

## RESEARCH ARTICLE

# Developing a mouse model of human coronavirus NL63 infection: comparison with rhinovirus-A1B and effects of prior rhinovirus infection

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## Abstract

Human coronavirus (HCoV)-NL63 causes respiratory tract infections in humans and uses angiotensin-converting enzyme 2 (ACE2) as a receptor. We sought to establish a mouse model of HCoV-NL63 and determine whether prior rhinovirus (RV)-A1B infection affected HCoV-NL63 replication. HCoV-NL63 was propagated in LLC-MK2 cells expressing human ACE2. RV-A1B was grown in HeLa-H1 cells. C57BL6/J or transgenic mice expressing human ACE2 were infected intranasally with sham LLC-MK2 cell supernatant or  $1 \times 10^5$  tissue culture infectious dose (TCID<sub>50</sub>) units HCoV-NL63. Wild-type mice were infected with  $1 \times 10^6$  plaque-forming units (PFU) RV-A1B. Lungs were assessed for vRNA, bronchoalveolar lavage (BAL) cells, histology, HCoV-NL63 nonstructural protein 3 (nsp3), and host gene expression by next-generation sequencing and qPCR. To evaluate sequential infections, mice were infected with RV-A1B followed by HCoV-NL63 infection 4 days later. We report that hACE2 mice infected with HCoV-NL63 showed evidence of replicative infection with increased levels of vRNA, BAL neutrophils and lymphocytes, peribronchial and perivascular infiltrates, and expression of nsp3. Viral replication peaked 3 days after infection and inflammation persisted 6 days after infection. HCoV-NL63-infected hACE2 mice showed increased mRNA expression of IFNs, IFN-stimulated proteins, and proinflammatory cytokines. Infection with RV-A1B 4 days before HCoV-NL63 significantly decreased both HCoV-NL63 vRNA levels and airway inflammation. Mice infected with RV-A1B prior to HCoV-NL63 showed increased expression of antiviral proteins compared with sham-treated mice. In conclusion, we established a mouse model of HCoV-NL63 replicative infection characterized by relatively persistent viral replication and inflammation. Prior infection with RV-A1B reduced HCoV-NL63 replication and airway inflammation, indicative of viral interference.

**NEW & NOTEWORTHY** We describe a mouse model of human coronavirus (HCoV) infection. Infection of transgenic mice expressing human angiotensin-converting enzyme 2 (ACE2) with HCoV-NL63 produced a replicative infection with peribronchial inflammation and nonstructural protein 3 expression. Mice infected with RV-A1B 4 days before HCoV-NL63 showed decreased HCoV-NL63 replication and airway inflammation and increased expression of antiviral proteins compared with sham-treated mice. This research may shed light on human coronavirus infections, viral interference, and viral-induced asthma exacerbations.

*asthma; coronavirus; rhinovirus; interference; interferon*

## INTRODUCTION

The human coronaviruses (HCoV) 229E and OC43 were first isolated from patients with respiratory illness in the 1960s (1–3). Volunteers inoculated with HCoV-229E and HCoV-OC43 developed a common cold (4). HCoV-NL63 was first isolated from a 7-mo-old patient with bronchiolitis and conjunctivitis in the Netherlands in 2003, and HCoV-HKU1 was isolated from a 71-yr-old patient with pneumonia in Hong Kong in 2004 (5, 6). These human coronaviruses are generally not life-threatening in healthy individuals, causing the common colds and croup in children and young adults. However, outbreaks of highly pathogenic human coronaviruses, severe acute respiratory syndrome (SARS)-CoV, Middle

East respiratory syndrome (MERS)-CoV, and SARS-CoV-2, emerged in 2002 (7), 2012 (8) and 2019 (9).

The family Coronaviridae comprises a large group of viruses with broad host tropism and is divided into two subfamilies and five genera. HCoV-229E and HCoV-NL63 are grouped into the genus *Alphacoronavirus*, and HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2 into *Betacoronavirus* (10). Coronaviruses are enveloped positive-stranded RNA viruses with extended spike proteins on the surface. The envelope consists of a lipid bilayer in which the membrane (M), envelope (E), and spike (S) structural proteins are anchored. Inside the envelope is the nucleocapsid, which is formed from multiple copies of the nucleocapsid (N) protein bound to the positive-sense single-stranded RNA

genome. Coronavirus genome size ranges from 26 to 32 kB, one of the largest among RNA viruses. The genome carries a 5' methylated cap and a 3' polyadenylated tail and harbors two large open reading frames (11, 12). ORF1a and ORF1b encode the 16 nonstructural proteins indispensable for viral replication [nonstructural protein (nsp)1–16]. Later ORFs encode the four structural proteins and multiple accessory proteins.

Coronavirus-19 (COVID-19) disease, caused by SARS-CoV-2, is now a leading cause of death in the United States. Although HCoV-NL63 is not considered life-threatening in healthy individuals, HCoV-NL63 carries some similarities to SARS-CoV-2. Like SARS-CoV and SARS-CoV-2, the HCoV-NL63 spike (S) uses angiotensin-converting enzyme 2 (ACE2) as a receptor albeit the NL63 spike has been shown to bind with less affinity (13). Furthermore, in immunocompromised patients, HCoV-NL63 may cause similar clinical syndromes as SARS-CoV-2. Infection with coronavirus may be more serious in immunocompromised or elderly adults (14–16). In a study of patients with acute respiratory disease warranting presentation to Queensland, Australia hospitals, HCoV-NL63 positive individuals often showed abnormal chest radiographs (56%), respiratory distress (50%), wheeze (44%), and rales (25%) (17). COVID-19 can spread rapidly in long-term residential care facilities (18). In an outbreak of HCoV-NL63 in a long-term care facility in Louisiana, 6 of 20 patients were hospitalized with pneumonia and 3 died (19). Finally, like SARS-CoV-2 (20), an association between HCoV-NL63 and systemic inflammation in children has been described (21). Since studies of SARS-CoV-2 require BSL-3 containment, we wanted to develop a system to study human CoVs using an informative BSL-2 animal model system.

We sought to establish a mouse model of HCoV-NL63 by infecting transgenic mice expressing human ACE2. These mice have been used to study SARS-CoV and SARS-CoV-2 infection in vivo (22, 23). We hypothesize that expression of human ACE2 in mice permits HCoV-NL63 infection.

We previously developed a mouse model of rhinovirus (RV)-A1B infection, a minor group virus which binds to the low-density lipoprotein receptor (LDL-R). Based on partial homology between human and mouse LDL-R, mice infected with RV-A1B show limited viral replication, as evidenced by interferon (IFN) production and the presence of double-stranded viral RNA (24). We compared the effects of RV-A1B and HCoV-NL63 on lung inflammation and gene expression.

Finally, RV infection blocks SARS-CoV-2 replication in cultured airway epithelial cells (25) and airway epithelial organoids (26). We therefore tested whether RV-A1B infection blocks HCoV-NL63 infection in our in vivo model.

## MATERIALS AND METHODS

### Antibodies to HCoV-NL63 Proteins

Rabbit polyclonal antibodies against HCoV-NL63 the nsp3 peptide sequence YIYDEEGGYDVSKPV and the nucleoprotein sequence DKPSQLKKPRWKRVP were generated by GenScript (Piscataway, NJ). Carboxy terminal cysteines were added to allow conjugation to hemocyanin. Purified primary antibodies were labeled for immunofluorescence using

AlexaFluor dye-conjugated N-hydroxy succinimidyl esters, as described (27).

### Viral Preparation

HCoV-NL63 and LLC-MK2 cells were obtained from BEI Resources (Manassas, VA). LLC-MK2 cells permissive for HCoV-NL63 infection (28, 29) were transformed with the human ACE2 lentiviral vector pLV[Exp]-mCherry:T2A:Puro-CMV > hACE2[NM\_021804.3] (VectorBuilder, Chicago, IL) and selected with puromycin (0.5 µg/mL). Lentivirus-infected cells stably expressing human ACE2 were easier to grow and maintain several days postinfection than the original LLC-MK2 cell line. Infection was confirmed by staining with anti-HCoV-NL63 nucleoprotein (Fig. 1A). Infected LLC-MK2-ACE2 cells were harvested by scraping the plates, lysed with a sterile ground glass homogenizer, and centrifuged at 10,000 g. Cell supernatants were used for infections. The viral cytopathic effect in LLC-MK2-ACE2 cells at 72 h postinfection was measured to determine the tissue culture infectious dose (TCID<sub>50</sub>) by the Spearman–Karber method (30). HCoV-NL63 infection was confirmed by staining with anti-HCoV-NL63 nucleoprotein (Fig. 1A). Fresh LLC-MK2-ACE2 supernatants usually held a TCID<sub>50</sub> of  $\sim 1 \times 10^7$ /mL. Primers from the HCoV-NL63 sequence (NC\_005831.2) were used to measure viral copy number (vRNA) by qPCR (Table 1). A plasmid containing bases 1–540 of the sequence was used as a copy number standard (GenScript).

RV-A1B viral stocks (ATCC, Manassas, VA) were grown in HeLa-H1 cells, concentrated, and partially purified by ultrafiltration with a 100 kD filter (viral particles do not pass through the filter), and titered by plaque assay as previously described (31, 32).

To evaluate for cross contamination of viral stocks, RNA samples from RV-A1B-infected HeLa-H1 cells and HCoV-NL63-infected LLC-MK2-ACE2 cells were analyzed by next-generation sequencing. Total RNA was prepared using Trizol extraction (Invitrogen, Carlsbad, CA) and purified using RNeasy spin columns (Qiagen, Germantown, MD). After on column DNase I digestion (Qiagen), samples were submitted for ribosomal RNA depletion, library production, and RNA sequence analysis to Azenta Life Sciences (Burlington, MA). The RV-A1B sample had 2749837 hits for the NC\_038311.1 genome corresponding to RV-A1B and no hits for NC\_005831.2 genome corresponding to HCoV-NL63. The HCoV-NL63 sample had 107 hits for the NC\_038311.1 (RV-A1) genome and 627,140 hits for the NC\_005831.2 (NL63) genome.

### Infection of Wild-Type C57BL/6 and Human ACE2 Transgenic Mice

All animal research was performed according to the Guide for the Care and Use of Laboratory Animals (8th ed., National Academies Press, 2011) and the American Veterinary Medical Association Guidelines for the Euthanasia of Animals. The protocols were approved by the University of Michigan Animal Care and Use Committee (Protocol No. PRO00010065). All mouse treatments were administered under isoflurane anesthesia. Experimental animals were humanely euthanized at defined end points by exposure to isoflurane vapors followed by thoracotomy.

