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Supporting Information for

SARS-CoV-2 nsp15 endoribonuclease antagonizes dsRNA-induced antiviral signaling

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MATERIALS & METHODS

Recombinant viruses

Recombinant SARS-CoV-2 viruses were derived from a bacterial artificial chromosome (BAC) vector containing the full-length SARS-CoV-2 USA-WA1/2020 genome. For SARS-COV-2 nsp15^{mut}, nucleotides 20,320 (C) and 20,321 (A) were mutated in order to substitute histidine 234 with alanine, and the mutant virus was rescued as previously described [1, 2]. MERS-CoV (HCoV-EMC/2012) was also derived from a BAC. MERS-CoV-nsp15^{H231A} and MERS-CoV-nsp15^{H231A}/ΔNS4a were generated as previously described [3]. Virus stocks were sequenced and compared to the publicly available wild-type sequences on NCBI. All virus stocks were generated via low MOI infections in VeroE6 cells (for SARS-CoV-2 viruses) and VeroCCL81 cells (for MERS-CoV viruses).

Cloning and protein purification

SARS-CoV-2 nsp15 wild type and H234A mutant were PCR amplified using cDNA clones with forward primer 5'-TTCAAGGATCCATGAGTTTAGAAAATGTGGCTTTTAATG-3' (with BamHI restriction site [underlined]) and reverse primer 5'-TTCAAGTCGACCTATTGTAATTTTGGGTAAAATGTTTC-3' (with SalI restriction site [underlined]). The SARS-CoV-2 nsp15 wild type cDNA template was a gift from Prof. Wang Pei-Hui (Shandong University, China). H234A mutant cDNA clone was obtained by lambda red recombination of the full-length BAC and restriction cloning into the expression vector [4]. The amplified DNA product was cloned into pGEX-6P1 vector at BamHI and SalI site. Nsp15 purification was performed by modifying methods previously described for glutathione S-transferase (GST) fusion proteins [5, 6]. Briefly, GST fusion proteins were expressed from pGEX-6P-1 constructs in *E. coli* strain BL21(DE3)/pLysS (ThermoFisher). Secondary cultures were grown to an optical density (OD) (at 600 nm) of 0.6 in a shaking

incubator at 37°C at 250 rpm. Cells were induced with 0.2 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 16 hours at 22°C. Induced cell pellets were harvested and suspended in buffer A (20 mM HEPES (pH 7.5), 1 M KCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 10% (v/v) glycerol, and EDTA-free Pierce protease inhibitor (ThermoFisher)). Pelleted cells were lysed by the addition of 200 µg/mL lysozyme and sonication. Supernatants were added to Pierce glutathione agarose (ThermoFisher) and incubated for 2 hours at 4°C, followed by washes with buffer A. Digestions to cleave the GST tag were performed with PreScission protease (Cytiva) in a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT for 16 hours at 4°C. Concentrated supernatants containing tag-less protein were loaded onto a Superdex 75 column on an Äkta pure 25L protein purification system (GE Healthcare) in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM DTT. Protein concentrations were estimated using the Bio-Rad protein assay reagent (Bio-Rad). All proteins were stored in buffer supplemented with 10% glycerol at −80°C until further use.

Endonuclease activity assay

Endonuclease activity of nsp15 was determined in a fluorescence based kinetic assay. Synthetic RNA substrate (6-FAM-UUA UCA AAU UCU UAU UUG CCC CAU UUU UUU GGU UUA-BHQ-1) with a FRET (Fluorescence resonance energy transfer) pair (6-FAM at the 5′-terminus and a black hole quencher-1 (BHQ1) at the 3′-terminus) was commercially obtained from IDT [7]. Final reaction mixtures contained 20 mM HEPES (pH 7.5), 1 mM DTT, 5 mM $MnCl_2$, 0.2 µM RNA substrate in the absence or presence of 50 nM wild type or H234A mutant nsp15 in a 50 µl volume. The reactions were set in a round bottom black polystyrene 96 well plate (Costar) and briefly spun. Reaction kinetics were monitored by measuring fluorescence using a Varioskan LUX microplate reader

(ThermoFisher) set at an excitation/emission wavelengths of 485nm/535nm (excitation bandwidth 12 nm and measurement time 100 ms). The kinetics were recorded at 5-minute intervals over a period of 60 minutes using SkanIt Software 6.0.1.

Cell lines

VeroE6 cells and VeroCCL81 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% L-glutamine and 4.5g/L D-glucose (Gibco, ThermoFisher) supplemented with 10% heat inactivated (HI) fetal bovine serum (FBS) (Hyclone, Cytiva) and 1X penicillin/streptomycin (pen/strep) (Gibco, ThermoFisher). Human A549 cells engineered to stably express angiotensin-converting enzyme 2 (ACE2) (A549-ACE2) were cultured in RPMI 1640 (Gibco, ThermoFisher) supplemented with 10% HI FBS and 1X pen/strep [8]. Human Calu3 cells were (ATCC) cultured in DMEM containing 10% L-glutamine and 4.5g/L D-glucose supplemented with 20% HI FBS and 1X pen/strep.

