Supplementary Materials for

SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo


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Other Supporting Online Material for this manuscript includes the following:
(available at science.sciencemag.org/cgi/content/full/science.abe8499/DC1)

MDAR Reproducibility Checklist (.pdf)

Correction
On page 4 of Materials and Methods, the description of the IRB number of COVID-19 patient sera was corrected. In addition, fig. S2F was revised to show an empty cage, to address animal welfare concerns. The original figure depicted two hamsters inside the cage. Various grammatical errors and typos have also been corrected.
Materials and Methods

Antibodies

Monoclonal SARS-CoV-2 RBD-binding neutralizing antibodies (nAb) B38 and H4 were synthesized at UNC Protein Expression and Purification core based on previously reported protein sequences (27). The nAbs S309, REGN10933, REGN10987, and JS016 were reported previously (28, 29) and were kindly provided by Adimab LLC. Monoclonal antibody targeting the cytoplasmic tail of SARS-CoV-2 S protein was purchased from Abcam (ab272504). Polyclonal antibodies targeting the SARS-CoV N protein PA1-41098 and ANT-180 were purchased from Invitrogen and Prospecl, respectively. Mouse antiserum targeting SARS-CoV-2 nucleocapsid protein was produced in our laboratory as described previously (10).

Cells and viruses

Simian kidney cell lines Vero-81 (ATCC # CCL81) and Vero-E6 (ATCC # CRL1586) were maintained in Eagle’s Minimum Essential Medium (Gibco) supplemented with 10% fetal calf serum (FBS, Hyclone). Human liver cell line Huh7 and A549-ACE2 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) with 10% FBS. A clonal A549-ACE2 stable cell line was generated by overexpressing human ACE2 in the human alveoli adenocarcinoma cell line A549 cell line (ATCC # CCL185) using the Sleeping Beauty Transposon System. The generation of primary human pulmonary cell cultures was described previously (8). Primary human nasal epithelial cells (HNE), human bronchial epithelial [large airway epithelial (LAE)] and bronchiolar [small airway epithelial (SAE)] cells were isolated from freshly excised normal human tissues obtained from transplant donors under UNC Institutional Review Board (IRB)-approved protocol (#03-1396) and cultured in air-liquid interface (ALI) media, as previously described (8, 30). The age and gender of the donors are: donor 1 (52 year-old male); donor 2 (49 year-old male); donor 3 (57 year-old male); donor 4 (51 year-old female); donor 5 (70 year-old male); donor A (27 year-old male); donor B (35 year-old female); donor C (21 year-old male); donor D (60 year-old female).
SARS-CoV-2 WA1 molecular clone, WT, and nLuc viruses were generated previously (8, 10). The genomic sequence of the WT recombinant SARS-CoV-2 WA1 strain was submitted to GenBank (accession # MT461669). To generate the D614G and D614G-nLuc variants, the amino acid substitution was introduced into the S gene in the plasmid F and coupled with plasmid G with or without nLuc insertion in the ORF7a, as illustrated in Fig 1A. Then, the seven genomic cDNA fragments spanning the entire SARS-CoV-2 genome were digested, purified, and ligated. Full-length RNA was transcribed and electroporated into Vero E6 cells. Virus stocks were verified by Sanger sequencing. The generation of recombinant SARS-CoV-2 D614G variants has been approved by UNC Institutional Biosafety Committee (IBC) under the laboratory safety plan schedule G # 78762. All viral infections were performed under biosafety level 3 (BSL-3) conditions at negative pressure, and Tyvek suits connected with personal powered-air purifying respirators.

**nLuc virus entry assay**

Monolayers of Vero-E6, Vero-81, A549-ACE2, and Huh7 cells were cultured in black-walled 96-well plates (Corning 3904) overnight. The cells were infected with WT-nLuc or D614G-nLuc viruses at an MOI of 0.5. After incubation for 1h, inocula were removed, and the cells were washed two times with PBS and maintained in DMEM containing 5% FBS and the mixture of SARS-CoV-2 nAbs REGN10933, REGN10987, and JS016 at a concentration of 1000 times of IC₅₀. After incubation at 37°C for 8h, viral entry was quantified by measuring nLuc activity using Nano-Glo Luciferase Assay System (Promega) according to the manufacturer specifications.