**Table 1.** Oligonucleotide primers used for qPCR

|                               | Sequence                                            |
|-------------------------------|-----------------------------------------------------|
| Human ACE2 genomic (forward)  | 5'-GGA TGG AGT ACC GAC TGG AG-3'                    |
| Human ACE2 genomic (reverse)  | 5'-CAT ATG CTT TAT CTC CAA GAG CTG-3'               |
| Human ACE2 genomic (probe)    | 5'-/56-FAM/ AGA CCA AAG CAT CAA AGT GAGG/3BHQ_1/-3' |
| Human ACE2 qPCR (forward)     | 5'-AGA AAG CAG TCT GCC ATC CC-3'                    |
| Human ACE2 qPCR (reverse)     | 5'-GCT GTC AGG AAG TCG TCC AT-3'                    |
| Mouse ApoB genomic (forward)  | 5'-CAC GTG GGC TCC AGC ATT-3'                       |
| Mouse ApoB genomic (reverse)  | 5'-TCA CCA GTC ATT TCT GCC TTT G-3'                 |
| Mouse ApoB genomic (probe)    | 5'-/5Cy5/ CCA ATG GTC GGG CAC TGC TCAA/3BHQ_2/-3'   |
| HCoV-NL63 (forward)           | 5'-GAT AGA GAA TTT TCT TAT TTA GAC TTT GT-3'        |
| HCoV-NL63 (reverse)           | 5'-TGC CAT AAC AAA TGA CAG CAC T-3'                 |
| RV (forward)                  | 5'-GTC CTC CGG CCC CTG AAT G-3'                     |
| RV (reverse)                  | 5'-GAA ACA CGG ACA CCC AAA GTA G-3'                 |
| Mouse <i>Ifna1</i> (forward)  | 5'-CCA TCC CTG TCC TGA GTG-3'                       |
| Mouse <i>Ifna1</i> (reverse)  | 5'-CCA TGC AGC AGA TGA GTC CTT-3'                   |
| Mouse <i>Ifng</i> (forward)   | 5'-TGG CTG TTT CTG GCT GTT AC-3'                    |
| Mouse <i>Ifng</i> (reverse)   | 5'-TCC ACA TCT ATG CCA CTT GAG TT-3'                |
| Mouse <i>Cxcl1</i> (forward)  | 5'-TGC ACC CAA ACC GAA GAA GTC AT-3'                |
| Mouse <i>Cxcl1</i> (reverse)  | 5'-CAA GGG AGC TTC AGG GTC AAG-3'                   |
| Mouse <i>Cxcl10</i> (forward) | 5'-GCT GCA ACT GCA TCC ATA TC-3'                    |
| Mouse <i>Cxcl10</i> (reverse) | 5'-TTT CAT CGT GGC AAT GAT CT-3'                    |
| Mouse <i>Ifnb1</i> (forward)  | 5'-CAG CCC TCT CCATCA ACT ATA AG-3'                 |
| Mouse <i>Ifnb1</i> (reverse)  | 5'-CCT GTA GGT GAG GTT GAT CTT TC-3'                |
| Mouse <i>Ifnl3</i> (forward)  | 5'-CTT GAG AAG GAC ATG AGG TGC AG-3'                |
| Mouse <i>Ifnl3</i> (reverse)  | 5'-GGT CAG GGC TGA GTC ATT TAT GTT-3'               |
| Mouse <i>Ifi35</i> (forward)  | 5'-CCG GAG CCA AGA TCC CAT T-3'                     |
| Mouse <i>Ifi35</i> (reverse)  | 5'-TGC CTG CCC TGC TTA GTT TG-3'                    |
| Mouse <i>Isg20</i> (forward)  | 5'-AAG CGC CTG CTA CAC AAG AAC-3'                   |
| Mouse <i>Isg20</i> (reverse)  | 5'-TAG AGC TCC ATT GTG GCC CTG-3'                   |
| Mouse <i>Oasl1</i> (forward)  | 5'-GAC ATA GTG GCT CAG CGG G-3'                     |
| Mouse <i>Oasl1</i> (reverse)  | 5'-ATA TCG GGT GCT CTC TTC ACC-3'                   |
| Mouse <i>Mx1</i> (forward)    | 5'-GCA GTC ATC AGA GTG CAA GC-3'                    |
| Mouse <i>Mx1</i> (reverse)    | 5'-TCA CCA GAA AGG ACA TCC TCG-3'                   |
| Mouse <i>Mx2</i> (forward)    | 5'-ACC AGG CTC CGA AAA GAG TT-3'                    |
| Mouse <i>Mx2</i> (reverse)    | 5'-TCT CGT CCA CGG TAC TGC TT-3'                    |
| Mouse <i>Gapdh</i> (forward)  | 5'-GTC GGT GTG AAC GGA TTT G-3'                     |
| Mouse <i>Gapdh</i> (reverse)  | 5'-GTC GTT GAT GGC AAC AAT CTC-3'                   |
| Mouse <i>Nlrp3</i> (forward)  | 5'-TGC TCT TCA CTG CTA TCA AGC CCT-3'               |
| Mouse <i>Nlrp3</i> (reverse)  | 5'-ACA AGC CTT TGC TCC AGA CCC TAT-3'               |
| Mouse <i>Tlr2</i> (forward)   | 5'-TCG TTC ATC TCT GGA GCA TC-3'                    |
| Mouse <i>Tlr2</i> (reverse)   | 5'-TTG ACG CTT TGT CTG AGG TT-3'                    |
| Mouse <i>Il1b</i> (forward)   | 5'-TGG CAG CTA CCT GTG TCT TTC-3'                   |
| Mouse <i>Il1b</i> (reverse)   | 5'-GGA TGG GCT CTT CTT CAA AGA TG-3'                |
| Mouse <i>Cxcl2</i> (forward)  | 5'-GCG CTG TCA ATG CCT GAA G-3'                     |
| Mouse <i>Cxcl2</i> (reverse)  | 5'-CGT CAC ACT CAA GCT CTG GAT-3'                   |
| Mouse <i>Il1f6</i> (forward)  | 5'-CTG TTC TGC ACA AAG GAT GGG-3'                   |
| Mouse <i>Il1f6</i> (reverse)  | 5'-AGA GAG AGG CTT TTA CAG GTT C-3'                 |
| Mouse <i>Il17a</i> (forward)  | 5'-GCC TGA GAG CTG CCC CTT CAC-3'                   |
| Mouse <i>Il17a</i> (reverse)  | 5'-GGC TGC CTG GCG GAC AAT CG-3'                    |
| Mouse <i>Il12b</i> (forward)  | 5'-CTC CTG GTT TGC CAT CGT TT-3'                    |
| Mouse <i>Il12b</i> (reverse)  | 5'-GGG AGT CCA GTC CAC CTC TA-3'                    |
| Mouse <i>Il21</i> (forward)   | 5'-TCC ACA AGA TGT AAA GGG GCA-3'                   |
| Mouse <i>Il21</i> (reverse)   | 5'-TGA TGG CTT GAG TTT GGC CT-3'                    |

5Cy5, 5' cyanine 5; 3BHQ, black hole quencher, 1 for FAM, 2 for Cy5; 56-FAM. 5' 6-carboxyfluorescein.

B6.Cg-Tg(K18-ACE2)2PrImn/J mice (Jackson Laboratories, Bar Harbor, ME) express the human ACE2 protein in epithelial cells using the keratin 18 promoter (22). Mice were genotyped using the vendor-recommended protocol and confirmed by measurement of human ACE2 mRNA expression by qPCR (primers are listed in Table 1). Eight-to-ten-week-old male and female K18-hACE2 or C57BL6/J mice were inoculated with 50  $\mu$ L of  $1 \times 10^5$  TCID<sub>50</sub> HCoV-NL63 or sham LLC-MK2-ACE2 supernatant intranasally and euthanized at 1–7 days posttreatment. The 10,000 g supernatant fluid of homogenized, uninfected LLC-MK2-ACE2 cells was used for sham infections.

Selected K18-hACE2 or C57BL6/J mice were infected intranasally with 50  $\mu$ L RV-A1B [ $1 \times 10^6$  plaque-forming units (PFU)] (24). In some experiments, mice were infected with RV-A1B or LLC-MK2-ACE2 supernatant 4 days prior to HCoV-NL63 infection.

#### Histology, Immunofluorescence, and Immunoblotting

After euthanasia, mouse right arteries were perfused with PBS + 5 mM EDTA. The tracheas were intubated, and the lungs were inflated with formalin and processed for paraffin sectioning. Sections were stained with hematoxylin and eosin or immunofluorescence imaging for

anti-HCoV nucleoprotein, anti-nsp3 (GenScript), and anti-double-stranded (ds)RNA (clone rJ2, Sigma-Aldrich, Burlington, MA). Lung lysates were also analyzed for nsp3 protein expression and  $\beta$ -actin by immunoblotting. Lungs were homogenized in lysis buffer containing 1% triton X-100 and protease inhibitors (Sigma-Aldrich). Insoluble particles were removed by centrifugation for 30 min at 4°C, and total protein was measured by bicinchoninic acid (BCA) assay (ThermoFisher, Waltham, MA). Cell extracts were resolved by 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% low-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (Sigma-Aldrich). For nsp3 immunoblotting, lanes were loaded with 50  $\mu$ g of total protein, and membranes were probed with 0.1  $\mu$ g/mL anti-nsp3 (GenScript). Signals were amplified and visualized with 0.01  $\mu$ g/mL goat anti-rabbit IgG-horseradish peroxidase conjugate (ThermoFisher) and chemiluminescence solution (Pierce, Rockford, IL). For  $\beta$ -actin immunoblotting, lanes were loaded with 20  $\mu$ g of total protein, membranes were probed with 0.01  $\mu$ g/mL mouse monoclonal anti- $\beta$ -actin (Sigma-Aldrich), and amplified with 0.01  $\mu$ g/mL rat anti-mouse IgG kappa chain-horseradish peroxidase conjugate (ThermoFisher).

### Bronchoalveolar Lavage

Mouse lungs were processed for bronchoalveolar lavage (BAL) using 1 mL PBS aliquots (33). Cytospins were stained with hematoxylin and eosin (Sigma-Aldrich), and differential counts were determined from 200 cells.

### Next-Generation RNA Sequencing

RNA library generation and next-generation sequencing were performed by the University of Michigan Advanced Genomic Core. Total RNA was prepared using Trizol extraction (Invitrogen). RNA was double purified using RNEasy spin columns (Qiagen) and enriched for mRNA using a Poly (A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA). cDNA libraries for next-generation sequencing were prepared with a xGen Broad-Range RNA Library Prep (IDT, Coralville, IA) with xGen Normalase UDI Primers (IDT). Samples were subjected to 151 bp paired-end sequencing using a NovaSeq S4 flow cell (Illumina, San Diego, CA) according to the Illumina NovaSeq manufacturer's protocol. Reads were mapped to the reference genome GRCm38 (ENSEMBL 102) using STAR v2/7.8a (34) and assigned count estimates to genes with RSEM v1/3.3 (35).

### Quantitative Polymerase Chain Reaction

Total RNA was prepared using Trizol extraction and RNAeasy purification (Qiagen). First-strand cDNA was prepared using the SuperScript IV system (Applied Biosystems, Waltham, MA). Transcript abundance relative to GAPDH was estimated by quantitative polymerase chain reaction (qPCR) using primers listed in Table 1. Viral copy number was measured as described earlier.

### Enzyme-Linked Immunosorbent Assay

Selected cytokines were measured in the bronchoalveolar lavage fluid by enzyme-linked immunosorbent assay (ELISA). We measured levels of IL-36 $\alpha$  (MyBioSource, San Diego, CA),

IL-1 $\beta$  (Invitrogen, Waltham, MA), IFN- $\alpha$ 1 (BioLegend, San Diego, CA), and IFN- $\lambda$ 2/3 (R&D Systems, Minneapolis, MN). In addition, CXCL1, CXCL2, and CXCL5 cytokine concentrations were measured by the University of Michigan Cancer Center Immunology Core using DuoSet ELISA kits (R&D Systems).

### Measurement of Airways Responsiveness

Mice were anesthetized, intubated, and ventilated with a Buxco FinePointe System (Data Sciences International, St. Paul, MN). Methacholine was administered by nebulizer as previously described (33).

### Data Analysis

For most experiments, statistical significance was assessed by the Kruskal–Wallis test for nonparametric data. Group differences were pinpointed by the Dunn's multiple comparisons test. For airways resistance data, statistical significance was assessed by two-way analysis of variance. Group differences were pinpointed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. A *P* value less than 0.05 was considered statistically significant. Adjusted *P* values were rounded according to the American Physiological Society guideline (36). All data are represented in box and whisker plots, showing the median, 25th and 75th percentiles (the hinges of the box), and min and max (location of the whiskers).

