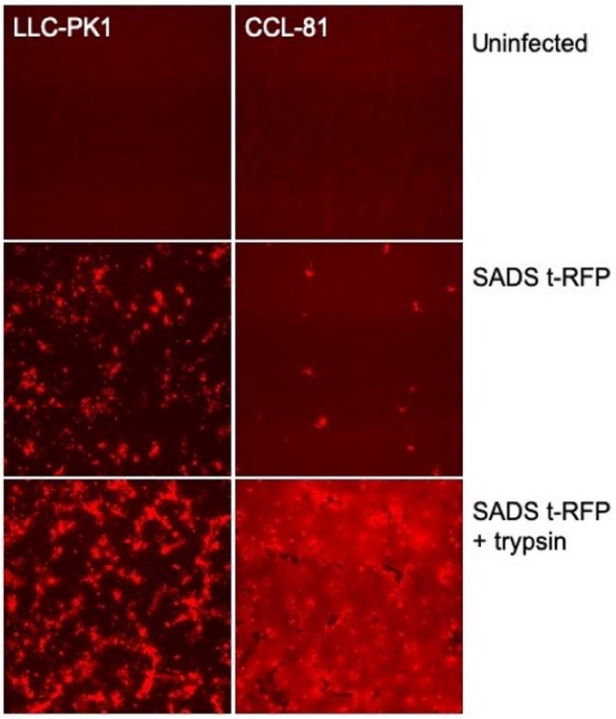
Poly-L lysine treated 96-well plates seeded with Huh7.5 cells were infected with SADS-CoV tRFP diluted to $2x10^5$ PFU in the presence of a dose response of remdesivir or DMSO for 1 hr at 37°C. Following infection, virus was removed, and cells were overlaid with serum-free media containing DMSO or the dose response of remdesivir. Cells treated with DMSO, but not virus, were included as a negative control to serve as the 100% inhibition marker. Cells were imaged for fluorescence and percent inhibition was calculated using the area of fluorescence.

Figure S1.



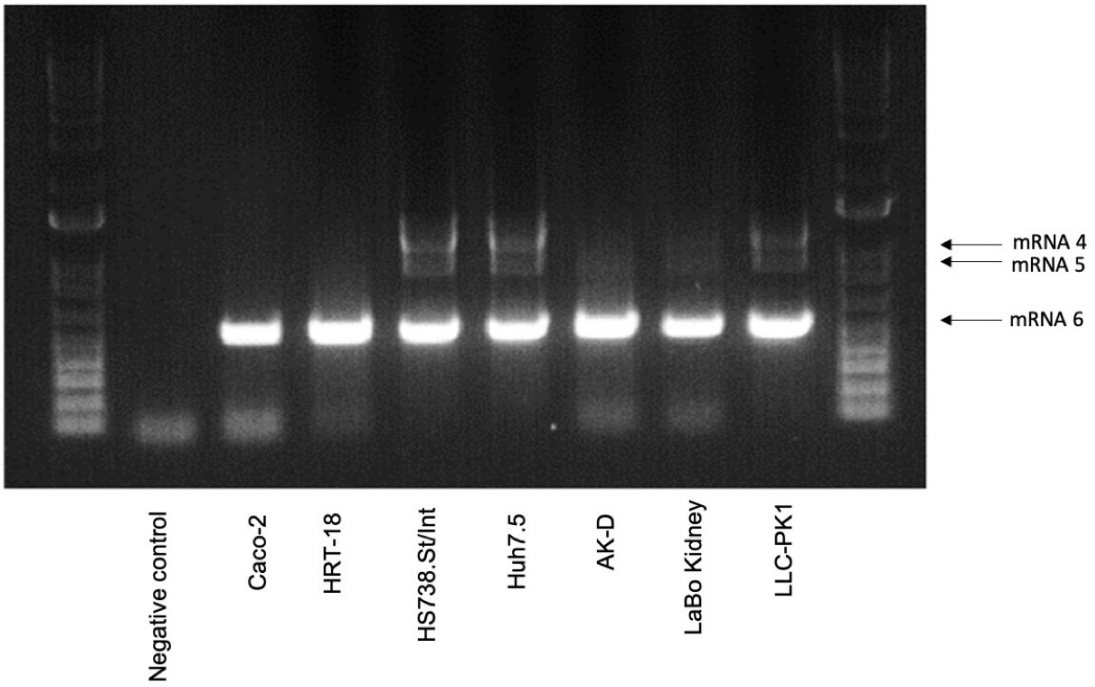
Plaque formation in Vero CCL-81 cultures. In Vero CCL-81 cultures, wildtype SADS-CoV grows to a titer exceeding 10^6 PFU/ml at 24 hours post infection as indicated by plaque assay.