Infections in A549-ACE2 and Calu3 cell lines

Viruses were diluted in serum-free (SF) DMEM or RPMI and added to cells for absorption for 1 hour at 37°C. All infections were performed at an MOI of 1 unless otherwise indicated. Cells were washed three times with phosphate-buffered saline (PBS) after the incubation period and DMEM supplemented with 4% HI FBS for Calu3 infections or RPMI supplemented with 2% HI FBS for A549-ACE2 infections was then added. 200 μL of supernatant was collected at the indicated time points for infectious virus quantification. The third PBS wash was collected and represents the zero hour time point to quantify remaining input virus. All infections, plaque assays, and virus manipulations were carried

out in a biosafety level 3 laboratory utilizing appropriate personal protective equipment and protocols.

Immunofluorescence and single-molecule fluorescent *in situ* hybridization (smFISH)

Calu3 cells were seeded on to glass cover slips (Fisher Scientific). Once the cells reached 80% confluence they were infected with the indicated virus at an MOI of 1. At 48 hours post infection (hpi), cells were fixed in 4% paraformaldehyde for 30 minutes and then stored in 75% ethanol for up to 4 days at 4ºC. Immunofluorescence assay and smFISH were performed as described in [9]. Cells were stained with K1 dsRNA antibody (Cell Signaling Technology 28764) at 1:1000 and DNA oligos targeting the SARS-CoV-2 genome RNA (5'UTR-ORF1a junction) labeled with ATTO-633 [10]. The cover slips were mounted on slides with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories; H-1200). Cells were imaged with a Nikon Eclipse Ti2 equipped with a Yokogawa CSU-W1 Spinning Disk Confocal and Gataca systems live super resolution using Nikon elements software. Images were processed in Image J and displayed at intensities ideal for visualization of relevant biological processes.

For quantification of SARS-CoV-2 genomes and dsRNA in infected cells, cells were imaged with a 20X air objective, super-resolution module, and large image (2x2) function. Each cells in a field of view was designated as a region of interest (ROI). Mean intensity values for SARS-CoV-2 genome and dsRNA were then measured within the entire ROI of each cell. Cells with dsRNA or genome intensity two-fold above the highest values measured in mock-infected cells were considered infected and were included in the data set displayed in **Figure 2**. For analysis of dsRNA puncta, images were captured with a 100X objective along 10 Z planes with 200 nm steps. Images were stacked using

maximum projection. Intensity display settings between WT and nsp15^{mut} SARS-CoV-2 images were normalized. Within a single cell, each dsRNA puncta was manually designated as an ROI, and the area, mean intensity, and integrated intensity were measured in Image J. Statistical analyses were performed using prism. Data reporting intensity values (dsRNA intensity, genome intensity, puncta mean intensity, and puncta integrated intensity) was normalized relative to the average intensity value during WT SARS-CoV-2 infection in each individual experiment in order to correct for experiment-to-experiment variability in raw intensity values.

Nasal air-liquid interface (ALI) culture growth and differentiation

Nasal specimens were obtained via cytologic brushing of patients in the Department of Otorhinolaryngology-Head and Neck Surgery, Division of Rhinology at the University of Pennsylvania and the Philadelphia Veteran Affairs Medical Center after obtaining informed consent. Patients with any history of systemic disease and those currently on immunosuppressive medications were excluded. The full study protocol, including acquisition and use of nasal specimens, was approved by the University of Pennsylvania Institutional Review Board (Protocol #800614) and the Philadelphia VA Institutional Review Board (Protocol #00781). ALI cultures were grown on semi-permeable transwell supports (STEMCELL Technologies) (0.4 μm pores) as previously described [11-13]. Nasal cells derived from 4-6 patients were pooled prior to seeding on to transwell inserts (STEMCELL Technologies). Nasal cells were grown to confluence with Pneumacult-Ex Plus growth medium (STEMCELL Technologies) present both apically and basally until reaching confluence. At that point apical growth medium was removed. Pneumacult-ALI medium (STEMCELL Technologies) was used to differentiate all nasal ALI cultures and

was replaced two times per week for 4 weeks prior to infection. Growth and differentiation of cultures was conducted at 37ºC.

Infection of nasal ALI cultures

After differentiation, nasal ALI cultures were allowed to equilibrate at 33ºC for at least 24 hours prior to infection in order to replicate conditions of the *in vivo* nasal airway as previously described [13, 14]. In brief: viruses were diluted in SF DMEM to achieve a total inoculum volume of 60 μL at an MOI of 1. The inocula were added apically to nasal ALI cultures for a 1 hour adsorption period. After 1 hour, cells were washed on the apical surface three times with PBS. The third PBS wash was collected and represents the 0 hour time point. Every 48 hours following infection, 200 μL PBS was added apically to each nasal ALI culture in order to collect shed virus for quantification.