For RNA sequencing (RNA-Seq) analysis, gene expression was estimated according to the expected count totals

**Table 2.** List of GO groups examined for fold change >1.5 after infection with HCoV-NL63 and RV-A1B

| Process       |                                                                    |
|---------------|--------------------------------------------------------------------|
| 1 GO:0001775  | Cell activation                                                    |
| 2 GO:0001776  | Leukocyte homeostasis                                              |
| 3 GO:0001816  | Cytokine production                                                |
| 4 GO:0002200  | Somatic diversification of immune receptors                        |
| 5 GO:0002252  | Immune effector process                                            |
| 6 GO:0002253  | Activation of immune response                                      |
| 7 GO:0002262  | Myeloid cell homeostasis                                           |
| 8 GO:0002339  | B cell selection                                                   |
| 9 GO:0002404  | Antigen sampling in mucosal-associated lymphoid tissue             |
| 10 GO:0002440 | Production of molecular mediator of immune response                |
| 11 GO:0002507 | Tolerance induction                                                |
| 12 GO:0002520 | Immune system development                                          |
| 13 GO:0002532 | Production of molecular mediator involved in inflammatory response |
| 14 GO:0002682 | Regulation of immune system process                                |
| 15 GO:0002683 | Negative regulation of immune system process                       |
| 16 GO:0002684 | Positive regulation of immune system process                       |
| 17 GO:0006955 | Immune response                                                    |
| 18 GO:0007017 | Microtubule-based process                                          |
| 19 GO:0007267 | Cell-cell signaling                                                |
| 20 GO:0019882 | Antigen processing and presentation                                |
| 21 GO:0023056 | Positive regulation of signaling                                   |
| 22 GO:0023057 | Negative regulation of signaling                                   |
| 23 GO:0031294 | Lymphocyte costimulation                                           |
| 24 GO:0045058 | T cell selection                                                   |
| 25 GO:0045321 | Leukocyte activation                                               |
| 26 GO:0050900 | Leukocyte migration                                                |
| 27 GO:0090713 | Immunological memory process                                       |

GO, gene ontology; HCoV, human coronavirus; RV, rhinovirus.

extracted through sequence alignment. Differential gene expression for HCoV-NL63 versus sham and RVA-1B versus sham was determined using R package DESeq2 v1/38.1 with default parameters (37). DESeq2 uses an empirical Bayes approach to integrate the dispersion and fold change estimates and tests the gene differential expression using the Wald test. The Wald test *P* values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. Genes with an adjusted *P* value less than 0.05 were considered statistically significant. Of note, sequencing of specimens collected 4 days after viral infection was performed at a later date than sequencing of *day 2* specimens, allowing the possibility of batch effects. Potential batch effects were considered to be negligible. Also, because of the small number of samples, we also noted genes as those with a fold change of  $\geq 1.5$  or  $\leq 0.66$  ( $\log_2$  fold change  $\geq 0.585$  or  $\leq -0.585$ ). Using the R package clusterProfiler v4/6.0 (38), differentially expressed genes were categorized into biological process gene ontology groups at level 3 specificity. We focused on 27 gene ontology groups related to the immune process (see Table 2). Individual upregulated genes of interest—for example, genes encoding interferons (IFNs), interferon-stimulated genes, and proinflammatory cytokines and chemokines—were selected for fold change comparison.

## RESULTS

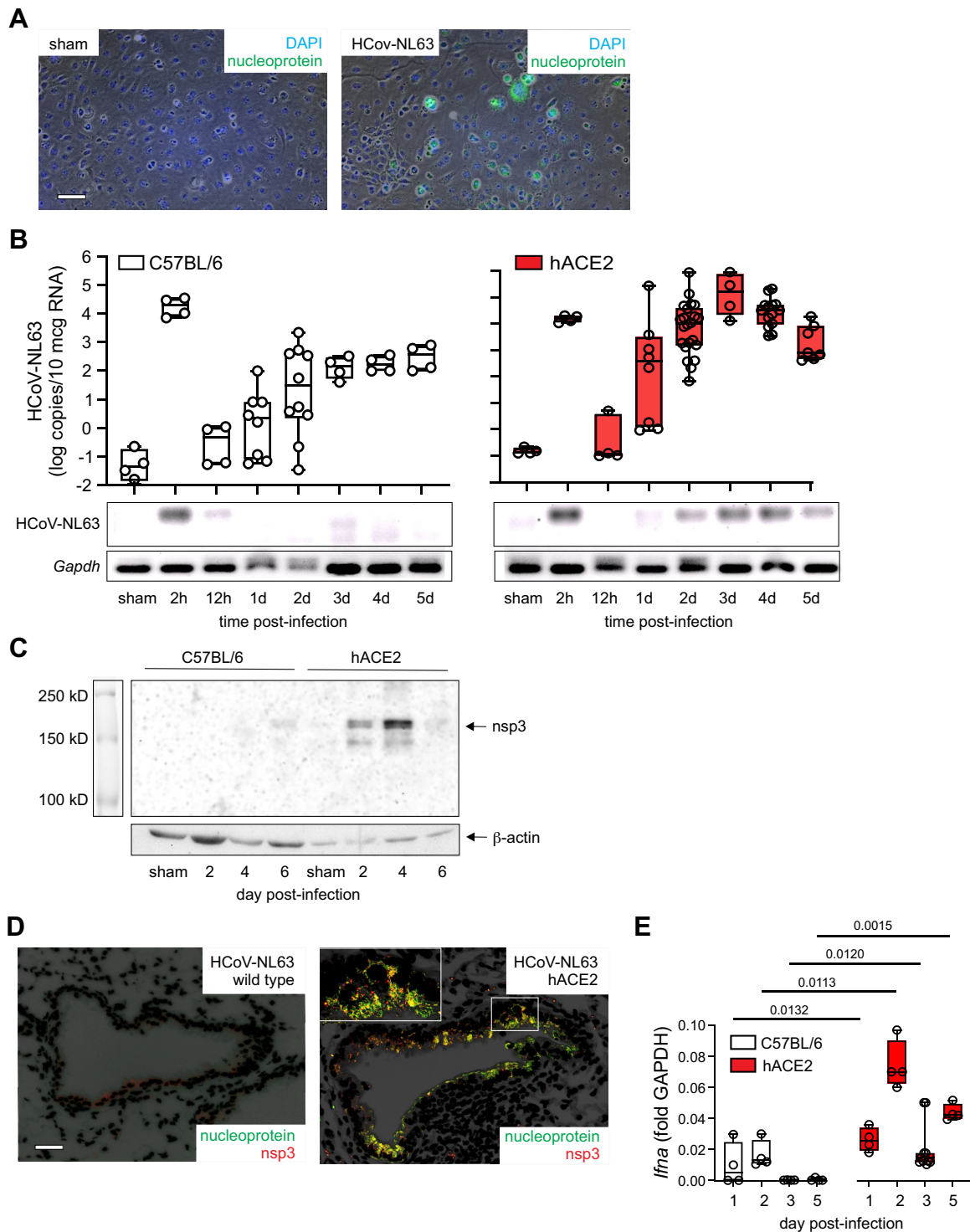
### HCoV-NL63 Propagation in K18-hACE2 Mice

Adult K18-hACE2 and C57BL/6J mice were infected with  $1 \times 10^5$  TCID<sub>50</sub> HCoV-NL63 or sham intranasally and euthanized at 0–6 days posttreatment. Viral copy number was assessed by qPCR and conventional PCR (Fig. 1B). The amount of “input” virus was measured 2 h after inoculation and did not differ between mouse strains. Because mice are relatively nonpermissive to human viral infections, viral copy number fell precipitously after inoculation of both C57BL/6J and hACE2 strains. Viral copy number appeared to increase with time in both strains, but median lung vRNA rose above the input amount only in the hACE2 mice (3 and 4 days after inoculation). Also, only samples from hACE2 mice showed a clear HCoV-NL63 PCR band 1–5 days after infection. The only solid band for the C57BL/6 mice occurred 2 h after inoculation, representing the viral input. Later time points for the C57BL/6 mice showed non-specific bands, which may have led to an elevation of the qPCR signal. Immunoblots of the HCoV-NL63 nonstructural protein nsp3 showed a similar pattern (Fig. 1C), with a strong band 2 and 4 days after inoculation only in hACE2 mice. HCoV-NL63 nucleoprotein was detected on the apical surface of airway epithelial cells of HCoV-NL63-infected K18-hACE2 mice but not sham-infected K18-hACE2 mice, and Nsp3 was detected in the cytoplasm of airway epithelial cells (Fig. 1D). Induction of lung IFN $\alpha$ 1 mRNA expression (Fig. 1E) was significantly higher in hACE2 mice than C57BL/6 mice, further evidence of viral replication (39). Together, these data demonstrate that meaningful viral replication occurred in HCoV-NL63-infected hACE2 mice but was negligible in C57BL/6 mice.

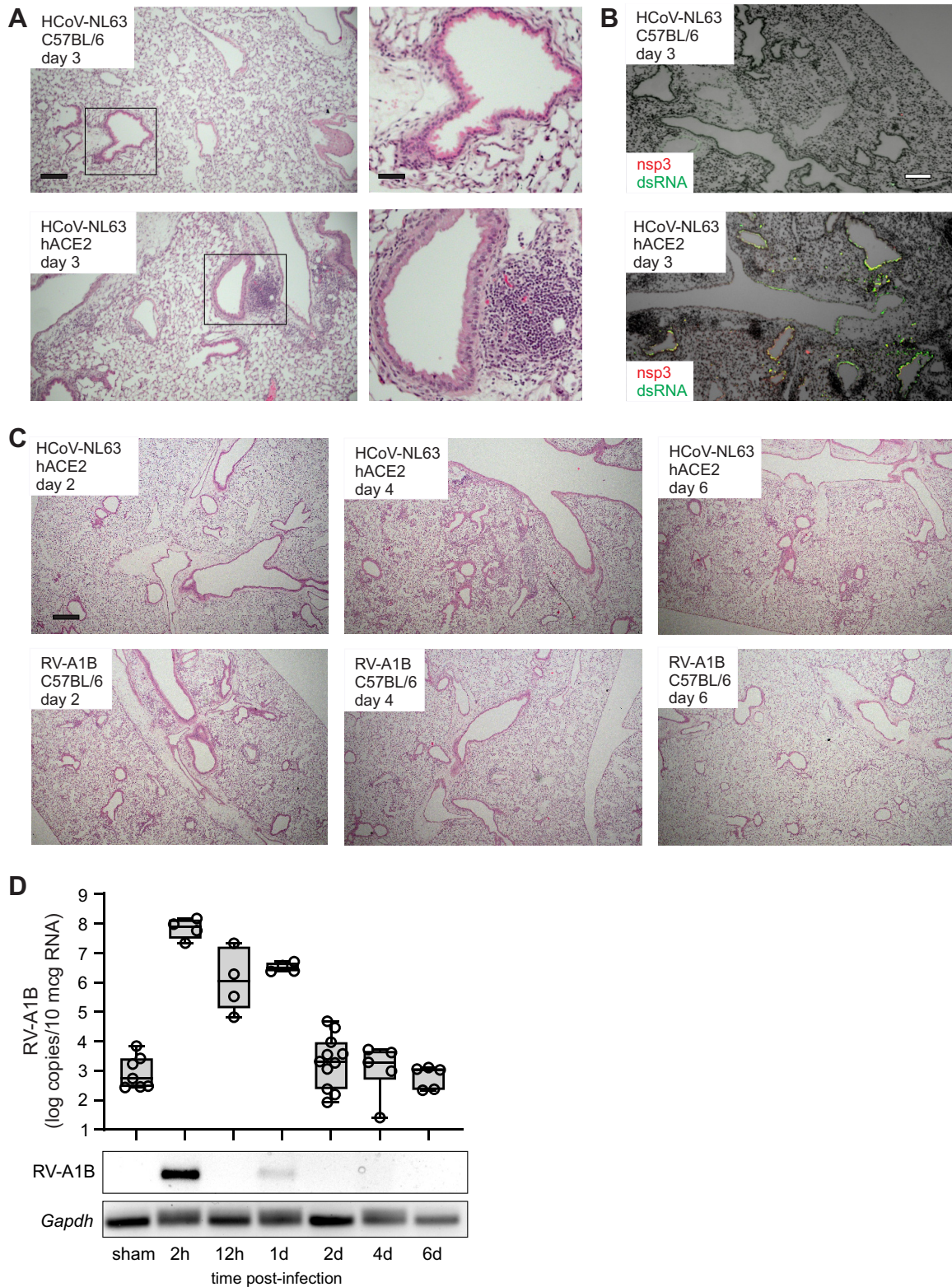
### HCoV-NL63 Infection Induces Airway Inflammation in hACE2 Mice

