Supplementary Materials

Materials and Methods

Ethics statement

All experiments involving live SARS-CoV-2 were conducted in the Animal Biosafety Level 3 (ABSL3) facility at the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). The Institutional Animal Care and Use Committee of KIZ, CAS, approved all protocols used in this study (Approval No: IACUC-RE-2022-04-001).

Virus strain and cells

The SARS-CoV-2 prototype (viral sequence available in the China National Microbiology Data Center under Accession No. NMDCN0000HUI) was kindly provided by Guangdong Provincial Center for Disease Control and Prevention (Guangdong, China) and has been described in our previous studies (Song et al., 2020; Xu et al., 2020; Zeng et al., 2022). The virus was amplified in Vero-E6 cells. Median tissue culture infective dose ($TCID_{50}$) was used to assess viral infectivity. Virus titers were calculated using the Reed-Muench method (Reed & Muench, 1938).

The Vero-E6 cells were obtained from the Kunming Cell Bank and grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and kept in a humidified 5% CO_2 incubator at 37 °C.

Animals and study design

A total of 41 Sprague-Dawley (SD) rats (four males and 37 females) aged 8–9 weeks were purchased from the Experimental Animal Center of Kunming Medical University. Rats were acclimated to the new environment for a week before SARS-CoV-2 infection. Animals were randomly allocated to different groups for the two experiments. Animals were anesthetized with isoflurane, then intranasally inoculated with 50 μL of SARS-CoV-2 (1×10^5 $TCID_{50}$). All infected animals were housed at the Animal Biosafety Level 3 (ABSL-3) animal core facility of the KIZ on a 12-h light/dark cycle, with free access to food and water.

Experiment I aimed to test whether SD rats could be infected with the SARS-CoV-2 prototype. In brief, 24 rats were infected with SARS-CoV-2 and randomly assigned into three groups (eight animals per group) selected for necropsy at 1 day, 3 days, and 5 days post-infection (dpi), respectively. Nine age-matched rats without treatment were assigned as the control group (mock).

Experiment II aimed to determine whether initial infection provided a protective effect against reinfection with SARS-CoV-2. In brief, we infected eight rats with the same amount of SARS-CoV-2 (1×10^5 $TCID_{50}$) following the same procedures as in Experiment I. We monitored the infected rats for 16 days, after which they received another challenge of SARS-CoV-2 (1×10^5 $TCID_{50}$) following the same procedures (termed reinfection group) as Experiment I, with five rats from the mock group of Experiment I also receiving an infective dose (1×10^5 $TCID_{50}$; termed initial infection

group). Animals in both the reinfection group (1 day post-reinfection (dpr)) and initial infection group (1 dpi) were euthanized for necropsy the day after infection. The remaining four rats in the mock group of Experiment I were used as the non-infected controls for all experiments.

We measured body weight and rectal temperature of all infected rats (32 animals at the beginning of infection) and uninfected rats (nine animals) in both experiments I and II daily at 10:00–11:00 am. We plotted daily changes in average body weight of all animals stratified by infection and non-infection status. The number of infected animals declined from 1 dpi, 3 dpi, and 5 dpi, as eight animals were euthanized for necropsy at each dpi for collecting tissue samples.

Animals were euthanized at indicated time points (Figure 1A) and throat swabs were collected. Each throat swab was placed into 1 mL of DMEM (Thermo Fisher Scientific, USA) and stored at −80 °C until viral load analysis. Gross images of lungs were obtained at time of necropsy. We collected nasal turbinate and lung lobe samples at the indicated time points of necropsy for virological and pathological analyses.