Figure S2.



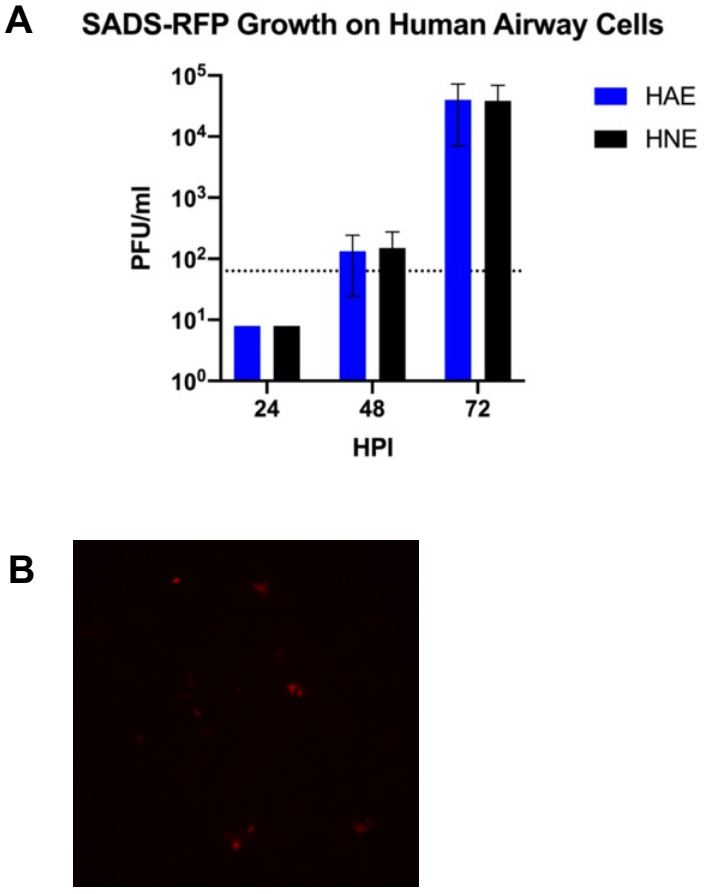
Effect of trypsin on SADS-CoV infectivity. Cultures of Vero CCL-81 and LLC-PK1 cells were infected with SADS tRFP and visualized for tomato red fluorescence to confirm the presence of virus infection. Cells were cultured both with and without trypsin to indicate increased infectivity in the presence of trypsin.

Figure S3.



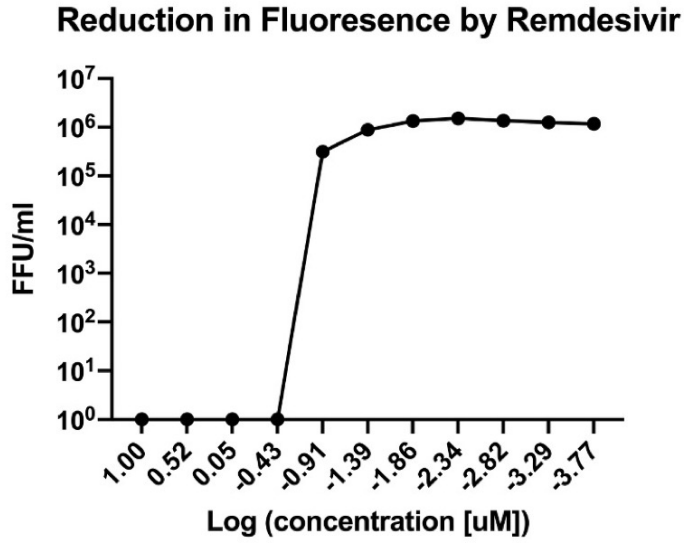
RT-PCR of leader containing transcripts in various cells types resolved by gel electrophoresis. Each sample contained appropriate bands to indicate presence of SADS-CoV leader N gene transcripts.