HCoV-NL63 infection did not cause mortality or weight loss in the mice, consistent with most human infections. To examine the effect of HCoV-NL63 infection on lung inflammation, lungs were perfused with saline, fixed with formalin, processed for paraffin sectioning, and stained with hematoxylin and eosin. Lungs from HCoV-NL63-infected hACE2 mice, but not C57BL/6 mice, showed bronchovascular and peribronchial inflammation (Fig. 2, A–C). Three days after inoculation of hACE2 mice, areas of inflammation appeared to correlate with immunofluorescent staining for nsp3 and dsRNA (Fig. 2B). We compared the histological appearance and viral copy number of lungs from NL63-infected mice with lungs from RV-A1B-infected mice (Fig. 2C). There was little or no inflammation 2 days after HCoV-NL63 infection; inflammation peaked 4 days after NL63 infection and was maintained 6 days after infection. In contrast, lung inflammation peaked 2 days after RV-A1B infection and was minimal or absent 6 days after infection. These data are consistent with the time course of viral replication in HCoV-NL63-infected mice (Fig. 1B) and RV-A1B-infected mice, for which vRNA peaked 1 day after inoculation (Fig. 2D). BAL neutrophils and lymphocytes were higher in hACE2 mice compared with wild-type mice (Fig. 3A). There was no significant difference in macrophages or eosinophils between wild-type and hACE2 mice. Lung expression of *Ifng*, *Cxcl1*, and *Cxcl10* was significantly increased after infection in HCoV-NL63-treated K18-hACE2 but not C57BL/6J mice (Fig. 3B). Compared with sham infection, hACE2 mice inoculated with HCoV-NL63 showed airway cholinergic hyperresponsiveness 4 days after inoculation (Fig. 3C). HCoV-NL63-infected hACE2 mice also showed significantly higher airways resistance than HCoV-NL63-infected C57BL/6 mice.

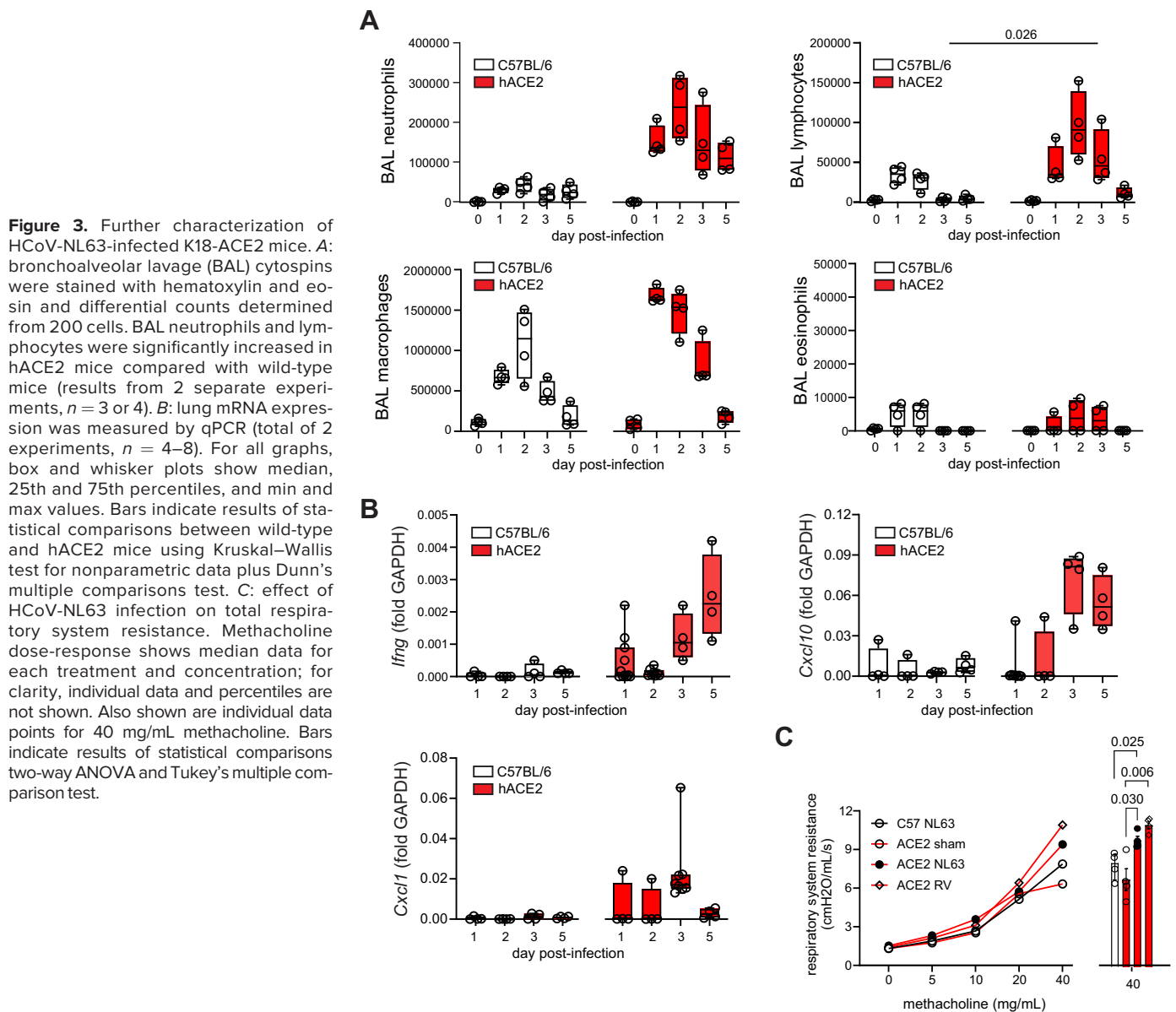
To further characterize the response of hACE2 mice to HCoV-NL63 infection, we analyzed lungs from sham and HCoV-NL63-infected mice using next-generation RNA sequencing. For comparison, we also examined gene expression in RV-A1B-infected mice. In this analysis, the following groups were studied: 1) *day 2* after sham infection (LLC-MK2-ACE2 supernatant, *n* = 3); 2) *day 2* after NL63 infection (*n* = 3); 3) *day 4* after HCoV-NL63 infection (*n* = 3), *day 2* after RV-A1B infection (*n* = 3), and *day 4* after RV-A1B infection (*n* = 3). In this experiment, mean viral copy number two days after infection for the two viruses was comparable ( $4.85 \times 10^3$  copies for NL63 and  $4.75 \times 10^3$  copies for RV-A1B), despite a 10-fold higher input for RV-A1B. Two days following HCoV-NL63 infection, 433 immune process genes were differentially expressed compared with sham (adjusted *P* value < 0.05), with 189 upregulated and 244 downregulated (volcano plots are shown in Fig. 4A). Of note, numerous IFN-stimulated genes were increased, including *Ifi27*, *Ifi35*, *Ifi44*, *Ifih1*, *Ifit1-3*, *Ifitm3*, *Isg15*, *Isg20*, *Mx2*, *Oasl1*, and *Oasl2*. Four days following HCoV-NL63 infection, 707 immune process genes were differentially expressed compared with sham, with 306 upregulated and 401 downregulated. In addition to antiviral genes, genes encoding proinflammatory cytokines were increased including *Il1a*, *Il1b*, *Il6*, *Il18*, *Il33*, *Nlrp3*, *Cxcl2*, *Cxcl3*, *Ccl6*, *Ccl9*, and *Ccl24*.



**Figure 1.** Human coronavirus (HCoV)-NL63 produces a replicative infection in K18-ACE2 mice. **A:** HCoV-NL63 was titrated by examining cytopathic effects in LLC-MK2-ACE2 cells. Cells were infected with sham cell supernatant (*left*) or HCoV-NL63 (*right*). Infection was confirmed by staining with anti-HCoV-NL63 nucleoprotein. The white scale bar is 100  $\mu$ m. **B:** K18-hACE2 or C57BL/6 mice were inoculated with 50  $\mu$ L of  $1 \times 10^5$  TCID<sub>50</sub> HCoV-NL63 intranasally and euthanized at 0–5 days postinfection. Mouse lung viral copy number was assessed by qPCR (total of 7 experiments,  $n = 4–22$ ) and standard PCR. The amount of “input” virus was measured 2 h after inoculation. **C:** lung lysates from sham- and HCoV-NL63-infected wild-type and K18-hACE2 mice were immunoblotted for nsp3 and  $\beta$ -actin. **D:** lung sections from wild-type (*left*) and K18-hACE2 mice (*right*) were stained for HCoV-NL63 nucleoprotein (green) and nsp3 (red). The white scale bar is 100  $\mu$ m. **E:** lung mRNA expression of IFN- $\alpha$  was measured by qPCR (total of 2 experiments,  $n = 4–8$ ). For all graphs, box and whisker plots show median, 25th and 75th percentiles (hinges of the box), and min and max values (whiskers). Bars indicate results of statistical comparisons between wild-type and hACE2 mice using Kruskal–Wallis test for nonparametric data plus Dunn’s multiple comparisons test. ACE2, angiotensin-converting enzyme 2; TCID<sub>50</sub>, tissue culture infectious dose.



**Figure 2.** Human coronavirus (HCoV)-NL63 induces lung inflammation in K18-ACE2 mice. K18-hACE2 or C57BL/6J mice were inoculated with HCoV-NL63 intranasally. **A:** lung sections from wild-type (top) and K18-hACE2 mice (bottom) were stained with hematoxylin and eosin 3 days after infection. In the left, the black scale bars are 200  $\mu$ m. In the right, the black scale bars are 100  $\mu$ m. **B:** lung sections from wild-type (top) and K18-hACE2 mice (bottom) were immunostained for nsp3 (red) and dsRNA (green). The white scale bar is 200  $\mu$ m. **C:** histological appearance of lungs infected with HCoV-NL63 (top) and rhinovirus (RV)-A1B (bottom). The black bar is 400  $\mu$ m. **D:** hACE2 mice were inoculated with 50  $\mu$ L of RV-A1B intranasally and euthanized at 0–6 days posttreatment. Mouse lung viral copy number was assessed by qPCR and conventional PCR. The amount of “input” virus was measured 2 h after inoculation. Box and whisker plots show median, 25th and 75th percentiles, and min and max values (total of 2 experiments,  $n = 5–10$ ). ACE2, angiotensin-converting enzyme 2.