Measurement of viral RNAs

RNA copies per mL of throat swabs or per μg of lung and nasal turbinate samples were determined by quantitative real-time polymerase chain reaction (RT-qPCR). We extracted total RNA from 200 μL of throat swab solution using a High Pure Viral RNA Kit (Roche, Germany). Extracted RNA was eluted in 50 μL of elution buffer. For lung tissues and nasal turbinate samples, we used Trizol Reagent (Thermo Fisher Scientific, USA) for total RNA isolation from homogenized tissues. Viral RNA copies were detected using a THUNDERBIRD Probe One-Step qRT-PCR kit (TOYOBO, Japan). A 25 μL reaction was established containing 8.7 μL of extracted RNA from throat swabs or 1 μg of total RNA from lung tissues and nasal turbinate samples in a total volume of 8.7 μL, 12.5 μL of 2×reaction buffer, 0.625 μL of DNA polymerase, 0.625 μL of RT Enzyme Mix, 0.75 μL of each specific primer (10 μM, Supplementary Table S1), 1 μL of specific probe (5 μM, Supplementary Table S1), and 0.05 μL of 50×ROX reference dye. The RT-qPCR was performed using the Applied Biosystems 7500 Real-Time PCR system with the following thermal cycle conditions: 10 min at 50 °C for reverse transcription, 60 s at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. In each run, serial dilutions of the SARS-CoV-2 RNA reference standard (National Institute of Metrology, China) were used in parallel to calculate copy numbers in each sample.

Viral titers

Lung tissues from each rat were collected at necropsy and homogenized in 1 mL of serum-free DMEM using a Servicebio Tissue Homogenizer (Wuhan Servicebio Technology Co., Ltd., China). The tissue homogenates were centrifuged at 1 000 *g* for 10 min at 4 °C to pellet cell debris. The homogenate supernatants were serially diluted (10-fold) in serum-free DMEM. Vero-E6 cells (2×10^4 cells/well) were grown overnight in a 96-well plate, then 10-fold serial dilutions (100 μL/well) were added to the cultured Vero-E6 cells. After the 96-well plates were incubated at 37 °C for 60

min, we added DMEM supplemented with 4% FBS (100 μL/well) to the Vero-E6 cells for growth. We monitored the cytopathic effect (CPE) each day, with scoring at 5 dpi. $TCID_{50}$ was calculated using the Reed and Muench method (Reed & Muench, 1938).

Pathological analysis

Different lung lobes of rats were fixed in 4% paraformaldehyde for 7 days, processed in paraffin (Leica EG1160), sectioned at a thickness of 3–4 μm (Leica RM2255), then stained with hematoxylin and eosin (H&E) followed the procedure described in our previous study (Xu et al., 2020). The slices were imaged using a Nikon Eclipse E100 microscope (Japan) and were blindly evaluated by two pathologists.

Immunohistochemical analysis of lung tissue was performed as described previously (Xu et al., 2020; Zeng et al., 2022). In brief, each section was baked at 65 °C for 30 min, followed by deparaffinization using xylene and subsequent hydration with decreasing concentrations of ethanol (from 100% to 75%). Heat-induced antigen retrieval was performed using sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 25 min and non-specific binding was blocked with 3% bovine serum albumin (BSA) for 30 min at room temperature. Specimens were incubated overnight at 4 °C with rabbit anti-SARS-CoV-2 nucleocapsid protein antibody (Cell Signaling Technology, Inc., USA, Cat No. #26369, 1:200). The slices were also incubated with rabbit immunoglobulin G (IgG) (Thermo Fisher Scientific, USA, Cat No. #31235) as the negative control for viral antigen staining. Subsequently, the lung slices were incubated with anti-rabbit IgG secondary antibody (Wuhan Servicebio Technology Co., Ltd., China, Cat No. #GB23303) for 50 min at room temperature and visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The slices were counterstained with hematoxylin, dehydrated and mounted on a slide, and viewed under an Olympus OlyVIA microscope (Japan).

Neutralizing assay

Blood samples were collected from rats at necropsy and centrifuged (3 000 *g* for 10 min at room temperature) to obtain serum samples. After heat inactivation at 56 °C for 30 min, the serum samples were serially diluted in DMEM supplemented with 3% FBS to final dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1 280, and 1:2 560 in a 96-well plate (100 μL/well; dilution plate). The virus was diluted in DMEM supplemented with 3% FBS to a final amount of 100 $TCID_{50}$ in 100 μL of solution. The diluted virus solution (containing 100 $TCID_{50}$) was mixed with each diluted serum sample to form serum-virus mixtures at 37 °C for 60 min.

The Vero-E6 cells (2×10^4 cells/well) were grown overnight in a 96-well plate (culture plate). After removal of culture medium, the serum-virus mixtures (200 μL/well) prepared in the dilution plate were transferred to the culture plate, with the cells then grown for 5 days. Plates were monitored daily for CPE. The neutralization titer endpoint was based on inhibition of CPE observed at 5 dpi.