Infectious virus quantification

Extracellular supernatants were titered via plaque assay on either VeroE6 cells for SARS-CoV-2 or VeroCCL81 cells for MERS-CoV using previously described plaque assay methods [15]. Briefly, the indicated cell type was seeded ~24 hours before use. The next day, 1:10 serial dilutions of viral supernatants were performed in SF DMEM. Growth media was removed from each well and selected dilutions were added. Plates were incubated for 1 hour at 37ºC. At the end of the 1 hour incubation, liquid overlay (DMEM with L-Glut (ThermoFisher) supplemented with 2% HI FBS, 1% sodium pyruvate (ThermoFisher), and 0.01% molecular grade agarose (ThermoFisher)) was added to each well (~1.5 mL/well in 12-well plates). Plates were incubated at 37ºC for 3 days for both SARS-CoV-2 and MERS-CoV. After 3 days, the overlay was removed and 4% paraformaldehyde (PFA) in PBS (ThermoFisher) was added to each well for at least 30 min to fix the monolayer and inactivate infectious virus. PFA was then removed and disposed of appropriately. The

plates were stained using crystal violet (1% crystal violet (Sigma) in 20% ethanol (Fisher Scientific) and 80% water) and rinsed with water before counting. Each sample was plated in technical duplicate. Titer (plaque forming unit (PFU) per mL) represents the average plaque count divided by the dilution factor and inoculum volume in mL.

Reverse transcriptase (RT)-quantitative PCR

At indicated times post infection, cells were lysed with buffer RLT Plus (Qiagen RNeasy Plus #74136) and RNA was extracted following the manufacturer's protocol. Methods were adapted from [15]. RNA was reverse transcribed to make cDNA using a High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, ThermoFisher) (200 ng RNA per 20 μL cDNA reaction). cDNA was amplified using specific PCR primers (see Table S2), iQ™ SYBR® Green Supermix (Bio-Rad), and the QuantStudio™ 3 PCR system (Thermo Fisher). Fold changes in ISG mRNA level relative to mock-infected samples were calculated using the formula $2^{-\Delta(\Delta Ct)}$ ($\Delta Ct = Ct_{gene\ of\ interest} - Ct_{18S}$) and expressed as fold changes over mock. Primer sequences for each gene analyzed are provided in **Table S2**. SARS-CoV-2 genome copy numbers were quantified with primers directed against nsp12 (RdRp) and calculated using a standard curve generated with a digested plasmid encoding SARS-CoV-2 nsp12.

Western blot analysis

Cell lysates were harvested at indicated times post infection with lysis buffer (50 mM Tris hydrochloride pH 8.0, 150 mM sodium chloride, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% nonyl phenoxypolyethoxylethanol (NP)-40, 0.02% sodium azide) supplemented with protease inhibitor cocktail (cOmplete mini EDTA-free protease inhibitor tablets, Roche) and phosphatase inhibitors (PhosSTOP phosphatase inhibitor cocktail

tablets, Roche). Lysates were mixed 3:1 with 4x Laemmli sample buffer (Bio-Rad). Samples were heated to 95°C for 10 minutes, resolved on gradient SDS/PAGE gels (Bio-Rad), and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Blots were blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) in Tris buffered saline with 0.1% Tween 20 (TBST) and probed with the indicated antibody at the specified concentration in its respective blocking buffer as listed in **Table 2**. Blots were visualized using SuperSignal West Femto Chemiluminescent substrate (ThermoFisher). Blots were stripped using Restore Western Blot Stripping Buffer (ThermoFisher) for at least 1 hour at room temperature. After stripping, blots were thoroughly washed with TBST and blocked prior to the addition of the next primary antibody.

Ruxolitinib treatments

48 hours prior to infection, basal media of nasal ALI cultures was supplemented with 10 μM ruxolitinib (Selleck Chem) in DMSO. Control cultures were treated with the same percentage of DMSO. Basal media containing fresh ruxolitinib or DMSO was replaced at 0, 48, and 96 hpi. Sample collection and analysis was performed as described here.

Measurement of cytotoxicity via lactate dehydrogenase (LDH) assay and trans-epithelial electrical resistance (TEER)

Apical surface liquid samples collected from nasal cell cultures treated with ruxolitinib were used for quantification of cytotoxicity via LDH assay (Roche) relative to nasal cultures treated with Triton X-100 as previously described [13-14]. Epithelial barrier integrity quantified by TEER (reported in Ohms-cm^2) was measured with an EVOM ohm-voltmeter (World Precision Instruments) as previously described [13-14].