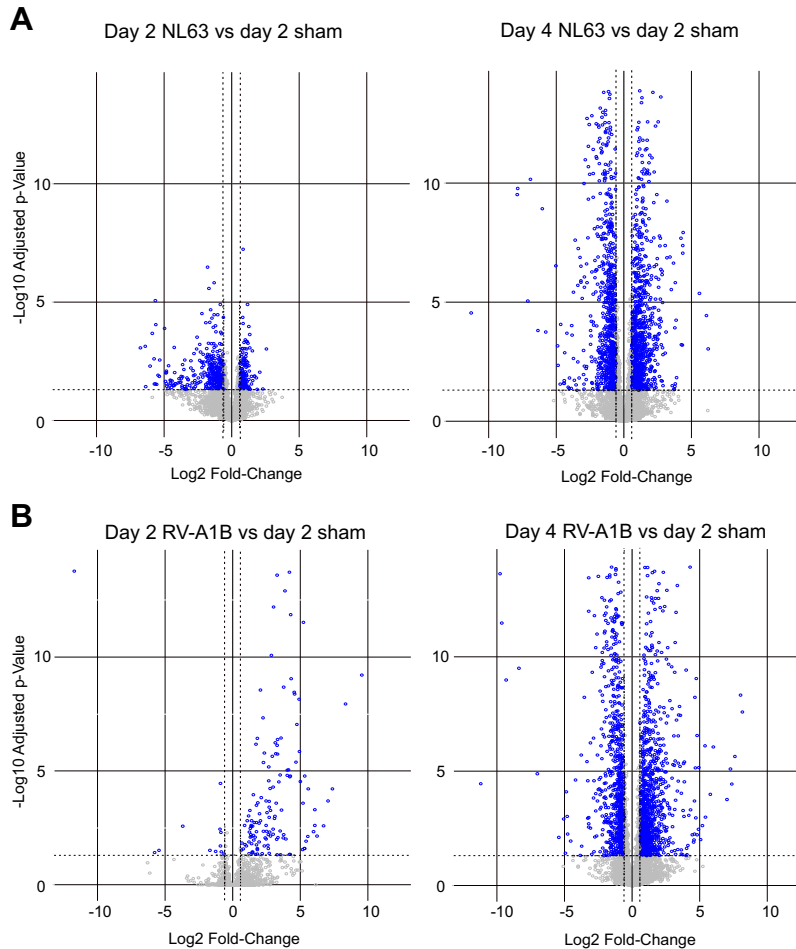


Two days following RV-A1B infection, 54 genes were differentially expressed compared with sham, with 48 upregulated and 6 downregulated (volcano plots are shown in Fig. 4B). Upregulated genes encoding proinflammatory cytokines included three members of the IL-36 family (*Il1f5*, *Il1f6*, and *Il1f8*) and *Cxcl3*. Four days following RV-A1B infection, 767 immune process genes were differentially expressed compared with sham, with 356 upregulated and 421 downregulated. In addition to the IL-36 family members, overexpressed cytokines included *Il1a*, *Il6*, *Il18*, *Il33*, *Cxcl2*, *Cxcl15*, *Ccl3*, *Ccl6*, *Ccl9*, *Ccl11*, and *Ccl24*. In addition, two interferon response genes were significantly upregulated (*Ifi30* and *Ifi207*).

We also directly compared gene expression patterns between HCoV-NL63 and RV-A1B. On day 2, 728 immune process genes were differentially expressed (adjusted  $P$  value  $< 0.05$ ), with 337 upregulated and 391 downregulated. Compared with RV-A1B, among the immune response genes significantly upregulated

by HCoV-NL63 were *Ifi27*, *Ifi35*, *Isg20*, *Ifit3*, and *Cxcl14*. In contrast, genes significantly downregulated compared with RV-A1B were proinflammatory, including *Il1f5*, *Ifi16*, *Il1f8*, *Il1a*, *Il33*, *Nlrp3*, *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl13*, *Cxcl5*, *Tlr1*, *Ccl3*, *Ccl4*, *Ccl9*, and *Ccl24*. However, by day 4, there was little difference in the response to the two viruses, with only 129 immune process genes differentially expressed (adjusted  $P$  value  $< 0.05$ ), with 33 upregulated and 96 downregulated. Among the immune response genes significantly upregulated by HCoV-NL63 on day 4 compared with RV-A1B were *Cxcl3*, *Il1b*, and *Nlrp3*. Genes significantly downregulated compared with RV-A1B included *Il1f5*, *Il1f6*, *Cxcl12*, and *Ccl11*.

We examined genes with greater than 1.5-fold changes in expression after viral infection, focusing again on expression of IFNs, antiviral IFN-stimulated proteins, and proinflammatory cytokines and chemokines. On day 2, HCoV-NL63 increased expression of more ISGs and fewer proinflammatory genes as compared with RV-A1B (Fig. 5A). The most



**Figure 4.** RNA sequencing (RNA-Seq) volcano plots comparing gene expression patterns in response to HCoV-NL63 infection (A) and rhinovirus (RV)-A1B infection (B). A: comparison between HCoV-NL63 infection and sham infection. B: Comparison between RV-A1B infection and sham infection.

highly expressed genes encoded the type I and III IFNs, *Ifnb1* and *Ifnl3*. In contrast, RV-A1B increased expression of more proinflammatory genes and fewer ISGs (Fig. 5B). The most highly expressed genes encoded the IL-36 isoforms, *Il1f6* (IL-36 $\alpha$ ) and *Il1f8* (IL-36 $\beta$ ). By day 4, both infections induced a mix of antiviral and proinflammatory genes. (Complete RNASeq data have been uploaded to NCBI Gene Expression Omnibus under GEO accession number GSE254967; see <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE254967>.)

We then measured transcription of selected genes by qPCR. Compared with sham treatment (LLC-MK2-ACE2 cell supernatant), both HCoV-NL63 and RV-A1B induced mRNA expression of IFNs, IFN-stimulated genes, and proinflammatory cytokines (Fig. 6). There were no statistically significant differences in gene expression between the two viruses except for *Ifi35*, which was greater after HCoV-NL63. Expression of *Mx2* also tended to be higher after HCoV-NL63 infection. Expression of *Il1f6*, *Il1b*, *Cxcl2*, and *Cxcl10* tended to be higher after RV-A1B infection.

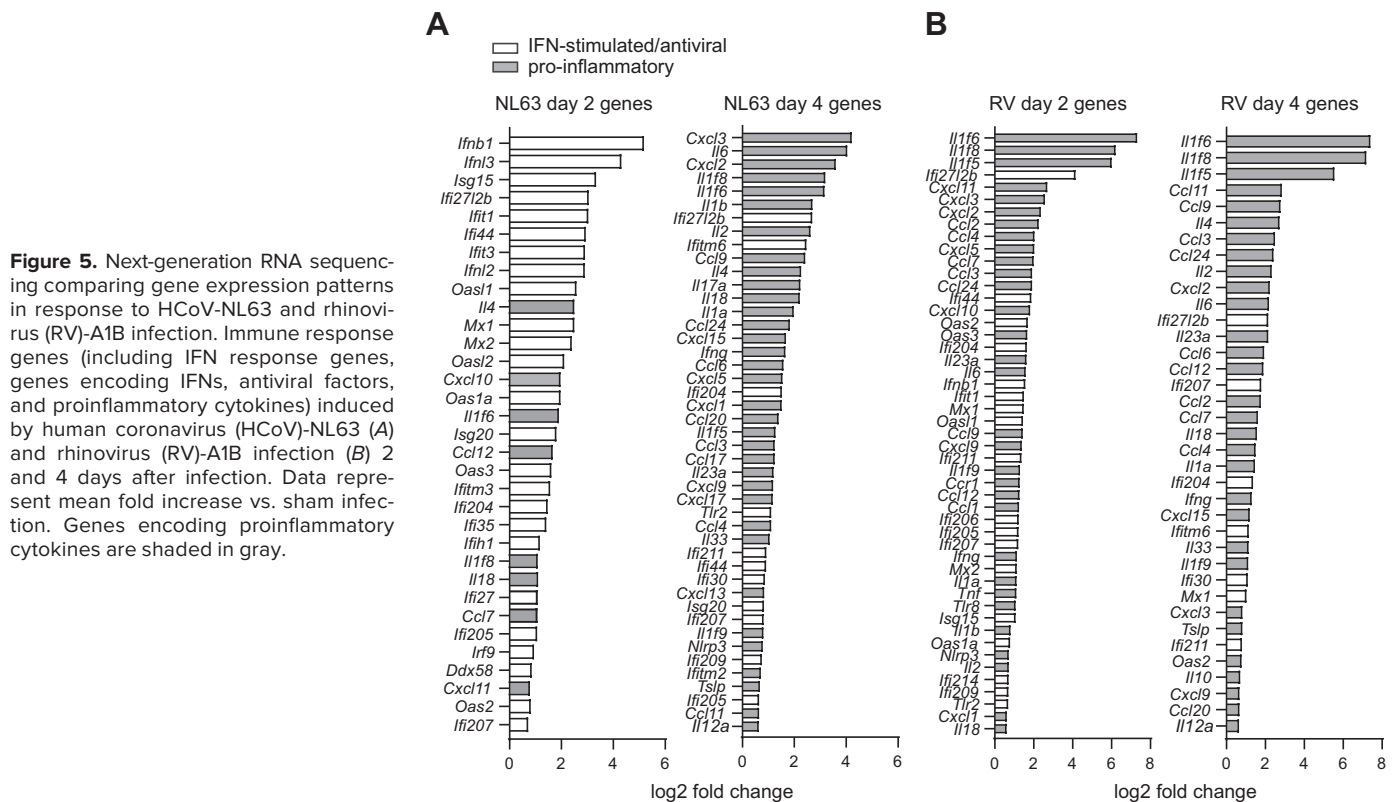
#### BAL Cytokines in HCoV-NL63-Infected and RV-A1B-Infected Mice

To compare the response to infection in HCoV-NL63-infected and RV-A1B-infected mice, we also measured the time course of selected cytokines in the BAL (Fig. 7). HCoV-

NL63 significantly increased expression of *Ifna* and *Ifnl*, and RV-A1B significantly increased expression of *Ifna*, *Il1f6*, and *Il1b*. Levels of the interferons IFN- $\alpha$ 1 and IFN- $\lambda$ 2/3 tended to be higher after HCoV-NL63 infection, and levels of the proinflammatory cytokines IL-36 $\alpha$  and IL-1 $\beta$  tended to be higher after RV-A1B infection, but there was no statistically significant difference in protein levels between the two viruses.