Statistical analyses

Statistical comparisons were performed using GraphPad Prism v8. Values of body weight were presented as mean±standard error of the mean (SEM) and viral copies were presented as mean±standard deviation (SD). Two-tailed Student's *t*-test was used to compare differences between infected and non-infected groups and $P<0.05$ was regarded as statistically significant.

References

Reed LJ, Muench H. 1938. A Simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, **27**(3): 493–497.

Song TZ, Zheng HY, Han JB, Jin L, Yang X, Liu FL, et al. 2020. Delayed severe cytokine storm and immune cell infiltration in SARS-CoV-2-infected aged Chinese rhesus macaques. *Zoological Research*, **41**(5): 503–516.

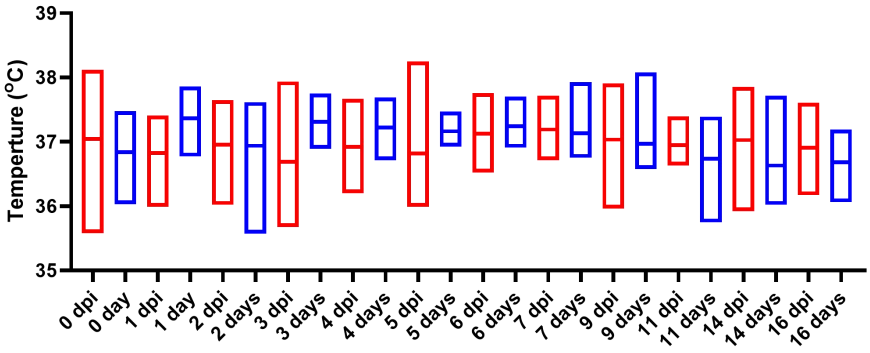
Xu L, Yu DD, Ma YH, Yao YL, Luo RH, Feng XL, et al. 2020. COVID-19-like symptoms observed in Chinese tree shrews infected with SARS-CoV-2. *Zoological Research*, **41**(5): 517–526.

Zeng JX, Xie XC, Feng XL, Xu L, Han JB, Yu DD, et al. 2022. Specific inhibition of the NLRP3 inflammasome suppresses immune overactivation and alleviates COVID-19 like pathology in mice. *eBioMedicine*, **75**: 103803.

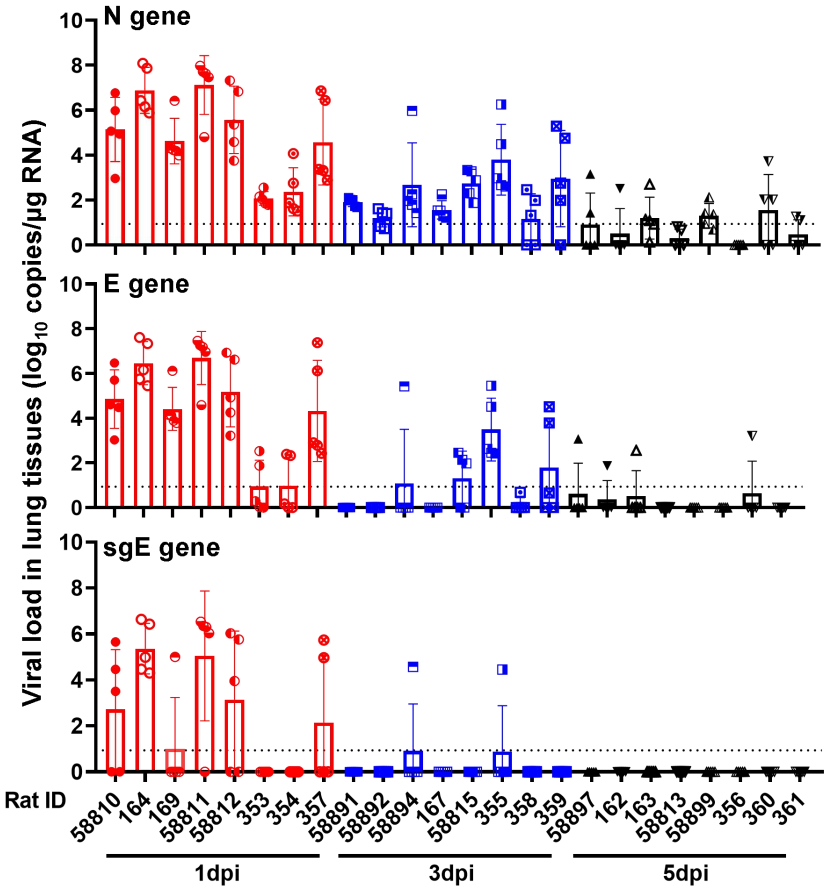
Supplementary Table S1 Primer pairs and probes for quantification of viral copies