Analyses of RNase L-Mediated rRNA degradation

Intracellular RNA was harvested with RLT plus buffer and extracted using Qiagen RNeasy Plus kit. RNA was prepared and analyzed on an RNA chip with the Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit according to manufacturer recommendations [16]. Poly(I:C) transfections were performed at 500 ng/mL of low molecular weight poly(I:C) (Millipore Sigma) applied either apically or both apically and basally (as indicated) using lipofectamine 2000 (ThermoFisher) and collected 24 hours post transfection, as previously described [17].

Statistics

Data was graphed and statistics were performed using GraphPad Prism. Data was graphed displaying either individual values or the mean +/- standard deviation. Unless otherwise stated, statistical significance was determined by comparing mutant viruses to WT using either a one-way ANOVA for single time point comparisons or a two-way ANOVA for experiments with multiple time points and/or viruses. Significance shown represents P values, where * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; and **** = $P < 0.0001$. Nonsignificant comparisons are not displayed in all figures.

Table S1. Primers used for qPCR analysis

Gene name	Primer orientation	Sequence
IFNL1	Forward	CGCCTTGGAAGAGTCACTCA
	Reverse	GAAGCCTCAGGTCCCAATTC
IFNB	Forward	GTCAGAGTGGAAATCCTAAG
	Reverse	ACAGCATCTGCTGGTTGAAG
IFIT1	Forward	TGGTGACCTGGGGCAACTTT
	Reverse	AGGCCTTGGCCCGTTCATAA

RSAD2	Forward	CACAAAGAAGTGTCCTGCTTGGT
	Reverse	AAGCGCATATATTCATCCAGAATAAG
CXCL10	Forward	CCTGCAAGCCAATTTTGTCC
	Reverse	ATGGCCTTCGATTCTGGATTC
ISG15	Forward	CATCTTTGCCAGTACAGGAGC
	Reverse	GGGACACCTGGAATTCGTTG
18S	Forward	TTCGATGGTAGTCGCTGTGC
	Reverse	CTGCTGCCTTCCTTGAATGTGGTA
SARS-CoV-2 nsp12 (RdRp)	Forward	GGTAACTGGTATGATTTCG
	Reverse	CTGGTCAAGGTTAATATAGG

Table S2. Antibodies used for western blotting

Primary Antibody	**Antibody species**	**Blocking buffer**	**Dilution**	**Catalog number**
p-PKR	Rabbit	5% BSA in TBST	1:1000	Abcam 32036
PKR	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 12297
p-eIF2α	Rabbit	5% BSA in TBST	1:1000	Cell Signaling 3398
eIF2α	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 9722
IFIT1	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 14769
Viperin	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 13996
MDA5	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 5321
p-STAT-1	Rabbit	5% BSA in TBST	1:1000	Cell Signaling 7649
STAT1	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 9172
STAT2	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 72604
IRF9	Rabbit	5% milk or 5% BSA in TBST	1:500	Santa Cruz SC-10793
IRF3	Rabbit	5% milk or 5% BSA in TBST	1:1000	Cell Signaling 11904
SARS-CoV-2 Nucleocapsid	Rabbit	5% milk or 5% BSA in TBST	1:2000	Genetex GTX135357
SARS-CoV-2 Nsp15	Rabbit	5% milk or 5% BSA in TBST	1:1000	Invitrogen MA5-47048
MERS-CoV Nucleocapsid	Mouse	5% milk or 5% BSA in TBST	1:2000	Sino Biological 40068-MM10
GAPDH	Rabbit	5% milk or 5% BSA in TBST	1:2000	Cell Signaling 2118
Secondary Antibody	**Antibody species**	**Blocking buffer**	**Dilution**	**Catalog number**
Anti-rabbit IgG HRP-linked	Goat	Same as primary	1:3000	Cell Signaling 7074
Anti-mouse IgG HRP-linked	Horse	Same as primary	1:3000	Cell Signaling 7076