#### Infection with RV-A1B Significantly Decreases HCoV-NL63-Induced Viral Replication and Airway Inflammation In Vivo

Previous studies revealed that RV-A16 blocks SARS-CoV-2 replication in cultured airway epithelial cells (25) and RV-A1A blocks SARS-CoV-2 replication in airway epithelial organoids (26). We therefore tested whether RV-A1B infection 4 days prior to HCoV-NL63 infection blocks viral replication in our model. Mice were infected with sham or RV-A1B 4 days before HCoV-NL63 infection. Lung RNA was harvested 2 days after sham or HCoV-NL63 infection. Thus, RV-A1B responses were measured 6 days after infection (Fig. 8A). There was no RV-A1B present at this time point (Fig. 2D). Prior RV-A1B infection significantly decreased HCoV-NL63 copy number in hACE2 mice (Fig. 8B). Prior RV-A1B infection also reduced neutrophils and lymphocytes in HCoV-NL63-infected hACE2 mice (Fig. 8C) and peribronchial inflammation (Fig. 8D). Finally, prior RV-A1B infection reduced viral



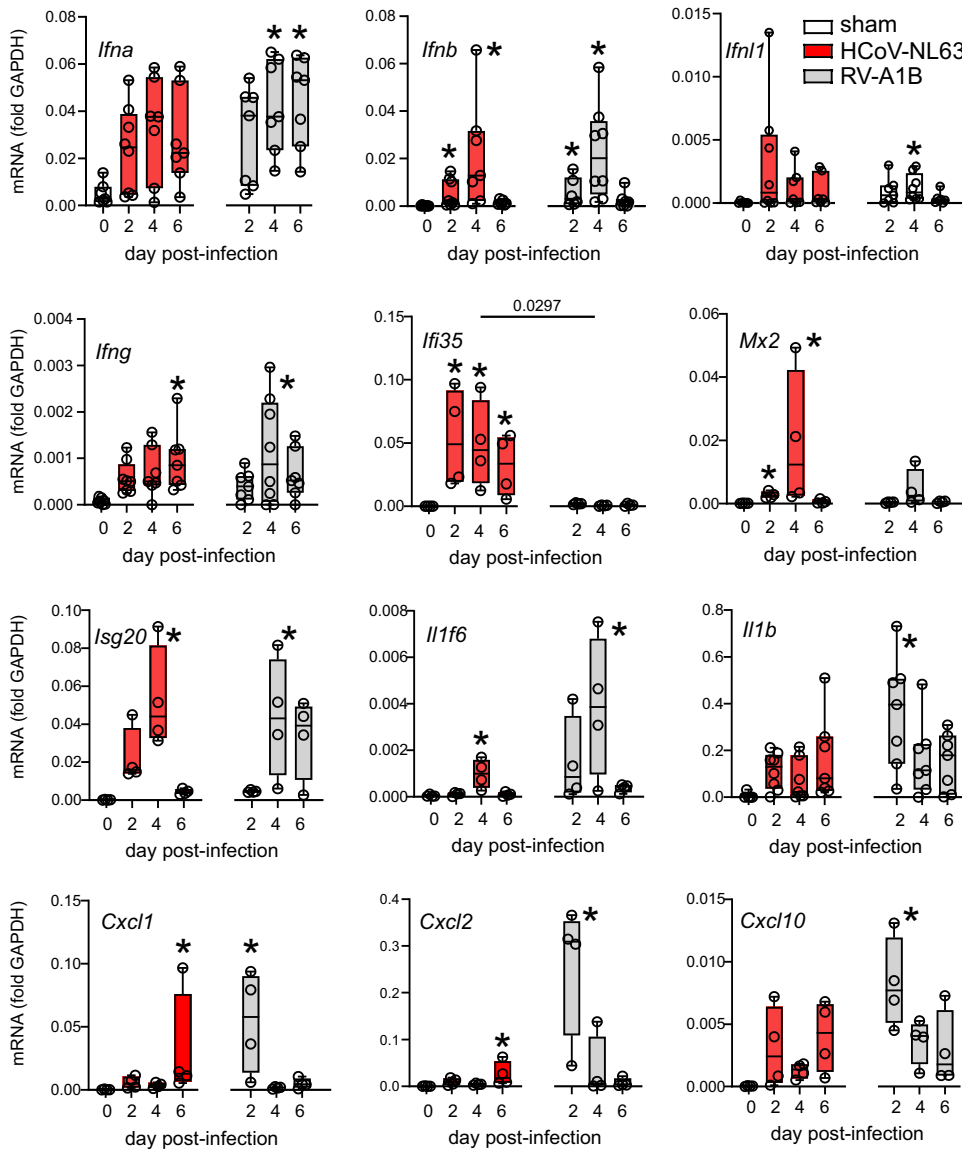
expression of nsp-3 (Fig. 8E). There was no effect of RV-A1B infection on hACE2 expression (Fig. 8F).

### Effect of Prior RV-A1B Infection on HCoV-NL63-Induced mRNA Expression

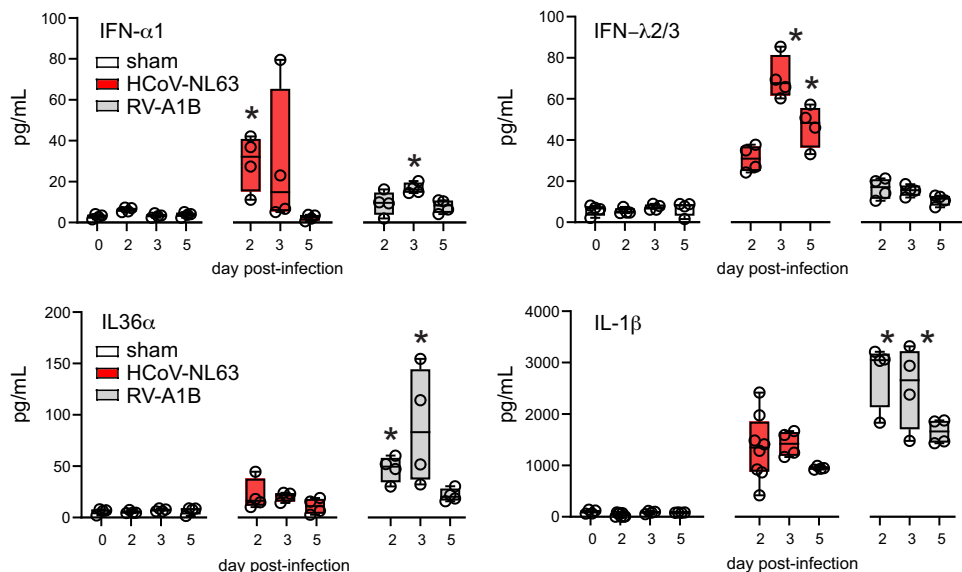
Using next-generation RNA sequencing, we compared lungs harvested from ACE2 mice pretreated with RV-A1B 4 days prior to HCoV-NL63 infection ( $n = 3$ ) with mice that underwent HCoV-NL63 infection ( $n = 3$ , described earlier). Lungs were harvested 2 days after HCoV-NL63 infection. Although we found statistically significant differences in gene expression between hCoV-NL63 and sham, RV-A1B and sham, and hCoV-NL63 and RV-A1B (see Fig. 4), there were no statistically significant differences in the expression of immune process genes between RV-A1B + hCoV-NL63 and hCoV-NL63 alone. This was in part because of the small number of samples. Therefore, we examined the immune response genes with the highest fold changes (all of which had log 2 fold change  $\geq 0.585$  or  $\leq -0.585$ ) (Fig. 9). Mice pretreated with RV-A1B showed greater than 1.5-fold reductions in the expression of IFN and IFN-stimulated genes, likely reflecting reduced viral replication. However, when we compared double-infected mice with sham-treated mice, we found that 30 genes were significantly upregulated, including increases in the expression of the antiviral IFN-stimulated genes *Mx2*, *Isg20*, and *Ifi35*. In addition, compared with sham, mice pretreated with RV-A1B showed greater than 1.5-fold increases in many IFN and IFN-stimulated genes including *Isg15*, *Ifi27*, *Ifi35*, *Ifi44*, *Ifi204*, *Ifi205*, *Ifi207*, *Ifi211*, *Ifi1*, *Ifi2*, *Ifi3*, *Ifih1*,

*Ifitm3*, *Irf7*, *Irf9*, *Isg20*, *Mx1*, *Mx2*, *Oasl1*, *Oasl1a*, *Oas2*, and *Oas3* (Fig. 7).

Next, we used qPCR to determine the effect of RV-A1B preinfection on selected genes. We compared four experimental groups: 1) mice inoculated with sham LLC-MK2-ACE2 supernatant 4 days before inoculation with a second dose of sham LLC-MK2-ACE2 supernatant, harvested 2 days after sham infection (sham); 2) mice infected with sham LLC-MK2-ACE2 supernatant 4 days before infection with HCoV-NL63, harvested 2 days after HCoV-NL63 infection (HCoV-NL63); 3) mice infected with RV-A1B 4 days before infection with LLC-MK2-ACE2 cell supernatant, harvested 6 days after RV infection and 2 days after sham infection (RV-A1B); and 4) mice infected with RV-A1B four days before infection with HCoV-NL63, harvested 6 days after RV-A1B infection and 2 days after HCoV-NL63 infection (RV-A1B + HCoV-NL63). Similar to previous results, HCoV-NL63 induced the expression of *Ifna*, *Ifnb1*, *Ifnl3*, and several ISGs including *Ifi35*, *Isg20*, *Oasl1*, and *Mx2* (Fig. 10). Increases in IFN and ISG expression were relatively small in response to RV-A1B, likely reflecting the fact that samples were collected 6 days after infection. When we examined HCoV-NL63-induced lung IFN and ISG mRNA expression in mice preinfected with RV-A1B, *Ifnb1*, *Ifnl3*, and *Ifi35* were significantly decreased compared with HCoV-NL63 alone. None of the IFNs or ISGs were increased by RV-A1B preinfection. However, compared with sham, mice infected with RV-A1B and HCoV-NL63 showed increases in *Ifi35*, *Isg20*, *Oasl1*, and *Mx2* despite the absence of HCoV-NL63 replication, indicating the influence of IFN production by prior RV-A1B infection.

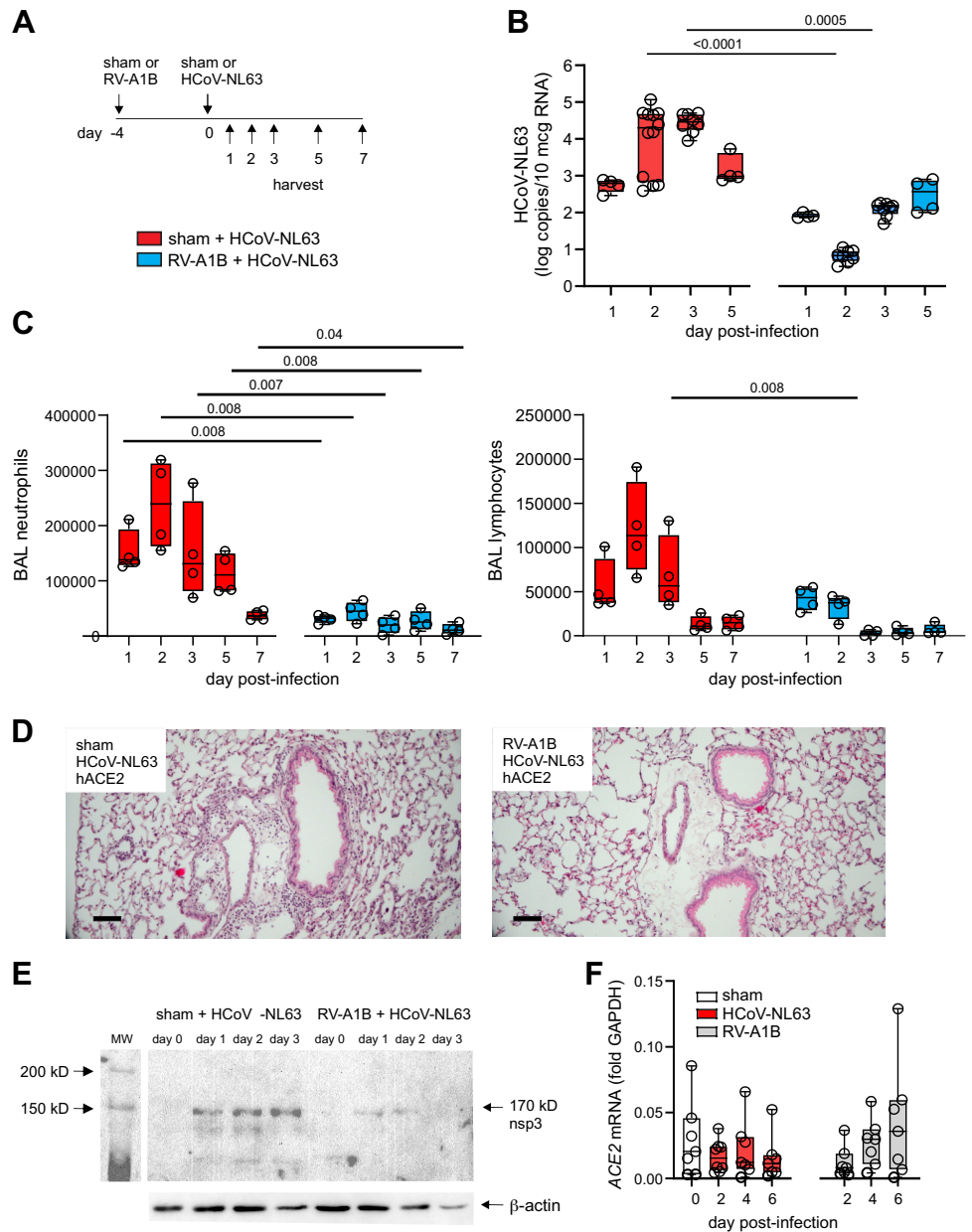


**Figure 6.** Lung mRNA expression by qPCR in HCoV-NL63- and rhinovirus (RV)-A1B-infected mice. Box and whiskers plots show median, 25th and 75th percentiles, and min and max values. Bars indicate results of statistical comparisons between HCoV-NL63 and RV-A1B. Significant increases vs. sham infection are also shown by the asterisks (\* $P < 0.05$ ). (One or two experiments each panel,  $n = 4-8$ ; Kruskal–Wallis test for non-parametric data plus Dunn’s multiple comparisons test.)



