Supplementary Materials

Supplementary Materials and Methods

Plasmids expressing SARS-CoV-2 spike protein

The codon-optimized Mu, Delta, and D614G spike genes with deletion of the C-terminal 18 amino acids were synthesized and cloned into pcDNA3.1 or were generated by site-directed mutagenesis using a Mut Express II Fast Mutagenesis Kit V2 (C214; Vazyme; China). The primers used for mutagenesis are listed in Supplementary Table S2.

Human subjects

This study was approved by the Medical Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences (Approval No. KIZRKX-2021-004). Serum samples were obtained from eight volunteers vaccinated with two doses by an inactivated vaccine.

Production and pseudotyped viral particles

We co-transfected 10 μg of pLVX-luciferase, 5 μg of psPAX2, and 2.5 μg of spike variant plasmids into 5×10^6 HEK-293T cells in a 10 cm dish using Lipofectamine 3000 (L30000015; Invitrogen; USA) according to the manufacturer's instructions. The medium was replaced with 10 ml of fresh medium at 6 h after transfection. At 72 h post-transfection, the supernatants containing the SARS-CoV-2 pseudotyped viruses were collected and filtered using a 0.45 μm filter. RNA in 100 μl of pseudotyped viruses was extracted using TRIzol reagent (Invitrogen; USA). Viral DNA was obtained by reverse transcription using M-MLV Reverse Transcriptase (M1705; Promega; USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify copies of pseudotyped viruses, while a known quantity of pLVX-luciferase vector gave rise to standard curves. The pseudotyped viruses were diluted with the same titer to perform experiments.

Infectivity assay

The HEK293T-ACE2 cells (1×10^4/100 μl/well) were seeded into 96-well cell culture plates in 5% CO_2 at 37 °C. On the second day, the medium in each well was replaced with 100 μl of Dulbecco's modified Eagle's medium (DMEM) containing 1×10^4 pseudotyped copies. The medium in each well was then replaced with 100 μl of fresh culture medium at 12 h post-incubation. At 48 and 72 h post-incubation, 100 μl of luciferase substrate buffer (Promega; USA) was added to each well and cell lysates were then transferred to a compatible opaque 96-well plate. Luciferase activity was detected using a Varioskan Lux Luminescence reader (Thermo Fisher Scientific; USA).

Cell-cell fusion assay

The plasmid encoding the SARS-CoV-2 spike protein was co-transfected with green fluorescent protein (GFP)-expressing plasmid into HEK293T cells using Lipofectamine 3000 (L30000015; Invitrogen; USA). In addition, the hACE2-expressing plasmid was also transfected into other HEK293T cells. The two groups of cells were mixed at a 1:1 ratio 24 h post-transfection and co-cultured in complete DMEM in 5% CO_2 at 37 °C. After 24 h of co-culture, the GFP signals were observed under a fluorescence microscope.

Neutralization by pseudotyped viruses

The HEK293T-ACE2 cells (1×10^4/100 μl/well) were seeded into 96-well cell culture plates. Serum was initially diluted with cell culture medium at 1:20 followed by 4-fold dilution. Equivalent pseudoviruses (5×10^3 copies in 50 μl) were mixed with serum dilutions and incubated for 60 min at 37 °C. The mixtures were added to infect the HEK293T-ACE2 cells. At 72 h post-infection, luciferase activity was measured to calculate neutralizing titer as a 50% inhibitory dose (ID_{50}).