SUPPLEMENTAL FIGURES

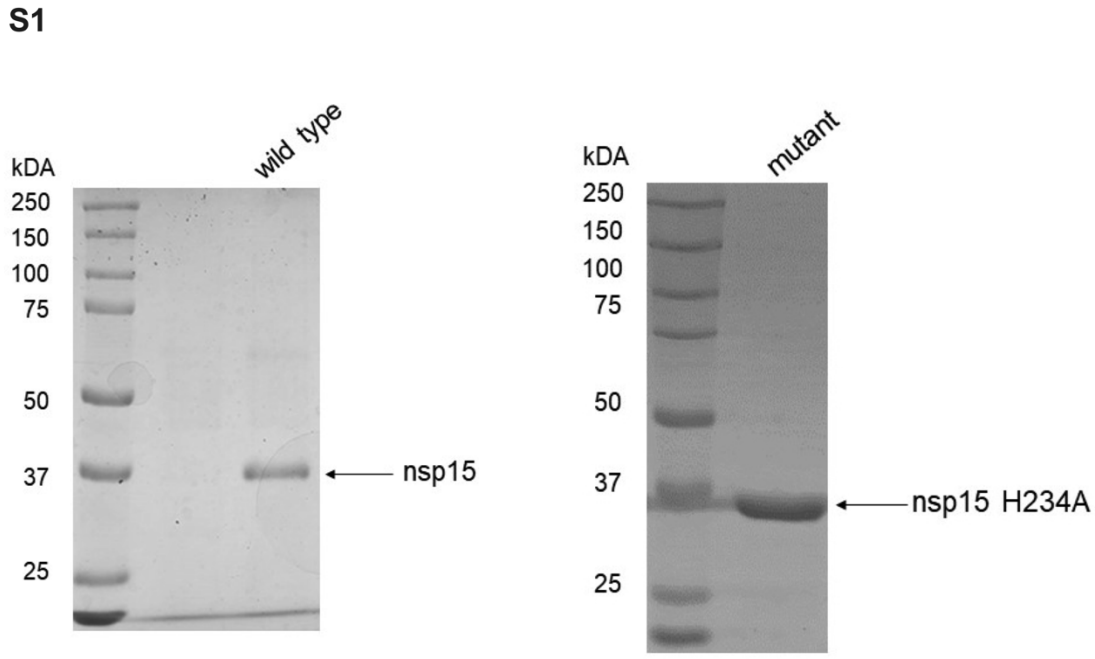

Figure S1. Purification of WT and H234A mutant nsp15 proteins

Each recombinant protein was purified and visualized on a gel to confirm homogeneity. See cloning and protein purification section of Materials & Methods for further detail.

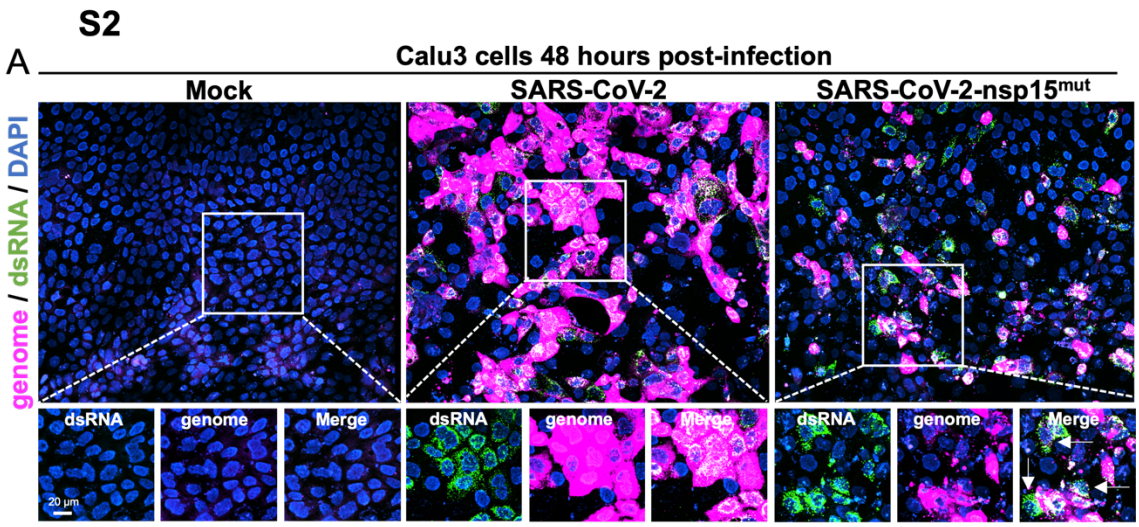

Figure S2. Representative widefield image of the smFISH performed in Figure 2. Immunofluorescence assay for dsRNA and smFISH for the SARS-CoV-2 genome in Calu3 cells. Intensity display for viral RNA genome was set to a threshold at which individual genomes could be observed. Arrows indicate SARS-CoV-2 nsp15^{mut}-infected cells with high levels of dsRNA but low levels of genomic RNA.