**Figure 7.** Bronchoalveolar lavage (BAL) cytokines in human coronavirus (HCoV)-NL63- and rhinovirus (RV)-A1B-infected mice. We measured the time course of selected cytokines in the BAL by ELISA. IL-36 $\alpha$  and IL-1 $\beta$  tended to be higher after RV-A1B infection, and IFN- $\alpha$ 1 and IFN- $\lambda$ 2/3 tended to be higher after HCoV-NL63 infection (2 experiments,  $n = 4-8$ ). Box and whisker plots show median, 25th and 75th percentiles, and min and max values. Bars indicate results of statistical comparisons between HCoV-NL63 and RV-A1B. In addition, significant increases vs. sham infection are also shown by the asterisks (\* $P < 0.05$ , Kruskal–Wallis test for non-parametric data plus Dunn’s multiple comparisons test).

**Figure 8.** Rhinovirus (RV)-A1B preinfection attenuates human coronavirus (HCoV)-NL63 replication and airway inflammation in K18-ACE2 mice. **A:** mice were infected with sham or RV-A1B 4 days before sham or HCoV-NL63 infection. **B:** lung HCoV-NL63 vRNA was harvested 2 days after HCoV-NL63 infection for viral copy number analysis by qPCR (total of 3 experiments,  $n = 4$ –12, the dotted line indicates the detection limit of the assay). **C:** bronchoalveolar lavage (BAL) cytopins were stained with hematoxylin and eosin and differential counts determined from 200 cells. BAL neutrophils (*left*) and lymphocytes (*right*) are shown (results from 2 separate experiments,  $n = 4$ /group). **D:** lung sections from hACE2 mice infected with sham and HCoV-NL63 (*left*) and hACE2 mice infected with RV-A1B and HCoV-NL63 (*right*) were stained with hematoxylin and eosin. The black scale bar is 100  $\mu$ m. **E:** lung lysates from sham- and HCoV-NL63-infected hACE2 mice (*left*) and RV-A1B and HCoV-NL63-infected hACE2 mice (*right*) were immunoblotted for nsp3. **F:** effect of HCoV-NL63 and RV-A1B infection on human ACE2 mRNA expression (total of 2 experiments,  $n = 7$ ). For all graphs, box and whisker plots show median, 25th and 75th percentiles, and min and max values. Bars indicate results of statistical comparisons between HCoV-NL63 and RV-A1B infections using Kruskal–Wallis test for nonparametric data plus Dunn’s multiple comparisons test.

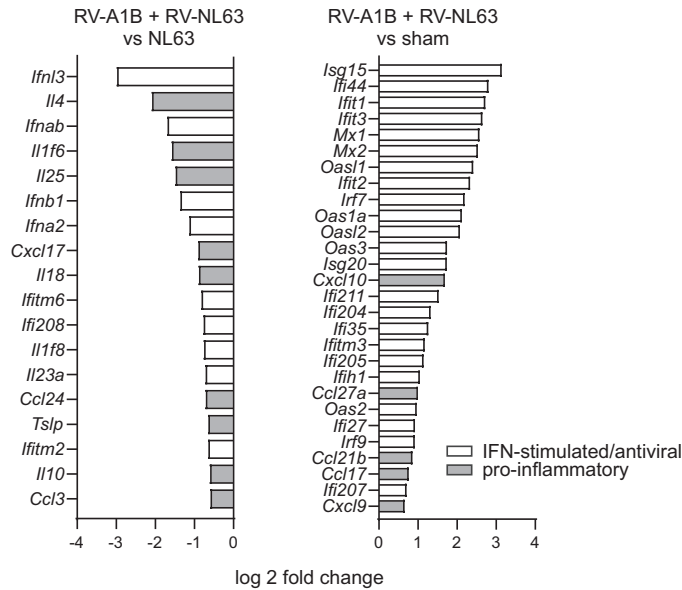


RV-A1B infection significantly increased the expression of proinflammatory mRNAs including *Il1f6*, *Tlr2*, *Il1b*, *Nlrp3*, *Ifng*, *Cxcl10*, and *Cxcl2* (Fig. 11A). Compared with RV-A1B alone, lungs from mice infected with RV-A1B and HCoV-NL63 showed reduced lung mRNA expression of *Il1f6*, *Nlrp3*, *Ifng*, *Cxcl10*, and *Cxcl2*, consistent with the reduced lung inflammation observed in these mice. On the other hand, we identified several transcripts in which RV-A1B preinfection increased HCoV-NL63-induced lung mRNA expression, including *Cxcl1* (Fig. 11B). To further examine the effect of RV-A1B preinfection on neutrophil chemoattractants, we examined the protein levels of CXCL1, CXCL2, and CXCL5 by ELISA (Fig. 12). We found that, although HCoV-NL63 alone significantly increased protein abundance of CXCL1, CXCL2, and CXCL5, mice preinfected with RV-A1B did not experience an increase in CXC chemokine expression over sham, consistent with the

reduction in lung neutrophils with double infection. The discrepancy between *Cxcl1* mRNA and protein levels of CXCL1 likely represents posttranscriptional regulation.

## DISCUSSION

We established a mouse model of HCoV-NL63 using mice expressing human ACE2 under control of the keratin 18 promoter. Compared with wild-type mice, hACE2 mice showed significantly higher levels of HCoV-NL63 vRNA and nsp3, a nonstructural viral protein that is produced in replicating virus. In addition, HCoV-NL63-infected hACE2 mice showed increased BAL neutrophils and lymphocytes, as well as peribronchial and perivascular infiltrates compared with mock-infected controls. Together, these data suggest that HCoV-NL63 causes a replicative infection in hACE2 transgenic



**Figure 9.** Next-generation RNA sequencing, examining the effect of rhinovirus (RV)-A1B preinfection on gene expression induced by human coronavirus (HCoV)-NL63. Immune response genes (including IFN response genes, genes encoding IFNs, antiviral factors, and proinflammatory cytokines) are shown. *Left* panel compares RV-A1B + HCoV-NL63 with HCoV-NL63 alone. *Right* panel compares RV-A1B + HCoV-NL63 with sham infection. Lung samples were taken 2 days after HCoV-NL63 or sham infection. Data represent mean fold increase vs. sham infection. Genes encoding proinflammatory cytokines are shaded in gray.

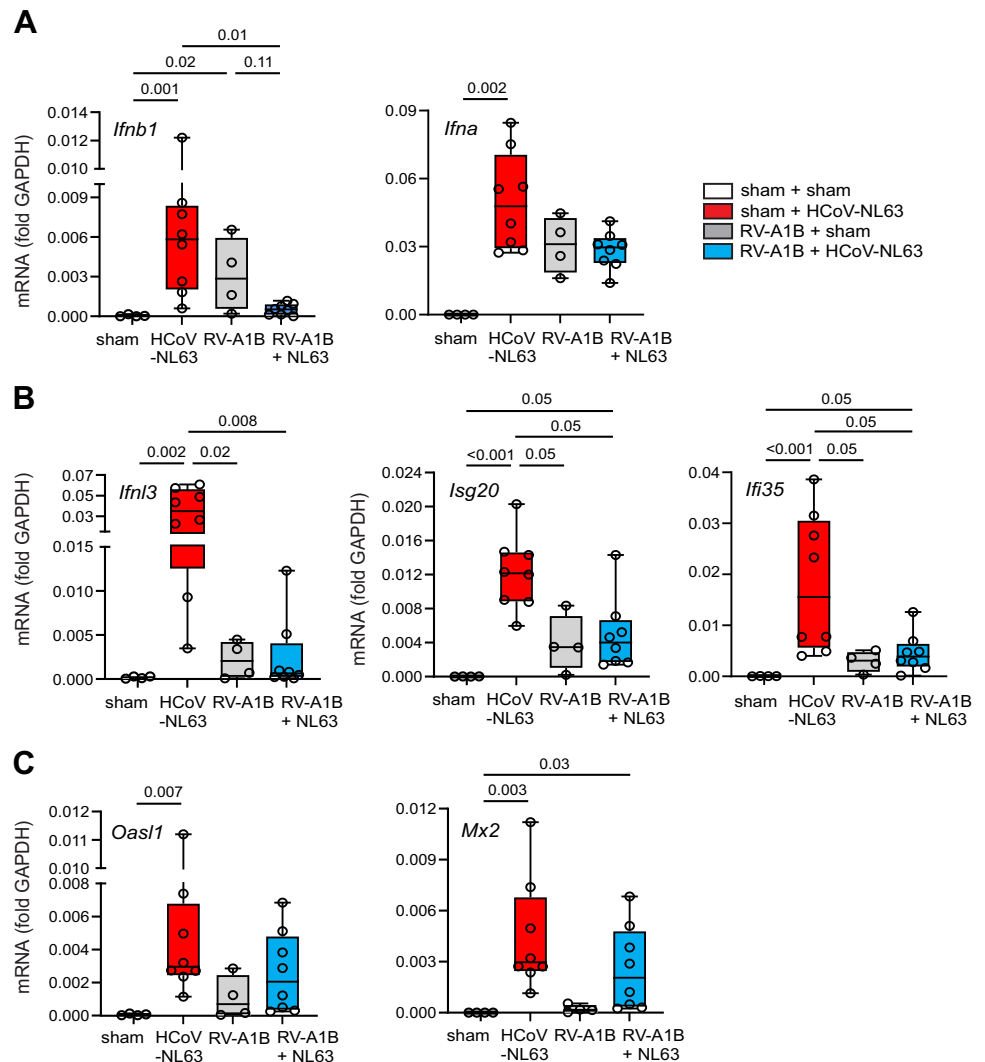
mice. Our work provides a BSL-2 animal model to study human CoV infection in mice.

