Deletion of the SARS-CoV-2 Spike Cytoplasmic Tail Increases Infectivity in Pseudovirus Neutralization Assays

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ABSTRACT

Pseudotyped viruses are valuable tools for studying virulent or lethal viral pathogens that need to be handled in biosafety level 3 (BSL-3) or higher facilities. With the explosive spread of the coronavirus disease 2019 (COVID-19) pandemic, the establishment of a BSL-2 adapted SARS-CoV-2 pseudovirus neutralization assay is needed to facilitate the development of countermeasures. Here we describe an approach to generate a single-round lentiviral vector-based SARS-CoV-2 pseudovirus, which produced a signal more than 2 logs above background. Specifically, a SARS-CoV-2 spike variant with a cytoplasmic tail deletion of 13 amino acids, termed SΔCT13, conferred enhanced spike incorporation into pseudovirions and increased viral entry into cells as compared with full-length spike (S). We further compared S and SΔCT13 in terms of their sensitivity to vaccine sera, purified convalescent IgG, hACE2-mIgG, and the virus entry inhibitor BafA1. We developed a SΔCT13-based pseudovirus neutralization assay and defined key assay characteristics, including linearity, limit of detection, and intra- and intermediate-assay precision. Our data demonstrate that the SΔCT13-based pseudovirus shows enhanced infectivity in target cells, which will facilitate the assessment of humoral immunity to SARS-CoV-2 infection, antibody therapeutics, and vaccination. This pseudovirus neutralization assay can also be readily adapted to SARS-CoV-2 variants that emerge.

IMPORTANCE

SARS-CoV-2 is the etiologic agent of the COVID-19 pandemic. The development of a high throughput pseudovirus neutralization assay is critical for the development of vaccines and immune-based therapeutics. In this study, we show that deletion of the cytoplasmic tail of the SARS-CoV-2 spike leads to pseudoviruses with enhanced infectivity. This SΔCT13-based pseudovirus neutralization assay should be broadly useful for the field.
INTRODUCTION

The emerging pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to an unprecedented need to develop vaccines and other countermeasures (1). To develop a successful vaccine candidate, identifying and screening the immune correlates of protection in a high-throughput assay is paramount. Neutralizing antibody responses may represent key immune correlates of protection for SARS-CoV-2 (2, 3). Therefore, it is essential to develop a high throughput in vitro neutralization assay that recapitulates key features of in vivo neutralizing activity. However, live SARS-CoV-2 research is restricted to biosafety level-3 (BSL-3) facilities, and traditional approaches such as plaque reduction assays are time-consuming and low-throughput.

Pseudotyped viruses have often been employed to evaluate the physiology of lethal viruses, such as Ebola virus (4), Marburg virus (5) and Lassa virus (6). Pseudoviruses encompass essential components for viral infection, typically the envelope proteins derived from viruses of interest and an inner core, but they lack intact viral genome and thus lose self-replication capacity (7). The conformational structure of pseudovirus surface proteins appears to resemble that of the native viral proteins (8), which may allow for a similar viral entry process between the pseudoviruses and infectious counterparts. As such, pseudoviruses are typically regarded as safe and effective virological tools. Pseudoviruses have been utilized for receptor identification, host tropism determination, and viral entry mechanistic studies (7); for identifying entry inhibitors by screening compound libraries (9); and for evaluating neutralizing antibody responses elicited by vaccination or from convalescent sera (10). Pseudoviruses may also be useful in assessments of antibody-dependent enhancement (11, 12). As such, it is important to develop a BSL2-adapted SARS-CoV-2 pseudovirus system.

SARS-CoV-2 encodes four structural proteins: spike (S), nucleocapsid (N), envelope (E) and membrane (M), and more than twenty non-structural proteins (13). S represents the only protein that mediates SARS-CoV-2 entry and serves as a key immunogen for inducing protective immunity (14). S is synthesized as a precursor in rough endoplasmic reticulum (ER) and further processed into amino-
terminal S1 and carboxyl-terminal S2 segments, which contribute to receptor binding and cell fusion, respectively. S has been utilized for generating pseudoviruses for SARS-CoV (SARS-CoV-1) (15) and Middle East respiratory syndrome coronavirus (MERS-CoV) (16), two highly pathogenic and closely related human coronaviruses. As of now, multiple platforms have been leveraged to investigate SARS-CoV-2 entry and/or to determine the induced antibody neutralizing activity, including Vesicular Stomatitis Virus (VSV)-based (17-22), Murine Leukemia Virus (MLV)-based (23-25), and Human Immunodeficiency Virus (HIV)-based pseudotypes (22, 26). In this paper, we observed a rather modest virus infection efficiency with full length spike protein (S)-based pseudovirus. In contrast, deletion of the last 13 amino acids of the S (SΔCT13) significantly increased spike incorporation into the pseudovirion and improved infectivity. This optimized SΔCT13-based SARS-CoV-2 neutralization assay will be useful in evaluating vaccines and immune-based therapeutics.
RESULTS