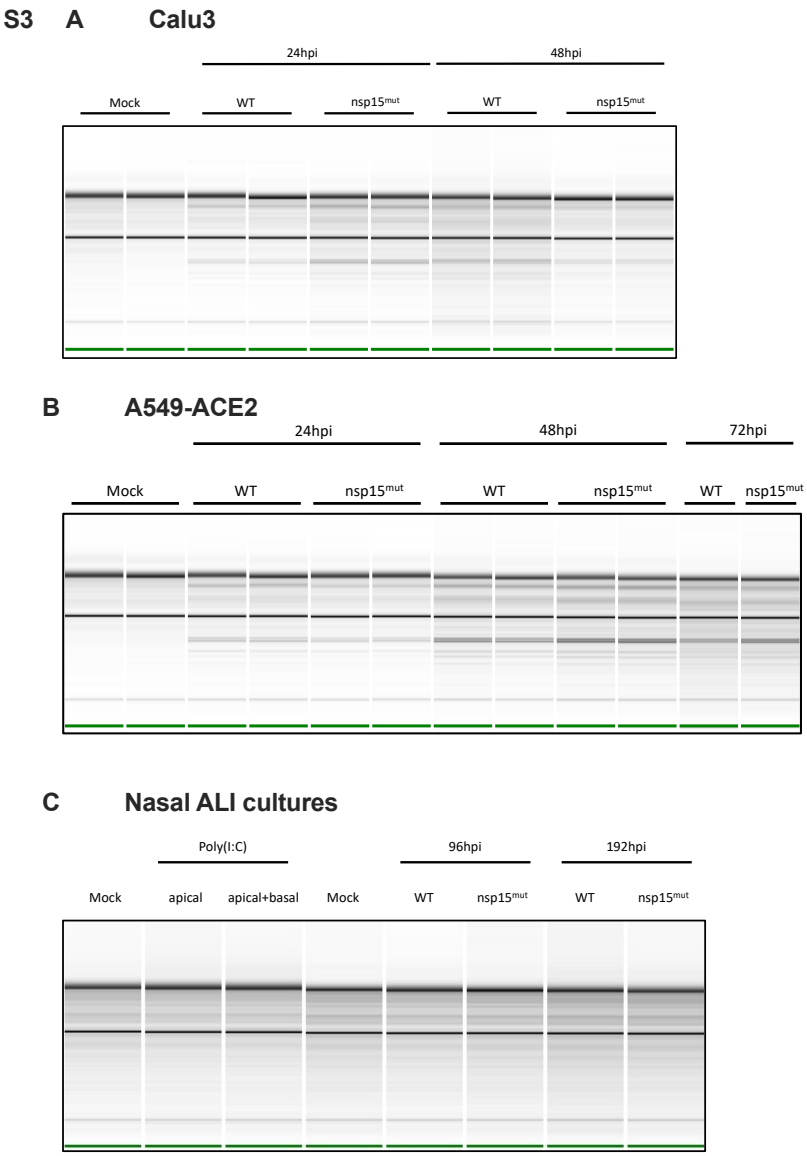

Figure S3 SARS-CoV-2 nsp15^{mut} does not induce the OAS/RNase L pathway above WT SARS-CoV-2 levels. Calu3 (A), A549-ACE2 (B), or nasal ALI cultures (C) were infected at MOI 1 with WT or nsp15^{mut} SARS-CoV-2. Total cellular RNA was collected at indicated times post infection and was assessed for RNase L activity using Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit. Nasal cell cultures were transfected with poly(I:C) apically or both apically and basally at 500ng/mL for 24 hours (see Methods).

S4

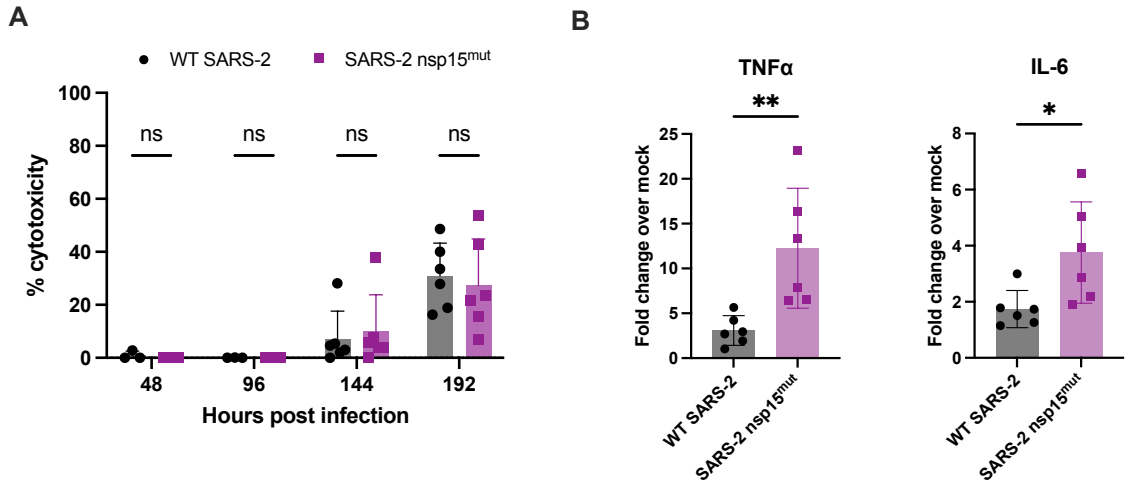

Figure S4 Inflammation during SARS-CoV-2 nsp15^{mut} infection of nasal ALI cultures. Nasal ALI cultures were infected at MOI 1 with WT or nsp15^{mut} SARS-CoV-2. (A) Apical surface liquid (ASL) samples were used for quantification of cytotoxicity via LDH assay, reported as % cytotoxicity relative to Triton X-100-treated cultures. (B) Total cellular RNA was collected at 192 hpi and mRNA expression of inflammatory mediators TNF-α and IL-6 was quantified. Data shown are from two experiments, performed in independent batches of pooled-donor nasal ALI cultures.