Viral infections trigger nearly 80% of asthma exacerbations in children and ~50% in adults (40, 41). An early study showed that HCoVs comprised 17% of all viral infections in children with asthma. We found a similar percentage of HCoV infections in children with asthma (42, 43). Viral infections also induce a substantial fraction of chronic obstructive pulmonary disease exacerbations (44, 45). Understanding of viral-induced exacerbations is incomplete, in part due to the paucity of animal models. We previously developed a mouse model of RV-A1B infection, a minor group virus that binds to LDL-R. Based on partial homology between human and mouse LDL-R, mice infected with RV-A1B show limited viral replication, as evidenced by IFN production and the presence of double-stranded viral RNA (24). However, viral replication is limited and peaks the day after inoculation (24, 46–48). In contrast, HCoV-NL63 replication peaked 3 days after infection of hACE2 mice and persisted for 1 wk. Accordingly, we found that mRNA expression of many IFNs and interferon-stimulated genes was greater for HCoV-NL63 than for RV-A1B, especially on *day 2* after infection. qPCR and ELISA data tended to confirm this general chronology. With regard to histological changes, inflammation peaked 4 days after HCoV-NL63 infection and was present 6 days after infection, in contrast with RV-A1B, which peaked 2 days after infection. Thus, by *day 4*, both viruses induced a proinflammatory gene expression profile. On the other hand, mRNA and protein expression of some proinflammatory genes tended to be higher after RV-A1B infection,

including members of the IL-36 family. Establishment of this mouse model of HCoV-NL63 infection may provide insights into the cellular and molecular mechanisms underlying endemic CoV infections and their potential role in the exacerbation of airway inflammation. In addition, the level of HCoV-NL63 replication in this model might offer advantages compared with RV when studying viral-induced asthma exacerbations. For example, although it has been posited that allergic sensitization interferes with the IFN response to viral infection (49, 50), this cannot easily be tested in the RV model because of limited viral replication and IFN response.

Previous studies have examined the influence of prior RV-A1B exposure on HCoV infection in cultured cells. RV-A1B blocks SARS-CoV-2 replication in cultured airway epithelial cells (25). Cells infected with RV and SARS-CoV-2 showed higher levels of MxA, the product of an interferon-stimulated gene (*Mx1*) that participates in the cellular antiviral response. As the *in vitro* efficacy of IFNs against CoVs is well established (51, 52), these data are consistent with the notion that RV-stimulated IFNs block SARS-CoV-2 replication. In addition, it has been shown that RV-A1A blocks SARS-CoV-2 replication in airway epithelial organoids (26). In organoid cultures, SARS-CoV-2 significantly induced ISG expression, but timing was delayed relative to viral replication. Organoids sequentially infected with RV and SARS-CoV-2 showed significantly higher levels of IFN- $\lambda$ 1 production and ISG expression compared with cultures infected with SARS-CoV-2 without prior RV exposure. Using nasal swabs, these investigators also showed that human patients demonstrate a significant enrichment of ISGs after SARS-CoV-2 expression (26). Finally, it has recently been shown that RV protects mice against mouse hepatitis virus strain 1 (MHV-1), a betacoronavirus that naturally infects the enteric tract (53). Mice preinfected with RV had less pulmonary inflammation and hemorrhage and improved survival. However, mice infected with MHV-1 alone had a brisk IFN- $\beta$  and *Mx1* response, and RV infection did not increase *Mx1* expression. Administration of a neutralizing antibody against the IFN  $\alpha/\beta$  receptor caused death in mice infected with a nonlethal dose of MHV-1, suggesting that IFNs are required but not sufficient for protection against MHV-1.

In our study, we examined the influence of prior RV-A1B exposure on HCoV-NL63 infection *in vivo*. We found that prior RV-A1B infection significantly reduced HCoV-NL63 replication and viral-induced peribronchial inflammation, an example of viral interference. However, when we measured IFN and interferon-stimulated gene expression in mice infected with RV-A1B 4 days before HCoV-NL63, there were no instances of increased HCoV-NL63-induced IFN or interferon-stimulated gene mRNA expression after RV-A1B coinfection, including that of *Mx1* or *Mx2*. However, we do not believe that this comparison tells the whole story, as it is possible that IFN and ISG expression was suppressed by the lower viral load in coinfecting mice. Indeed, even in the absence of RV-A1B or HCoV-NL63 viral copies, coinfecting mice showed higher IFN and ISG mRNA expression, including that of *Mx1* and *Mx2*, than sham-infected mice, as evidenced by both RNA-Seq and qPCR. In addition, although there was no appreciable RV-A1B present 4 days after infection, RV-A1B-induced IFN production persisted until that time. Thus,



in our model, we believe that the preponderance of evidence demonstrates that RV-A1B interferes with HCoV-NL63 infection by enhancing IFN production. We would also like to note that, if RV-A1B attenuates HCoV-NL63 replication by enhancing IFN production, it is conceivable that viral mimetics such as poly I:C (54) or TLR2 agonists (27, 55) could have similar effects on a subsequent viral infection.

In conclusion, we established a mouse model of HCoV-NL63 replicative infection that facilitates studies of the host immune response to endemic human CoV infection. Prior infection with RV-A1B reduced HCoV-NL63 replication and airway inflammation. This model may provide insights into the mechanisms underlying viral-induced asthma exacerbations and viral interference.

## DATA AVAILABILITY

Source RNASeq data are openly available at NCBI Gene Expression Omnibus under GEO accession number GSE254967 (see <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE254967>). Additional data are available upon reasonable request.

## ACKNOWLEDGMENTS

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## GRANTS

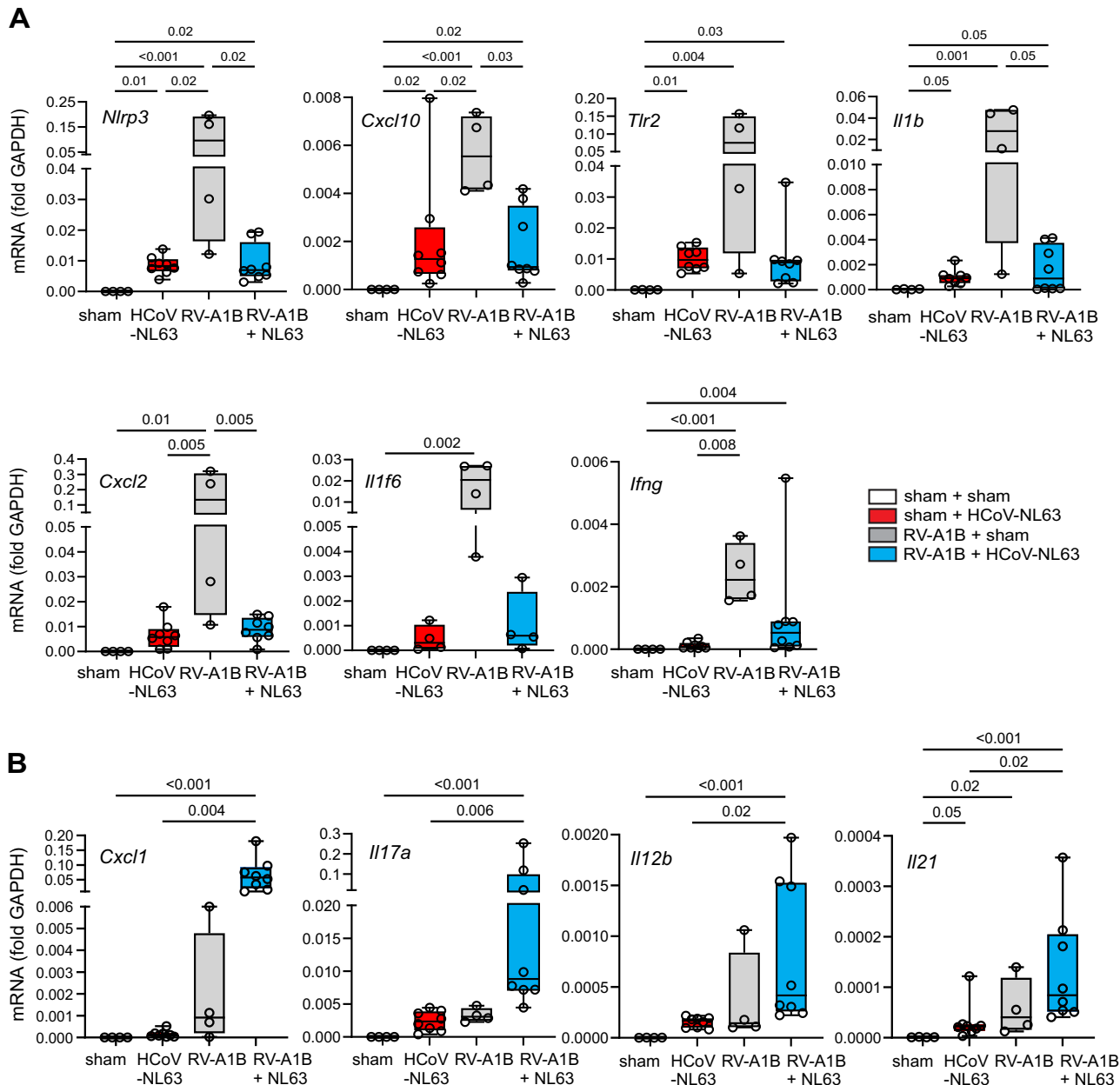
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## DISCLAIMERS

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.



**Figure 11.** Effect of prior rhinovirus (RV)-A1B infection on human coronavirus (HCoV)-NL63-induced mRNA expression, continued. A: lung mRNA expression of selected proinflammatory genes was measured by qPCR. B: mRNAs showing an additive synergistic effect of RV-A1B and HCoV-NL63. Box and whisker plots show median, 25th and 75th percentiles, and min and max values. Total of 2 experiments,  $n = 4-8$ ; bars indicate results of statistical comparisons using Kruskal–Wallis test for nonparametric data plus Dunn’s multiple comparisons test.

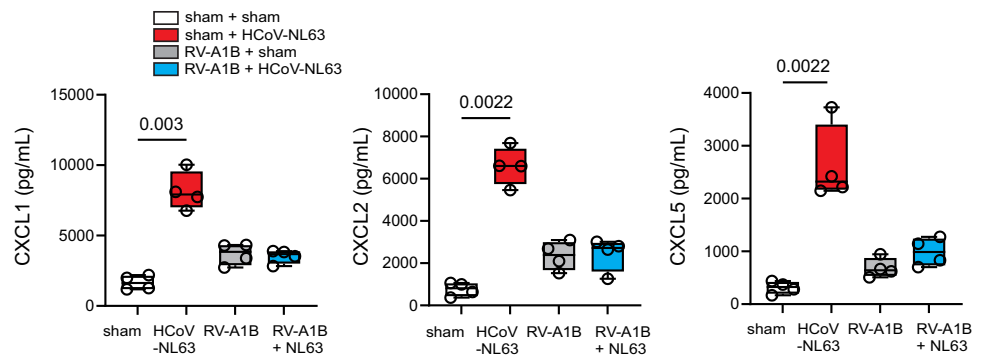
## AUTHOR CONTRIBUTIONS

J.K.B., C.N.L., and M.B.H. conceived and designed research; J.K.B., J.E.K., H.A.B., S.S., and J.L. performed experiments; J.K.B., J.E.K., H.A.B., Y.L., and M.B.H. analyzed data; J.K.B. and M.B.H. interpreted results of experiments; J.K.B., J.E.K., H.A.B., and M.B.H. prepared figures; J.K.B. and M.B.H. drafted manuscript; J.K.B., S.C.B., and M.B.H. edited and revised manuscript; J.K.B. and M.B.H. approved final version of manuscript.

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**Figure 12.** Effect of prior rhinovirus (RV)-A1B infection on human coronavirus (HCoV)-NL63-induced C-X-C chemokine protein abundance measured by ELISA. Box and whisker plots show median, 25th and 75th percentiles, and min and max values (1 experiment,  $n = 4$ ). Bars indicate results of statistical comparisons using Kruskal–Wallis test for non-parametric data plus Dunn's multiple comparisons test.



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