Primer	Sequence (5’-3’)	Target gene
N-F/N-R	5’-GGGGAACTTCTCCTGCTAGAAT-3’ /	genomic N gene
	5’-CAGACATTTTGCTCTCAAGCTG-3’	
N-P	5’-FAM-TTGCTGCTGCTTGACAGATT-TRMRA-3’	
E-F/E-R	5’-ACAGGTACGTTAATAGTTAATAGCGT-3’ /	genomic E gene
	5’-ATATTGCAGCAGTACGCACACA-3’	
E-P	5’-FAM-ACACTAGCCATCCTTACTGCGCTTCG-TRMRA-3’	
sgE-F/sgE-R	5’-CGATCTCTTGTAGATCTGTTCTC-3’ /	subgenomic E
	5’-ATATTGCAGCAGTACGCACACA-3’	gene (sgE)
sgE-P	5’-FAM-ACACTAGCCATCCTTACTGCGCTTCG-TAMRA-3’	

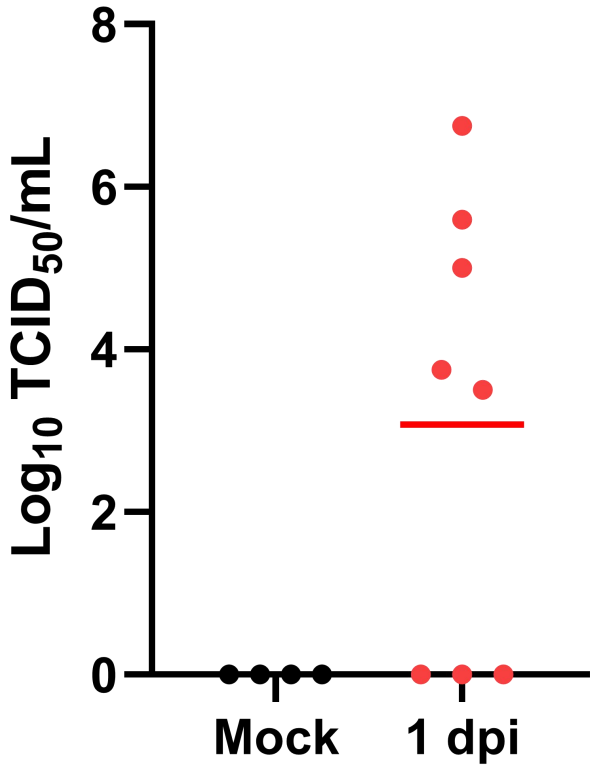
Forward (F) and reverse (R) primers were annotated by adding “-F” and “-R” in each primer name. Probes were annotated by adding “-P” in each probe name. All probes contained 5’ fluorescent reporter dye (FAM) and 3’ fluorescent quencher (TRMRA).