Immunoblotting

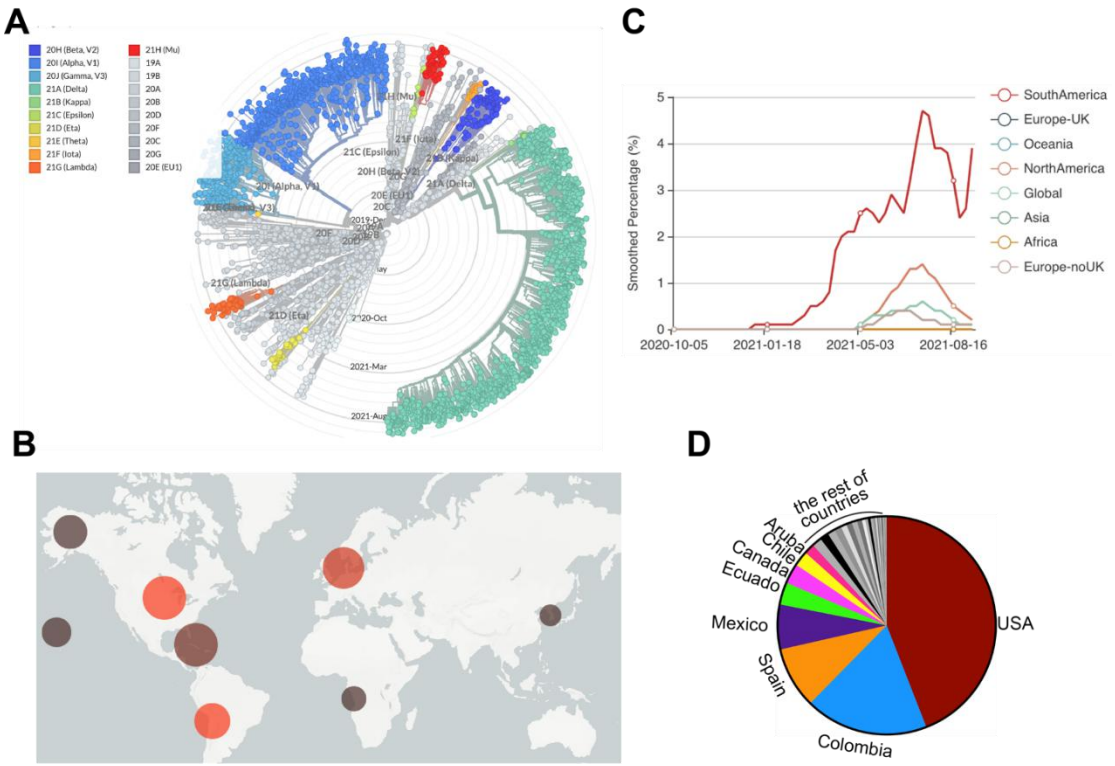
Immunoblotting was performed as described previously (Zeng et al., 2020). In brief, target cells were lysed in 1% NP40 buffer (Beyotime; China) with protease inhibitor cocktail (MedChemExpress; USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes using semi-dry transfer at 25 V for 30 min. The membranes were blocked in 5% milk in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 1 h and incubated overnight with mouse monoclonal anti-flag antibody (M220008; Abmart; China) in 5% bovine serum albumin at 4 °C. The membranes were then incubated with anti-mouse horseradish peroxidase-conjugated secondary antibodies in 5% milk for 1 h at room termperature and bands were developed using Chemi-Doc XRS imaging (Bio-Rad; USA)

Statistical analysis

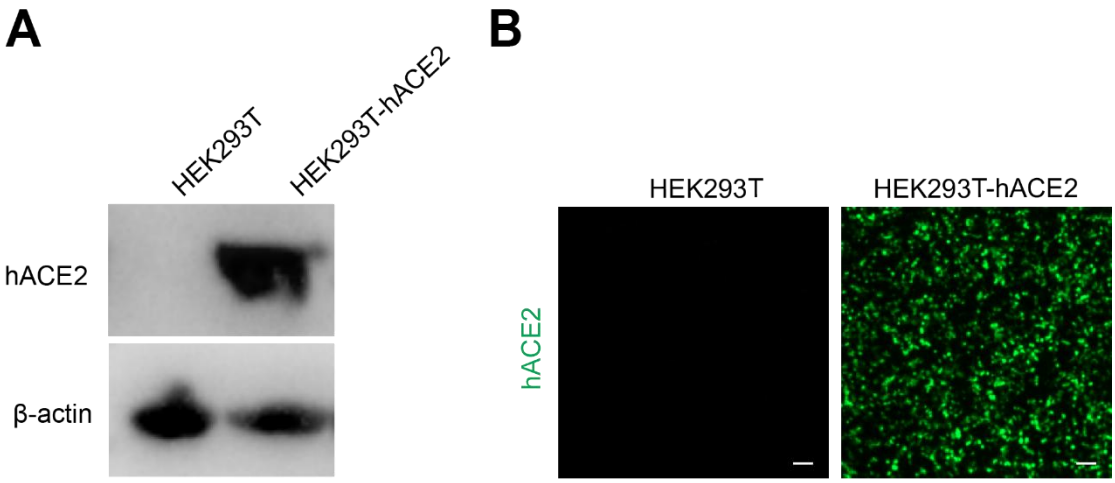
GraphPad Prism 8 was used for plotting and statistical analysis (mean±standard deviation (SD)). One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used, and $P<0.05$ was considered significant.