S5

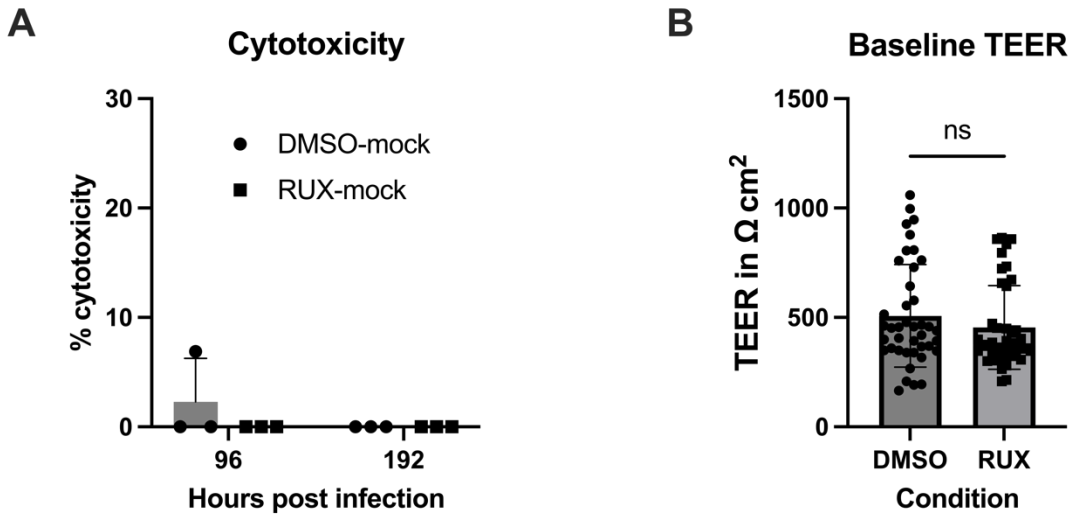

Figure S5. Ruxolitinib treatment of nasal ALI cultures is not associated with cytotoxicity or epithelial barrier disruption. Nasal ALI cultures were treated with ruxolitinib (10μM) or DMSO for 48 hours. (A) Apical surface liquid (ASL) samples were used for quantification of cytotoxicity via LDH assay, reported as % cytotoxicity relative to Triton X-100-treated cultures. Data shown is from one set of pooled-donor nasal cell cultures. (B) Cultures underwent TEER measurement to quantify epithelial barrier integrity. Data shown is from 4 independent sets of pooled-donor nasal cell cultures.

S6

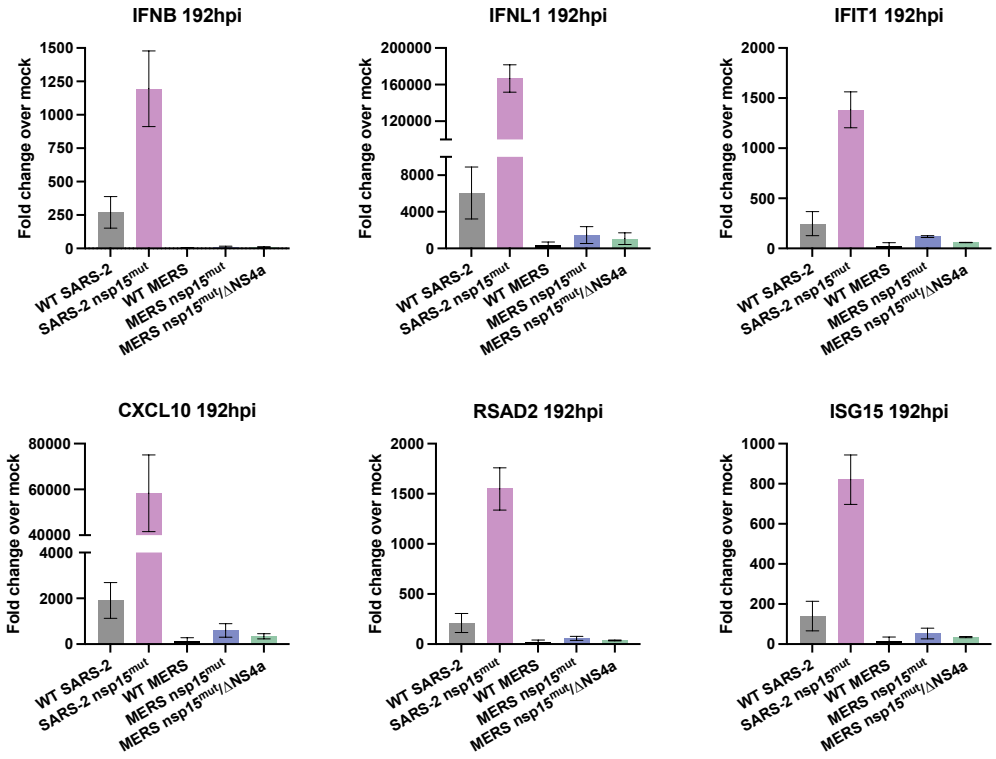

Figure S6. IFN and ISG mRNA expression by SARS-CoV-2 and MERS-CoV mutants at 192 hours post infection in nasal ALI cultures