**COVID-19 patient sera**
De-identified COVID-19 recovered patient serum samples were provided with informed consent through a UNC Biomedical IRB-reviewed protocol (#20-1141)

**SARS-CoV-2 neutralization assay**

Vero E6 cells were plated at 20,000 cells per well in black-walled 96-well plates (Corning 3904). Human serum samples were tested at a starting dilution of 1:40 and mAb samples were tested at a starting concentration of 30 to 0.1 μg/ml and were serially diluted 3-fold up to eight dilution spots. Diluted antibodies and sera were mixed with 87 PFU/well WT-nLuc or D614G-nLuc virus, and the mixtures were incubated at 37°C with 5% CO₂ for 1 hour. Following incubation, growth media was removed, and virus-antibody mixtures were added to the cells in duplicate. Virus-only controls were included in each plate. Following infection, plates were incubated at 37°C with 5% CO₂ for 48h. After the 48h incubation, cells were lysed, and luciferase activity was measured via Nano-Glo Luciferase Assay System (Promega) according to the manufacturer specifications. Neutralization titers were defined as the sample dilution at which a 50% reduction in the relatively light unit (RLU) was observed relative to the average of the virus control wells.

**WT and D614G competition assay and BtsCI digestion**

LAE cultures from the donor D were infected with MOI of 0.5 of WT and D614G mixture at 1:1 or 10:1 ratios. Following 1h incubation, the cultures were washed three times with PBS and cultures for 72h in the air-liquid interface condition. To passage the progeny viruses, 100uL PBS was added to each LAE surface for 10 min incubation and was added to naïve cultures surface for infection. The virus samples were continuously passaged three times in LAE culture, and cellular RNA samples from all the passages were extracted using TRIzol reagent (Thermo Fisher). A 1547bp fragment containing the D614G site was
amplified from each RNA sample by RT-PCR using primer set: 5’-GTAATTAGAGGTGATGAAGTCAGAC-3’ and 5’-GAACATTCTGTGTAACTCCAATACC-3’. The amplicon was purified by agarose gel electrophoresis and digested with BtsCl restriction enzyme (NEB) overnight. The digested products were analyzed on agarose gel electrophoresis.

**hACE2 mice infection and titration**

Mouse studies were performed in accordance with UNC Animal Care and Use Committee (IACUC) protocol # 20-182. Ten-week-old *HFH4-hACE2* transgenic mice (six male and four female) were bred and maintained at UNC. Mice were infected with 10⁵ PFU of WT or D614G viruses intranasally under ketamine/xylazine anesthesia. At indicated time points, a subset of mice was euthanized by isoflurane overdose, and tissue samples were harvested for viral titer analyses. The right caudal lung lobe, brain, and nasal turbinates were collected and stored at -80 °C until homogenized in 1mL PBS and titrated by plaque assay. Briefly, the supernatants of homogenized tissue were serially diluted in PBS, 200 µL of diluted samples were added to monolayers of Vero-E6 cells, followed by agarose overlay. Plaques were visualized by day 3 post staining with neutral red dye.

**Whole-mount immunostaining and imaging**

WT- or D614G-infected LAE ALI cultures were fixed twice for 20 minutes in 4% formaldehyde in PBS and stored in PBS. The SARS-CoV-2 N antigen was stained with polyclonal rabbit anti-SARS-CoV N protein (Invitrogen PA1-41098, 0.5 ug/mL), and using species-specific secondary antibodies as previously described (8). The cultures were also imaged for α-tubulin (Millipore MAB1864; 3ug/mL) and MUC5AC (Thermo Scientific 45M1; 4ug/mL) as indicated. Filamentous actin was localized with phalloidin (Invitrogen A22287), and nuclei were visualized with Hoechst 33342 staining (Invitrogen). An Olympus
FV3000RS confocal microscope in Galvo scan mode was used to acquire 5-channel Z stacks by a 2-phase sequential scan. Representative stacks were acquired and are shown as Z-projections and XZ cross-sections to distinguish individual cell features and to characterize the infected cell types. ImageJ was used to measure the relative apical culture surface covered by multiciliated cells.

**Western blot analysis of spike protein cleavage**

Exocellular SARS-CoV-2 virions were collected from WT or D614G infected HNE cultures by gently washing intact apical surface with 100uL of PBS. Samples from the triplicated cultures were pooled, lysed with modified RIPA buffer, and inactivated at 98°C. Protein samples were electrophoresed in 4-20% continuous SDS-PAGE gel (Bio-Rad) and transferred onto a PVDF membrane (Bio-Rad). SARS-CoV-2 S protein was probed using a mAb targeting SARS-CoV-2 S mAb (Abcam, ab272504) and the N protein was probed using a mouse antiserum produced in our laboratory. The Western blot images were captured and quantified using the Thermo Fisher iBright imaging system and software.