REFERENCES

Zeng JX, Dong SP, Luo ZF, Xie XC, Fu BS, Li P, et al. 2020. The zika virus capsid disrupts corticogenesis by suppressing dicer activity and miRNA biogenesis. *Cell Stem Cell*, **27**(4): 618–632.e9.



Supplementary Figure S1. Geographical epidemiology of B.1.621/Mu variant.
A: Genomic epidemiology of prevalent SARS-CoV-2 variants between December 2019 and September 2021. Red circle indicates Mu variant sampled globally as of 15 September 2021 from Nextstrain database. B: Map of tracked Mu variant occurrence as of 15 September 2021 from GISAID database. Circle size is proportional to number of variant genomes and color by recency (red being most recent). C: Relative Mu variant genome frequency per region between October 2020 and September 2021 from GISAID database (as of 15 September 2021). D: Number of sequenced Mu variants as of 15 September 2021 from GISAID database.



Supplementary Figure S2. Identification of HEK193T-ACE2 cells.

A: Immunoblot of flag-tagged human ACE2 in lysates of HEK293T or HEK293T-ACE2 cells.
B: Immunofluorescence of flag-tagged human ACE2 in HEK293T or HEK293T-ACE2 cells.
Scale bar: 20 μm.

Supplementary Table S1. Number of sequenced Mu variants from GISAID database (as of 15 September 2021).

Country	Total #Mu GH (B.1.621+B.1.621.1)	#Mu GH (B.1.621+B.1.621.1) in past 4 weeks	%Mu GH (B.1.621+B.1.621.1) in past 4 weeks
USA	2,513	49	0.1
Colombia	1,042	1	100
Spain	518	1	0.1
Mexico	379	4	0.4
Chile	189	19	20
Ecuador	170	0	0
Canada	128	0	0
Aruba	88	1	0.8
Italy	82	0	0
Netherlands	75	1	0.1
United Kingdom	62	0	0
Costa Rica	61	0	0
Austria	49	0	0
Switzerland	48	0	0
Dominican Republic	46	0	0
Belgium	41	3	0.1
Peru	27	0	0
Portugal	24	0	0
France	22	1	0.2
British Virgin Islands	21	0	0
Curacao	20	0	0
Germany	14	0	0
Brazil	11	0	0
Bonaire	8	0	0
Denmark	7	0	0
Haiti	6	0	0
Poland	6	0	0
Venezuela	5	0	0
Sweden	4	0	0
Ireland	4	0	0
Guatemala	3	0	0
Finland	3	0	0
Sint Maarten	3	0	0
Slovakia	3	0	0
Luxembourg	2	0	0
China	2	0	0
Turkey	2	0	0
Cayman Islands	2	0	0

Country	Total #Mu GH (B.1.621+B.1.621.1)	#Mu GH (B.1.621+B.1.621.1) in past 4 weeks	%Mu GH (B.1.621+B.1.621.1) in past 4 weeks
Japan	2	0	0
Liechtenstein	1	0	0
Gibraltar	1	0	0
Barbados	1	0	0
Romania	1	0	0
Czech Republic	1	0	0
Turks and Caicos Islands	1	0	0
South Korea	1	0	0
Israel	1	0	0
Malta	1	0	0

Supplementary Table S2. Primers used in this study.

Primer name	Primer sequence (5'-3')
S-D614G-F	GTACCAGggaGTGAATTGCACCGAGGTGCCA
S-D614G-F	AATTCACtccCTGGTACAGCACGGCCACCTGA