Figure S4.



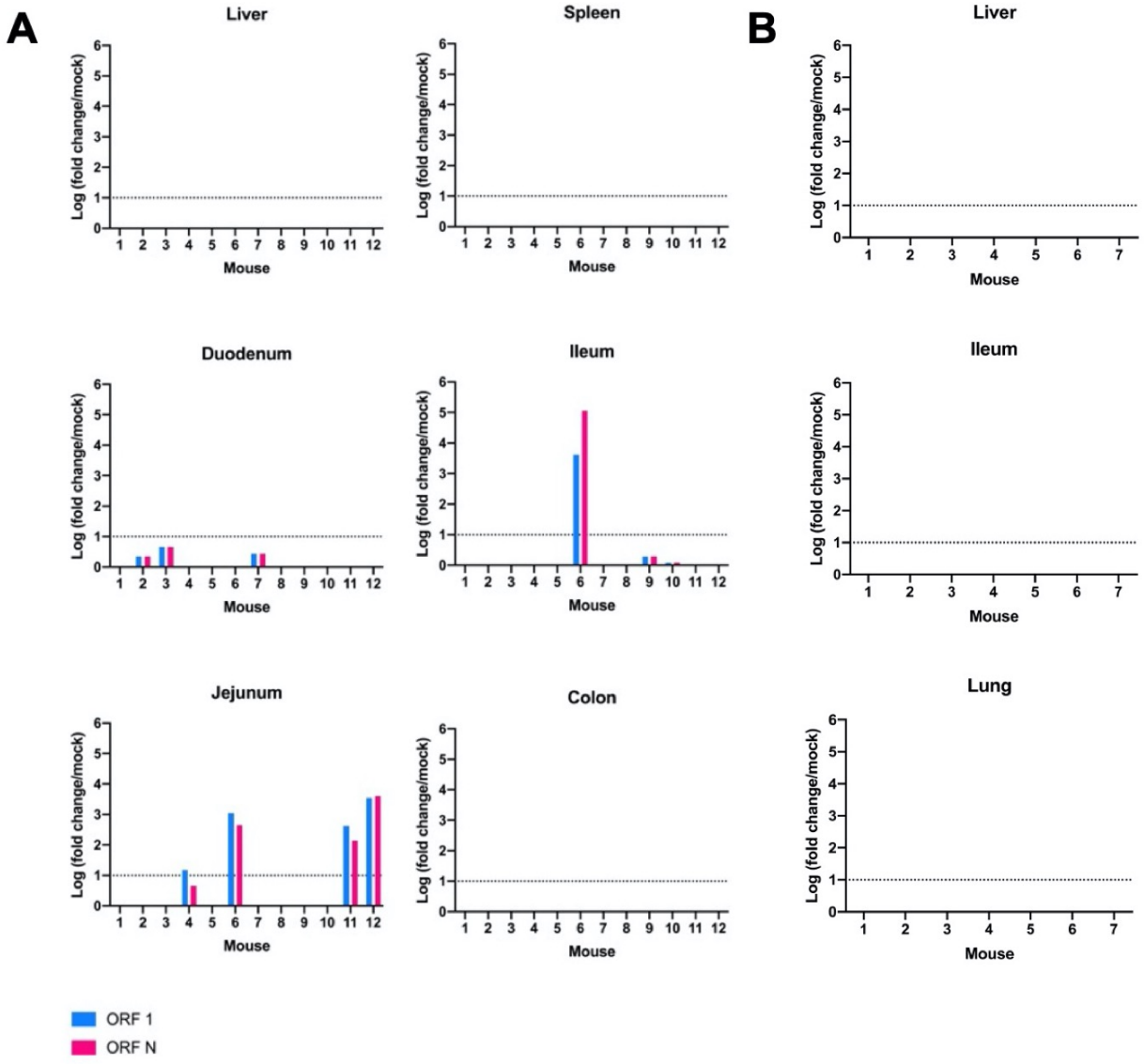
Primary cell titers and Imaging. (A) In HAE and HNE cultures, SADS-RFP replicated to about 10^5 PFU/ml by 72 hours post infection. The limit of detection is indicated by the dotted line at $10^{1.8}$. Samples with no detectable titers are presented as half the limit of detection (24 hours post infection). **(B).** An additional fluorescent image of gut cultures infected with SADS-RFP demonstrates productively infected cells, expressing reporter proteins.