**EM imaging and Spike quantification**

WT- or D614G- infected primary cell cultures were submerged in fixative (4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate) overnight. For SEM, samples were rinsed, fixed with 1% OsO4 (Electron Microscopy Sciences) in perfluorocarbon FC-72 (Thermo Fischer) solution for 1 hour. After dehydration and mounted on aluminum planchets, samples were imaged using a Supra 25 field emission scanning electron microscope (Carl Zeiss Microscopy). For TEM, fixed samples were rinsed and post-fixed with potassium-ferrocyanide reduced osmium (1% osmium tetroxide/1.25% potassium ferrocyanide/0.1 sodium cacodylate buffer. The cells were dehydrated and embedment in Polybed 812 epoxy resin (Polysciences). The cells were sectioned perpendicular to the substrate at 70nm using a
diamond knife and Leica UCT ultramicrotome. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate followed by Reynolds’ lead citrate. Samples were observed using a JEM-1230 transmission electron microscope operating at 80kV and images were taken using a Gatan Orius SC1000 CCD camera (Gatan). The number of spikes on each virion projection was quantified using ImageJ software. SEM images of infected cultures from 3 donors were imaged, at least 10 different micrographs (>100k ×) were analyzed using the multi-point counting tool on individual virions.

**Hamster infection, tissue collection, and transmission studies**

Hamster studies were performed in accordance with University of Wisconsin-Madison IACUC protocol # V00806. Syrian hamsters (females, 4-6 weeks old) were purchased from Envigo (Madison, WI) and allowed to acclimate for a minimum of three days at BSL-3 agriculture containment at the Influenza Research Institute (University of Wisconsin). Hamsters were infected with 10³ PFU of WT or D614G viruses intranasally under isoflurane anesthesia. At the indicated timepoints, a subset of hamsters was euthanized by deep anesthesia by isoflurane inhalation and cervical dislocation and tissue samples were harvested for virus titer and histopathology analysis. Weights of one group of hamsters were recorded for 14 days after infection. To evaluate the competitive fitness between WT and D614G viruses *in vivo*, four hamsters were infected intranasally with 10³ PFU of a mixture of 1:1 ratio of both viruses. Lung tissues were harvested at 3 days post-infection and passaged independently two more times in four independent lines of naïve animals. Viral RNA samples were extracted from lung tissues of infected animals for sequencing and BtsCI digestion analyses.
To evaluate indirect virus transmission between hamsters, groups of hamsters (n=8 per group) were infected with $10^3$ PFU of WT or D614G viruses intranasally under isoflurane anesthesia. Infected animals were placed in specially designed cages (Figure S2B) inside an isolator unit (II). Twenty-four hours later, naïve hamsters were placed on the other side of the cage with 5 cm separation by a double-layered divider to allow free airflow. The isolator unit provided one-directional airflow; therefore, the infected hamsters were placed in the front of the isolator unit. Metal shrouds were placed over the cages so only the front and back of the cage was open. Nasal washes were collected at 3-day intervals for the infected hamsters and 2-day intervals for the exposed animals starting on day 2 after infection or exposure (Fig. S2A).

**Pathological examination**

Tissues fixed for at least seven days in 10% formalin were trimmed and embedded in paraffin. The paraffin blocks were cut into 3 μm-thick sections and mounted on silane-coated glass slides. One section from each tissue sample was stained using a standard hematoxylin and eosin procedure. To detect SARS-CoV-2 Nucleocapsid protein in immunohistochemistry (IHC), tissue sections were incubated with a rabbit polyclonal antibody (Prospec, ANT-180) as the primary antibodies and peroxidase-labeled polymer-conjugated anti-rabbit immunoglobulin (EnVision/HRP, DAKO) as the secondary antibody. Immunostaining was visualized by 3,3’-diaminobenzidine tetrahydrochloride staining. Hematoxylin (Modified Mayer’s) was used as a nuclear counterstain for IHC.
Fig. S1. Additional data on WT and D614G infected primary human airway epithelial cells and neutralization assays.

(A) Individual growth curves of WT and D614G viruses in primary nasal (i), large airway (ii), and small airway (iii) epithelial cells relating to Fig. 1 D to 1F, MOI = 0.1; plaque assay detection limit: 1.7 log_{10} PFU/mL. (B) Comparison of WT and D614G titer at 96h on HNE, LAE, and SAE. (C) Whole-mount
staining of WT- and D614G-infected LAE cultures, blue: Hoechst (nuclei), red: phalloidin (F-actin), white: cilia (α-tubulin); yellow: MUC5AC, Green: SARS-CoV-2 N protein, scale bar: 50µm. Neutralization curves of the 25 human serum samples against equal PFU of the WT-nLuc (D) and D614G-nLuc (E) viruses.
Fig. S2. Additional information on hamster pathogenesis and transmission studies.

(A) Macroscopic images of lungs harvested from infected hamsters. (B) Schematic of a competition replication study in hamsters. BtsCI digestion (C) and Sanger sequencing chromatogram (D) of the S gene fragment amplified from viral samples in the hamster competition assay. (E) Timeline of nasal wash
sampling from infected and exposed animals. (F) Image of the cage setting in the hamster transmission study. The infected animal and the exposed animal are separated by a 5-cm space between them to avoid direct contact.
References and Notes