Nasal cell cultures were infected at MOI 1 with either WT SARS-CoV-2, SARS-CoV-2 nsp15^{mut}, WT MERS-CoV, MERS-CoV nsp15^{mut}, or MERS-CoV nsp15^{mut}/ΔNS4a in triplicate. Total cellular RNA was collected from infected cultures at 192 hpi and the $2^{-\Delta(\Delta Ct)}$ formula was to to calculate fold changes in gene expression relative to mock cultures.

S7

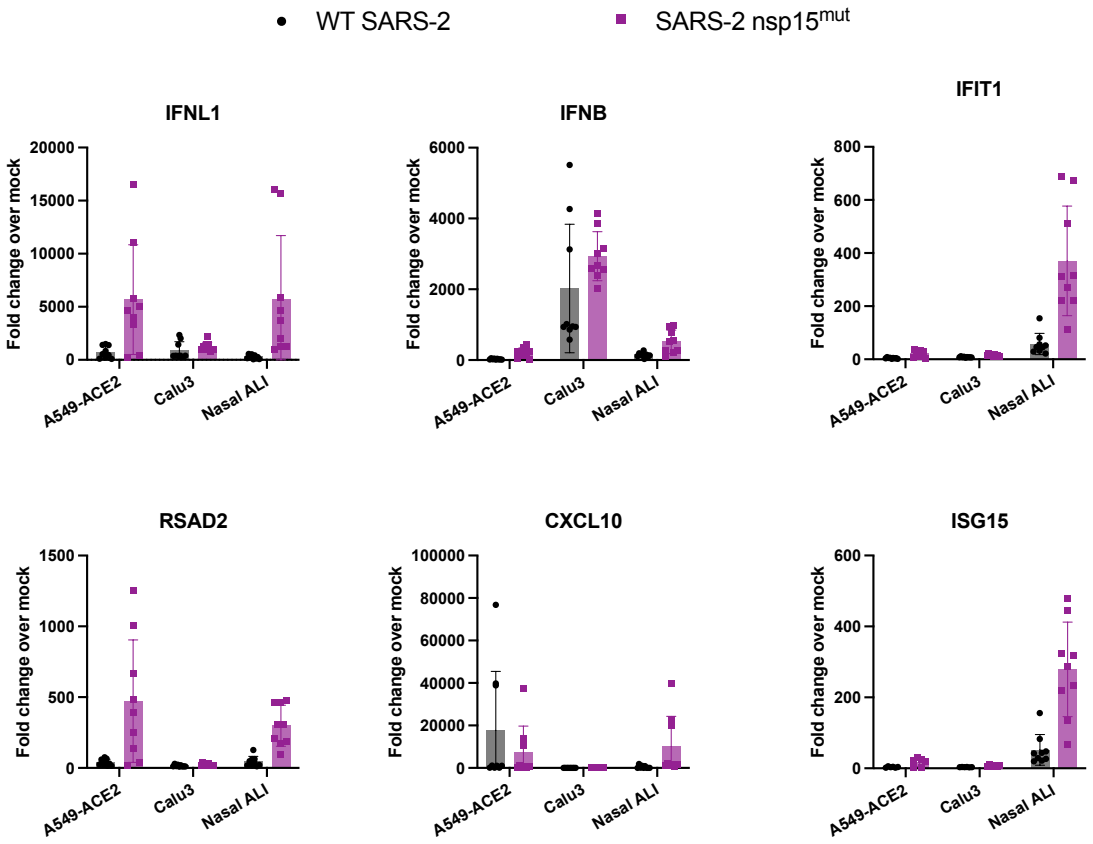

Figure S7. Comparison of fold change induction of IFN and ISG mRNAs in A549-ACE2 cells, Calu3 cells, and nasal ALI cultures

Cells from the indicated culture system were infected with either WT or nsp15^{mut} SARS-CoV-2. RNA was collected in triplicate for analysis of fold change in mRNA expression above mock-infected levels, calculated using the $2^{-\Delta(\Delta Ct)}$ formula for indicated IFN or representative ISG mRNA. Fold changes from 3 independent experiments (triplicate values from each experiment) were plotted for the time point of maximal fold change induction in IFN and ISG expression in that cellular system (72 hpi for A549-ACE2, 24 hpi for Calu3, 192 hpi for nasal ALI cultures). Fold changes over mock are plotted on the same graph for each gene in order to compare degree of induction in each cellular system.

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