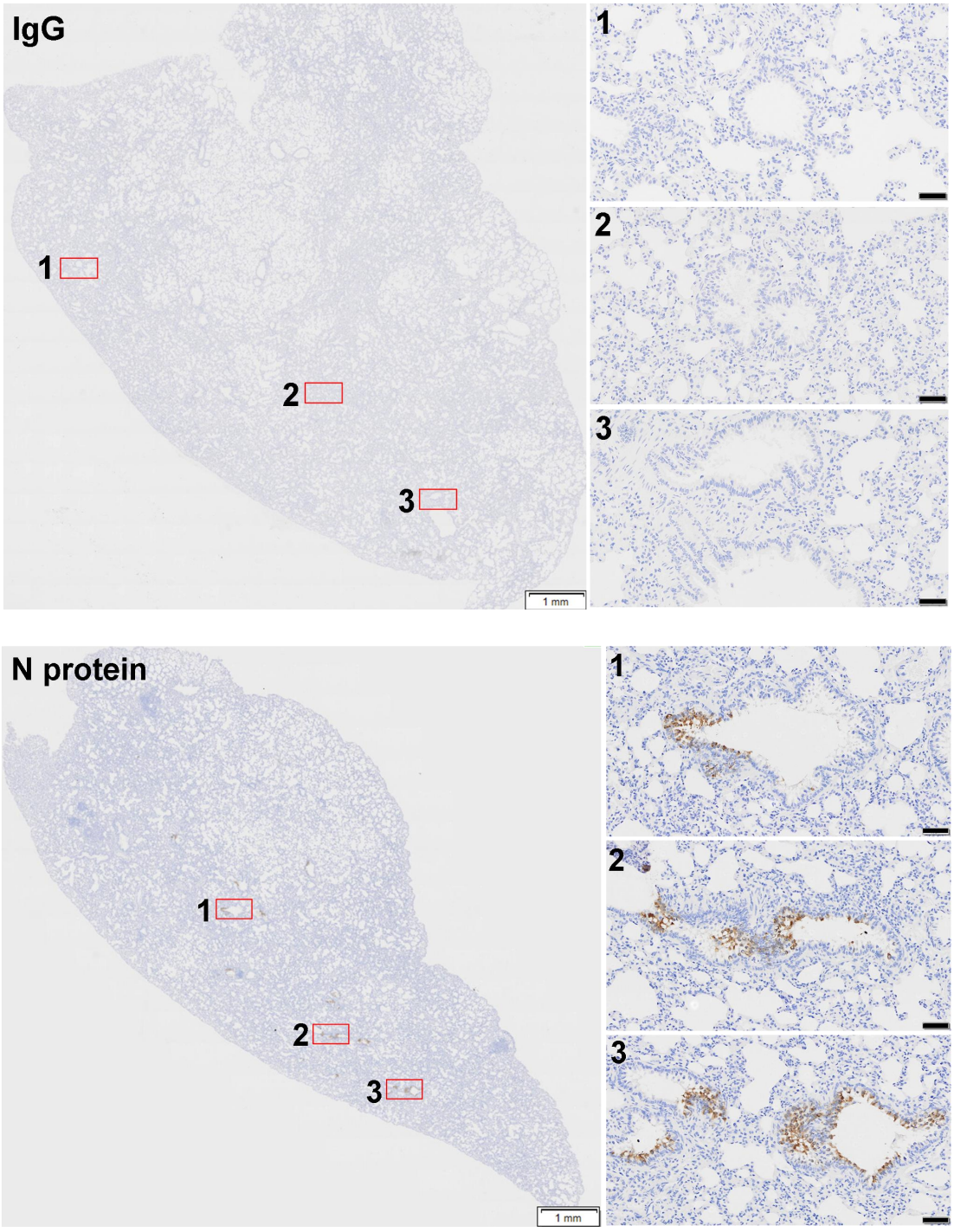
Supplementary Figure S1 Changes in body temperature over time in SARS-CoV-2-infected rats (infection group, red bars) and uninfected rats (mock group, blue bars). Data are mean±SEM of the infection and mock groups at different days post-infection (dpi).



Supplementary Figure S2 Viral RNA in five lung lobes of each infected rat at 1 dpi, 3 dpi, and 5 dpi (*n*=8 rats for each time point). Rat lungs have five lobes. We collected five lung tissue samples from each animal, with one tissue representing one lung lobe. Points in bar refer to viral copy values of five different lung lobes from the same individual. Dotted lines represent limit of quantification (8.7 copies/μg total RNA of lung tissue). Data are mean±SD.



Supplementary Figure S3 Viral titers in lung tissues of rats in mock group (black circles, *n*=4) and infection group at 1 day post-infection (1 dpi, red circles, *n*=8).



Supplementary Figure S4 Immunohistochemical analyses showing staining of IgG (negative control; upper) and SARS-CoV-2 N protein (below) in lung tissues of infected rats at 1 dpi. Scale bars, 1 mm for entire lung lobe section and 50 μm for enlarged view of boxed areas labeled by numbers.