Figure S5.



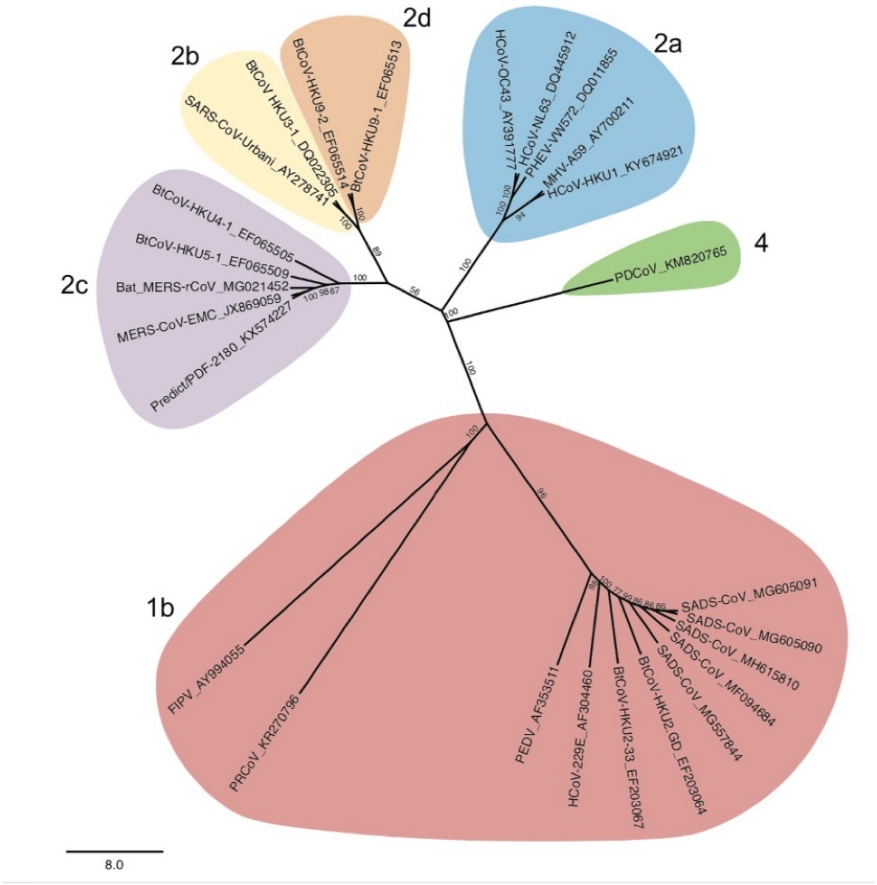
Reduction of SADS-RFP fluorescence by remdesivir. Reduction in SADS-CoV fluorescence is noted between 0.37uM and 0.12uM. Ultimately, SADS-CoV was reduced from 10^6 FFU/ml, in untreated control cells.

Figure S6.



SADS-CoV infection in Balb/c and IFNR mice. (A) IFN Type I/II deficient mice infected with 10^5 PFU of SADS-CoV and analyzed using qRT-PCR. Across each tissue type, only one mouse (1/12) showed positive levels of virus in multiple tissue sections, demonstrating a poorly reproducible model characterized by extensive non-productive infections in immunodeficient mice. **(B)** Balb/c mice infected both intranasally and by oral gavage demonstrated no levels of virus in the lungs, liver, or ileum.

Figure S7.



Phylogeny of nsp12 proteins of known coronaviruses. The nsp12 protein sequence of selected coronaviruses were aligned and phylogenetically compared. Coronavirus genera are grouped by classic subgroup designations (1b, 2a-d, and 4). Branches in each tree are labeled with consensus support values (in %). Sequences were aligned using free end gaps with the Blosum62 cost matrix in Geneious Prime. The tree was constructed using the neighbor-joining method based on the multiple sequence alignment, also in Geneious Prime. Numbers following the underscores in each sequence correspond to the GenBank Accession number. The radial phylogram was exported from Geneious and then rendered for publication using Adobe Illustrator CC 2019.

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