Deletion of the last 13 amino acids in spike protein enhances SARS-CoV-2 pseudovirus infection

Two main forms of lentiviral vector-based pseudovirus production strategies have been reported, involving a two-plasmid system or a three-plasmid system (8). The former requires a backbone plasmid that expresses a full set of HIV-1 gene products except for the HIV-1 envelope (Env), and a second plasmid that encodes heterologous Env of interest. In contrast, the three-plasmid system separates the backbone plasmid into two individual constructs, one packaging plasmid and one transfer plasmid (8).

The three-plasmid system was generally regarded as a safer method since multiple virulent genes have been removed (27). For this reason, we chose the three-plasmid system to generate the SARS-CoV-2 pseudovirus, with which we established a pseudovirus-based neutralization assay. The HIV core (backbone) is formulated by the packaging plasmid psPAX2 that expresses the HIV gag and pol proteins and the transfer plasmid pLenti-puro CMV-Luc that harbors the reporter firefly luciferase gene and essential HIV genomic cis-acting regulatory elements allowing proper encapsulation into the HIV capsid (28). During preliminary experiments, the SARS-CoV-2 full-length S demonstrated a rather modest viral entry (2-20 fold above cell-only background, data not shown), which limited its utility in evaluating neutralizing activity, and thus warranted assay optimization.

Deletion of the last few amino acids in the cytoplasmic tail of SARS-CoV-1 S generated pseudoviruses with greater infectivity (29). We thus sought similar strategy, deleted the last 13 amino acids of the SARS-CoV-2 S (termed CoV-2 SΔCT13) and evaluated it in HEK293T-hACE2 cells (Fig. 1A). Interestingly, we noticed significantly increased viral entry for CoV-2 SΔCT13 compared with CoV-2 S (Fig. 1A). Similarly, SARS-CoV-1 full-length (CoV-1 S) and cytoplasmic tail truncated version (CoV-1 SΔCT13) were included in parallel (Fig. 1A). Both SARS-CoV-1 and SARS-CoV-2 demonstrated augmented virus infection when the last 13 amino acids were ablated, suggesting a possible general mechanism of the enhancement. In particular, SARS-CoV-2 appeared to rely more on CT deletion than did SARS-CoV-1, with a 24-fold and 2.4-fold increase of infectivity, respectively. The SARS-CoV-1 S showed more than 200-fold enhancement of viral entry compared with SARS-CoV-2 S, despite the two
sharing the same entry receptor hACE2. To further explore the determinants of the CoV-2 S, we generated an additional CT variant with the last 6 amino acids deleted (CoV-2 S\(\Delta CT_6\)). As shown in Fig. 1A, CoV-2 S\(\Delta CT_6\) displayed similar viral infection efficiency to CoV-2 S, suggesting that a potential spike incorporation motif exists in the heptad peptides “SEPVLKG”. We further evaluated the spike protein mediated cell-cell fusion between HEK293T/Spike and TZM-bl/hACE2 cells. Deletion of the last 13 amino acids significantly improved CoV-1 S mediated cell-cell fusion, while CoV-2 S\(\Delta CT_{13}\) did such to a less extent (Fig. 1B). Consistent with viral infection result, CoV-2 S\(\Delta CT_6\) induced cell-cell fusion similarly to CoV-2 S (Fig. 1B). To gain mechanistic insight, we performed western blot analysis of the proteins expressed in virus producing HEK293T cells. As shown in Fig. 1C, the transfected cells showed a similar expression level of loading control β-actin and HIV core structural protein p24, suggesting comparable transfection efficiency. However, the S1+S2 precursor appeared comparable for CoV-1 S, CoV-1 S\(\Delta CT_{13}\), CoV-2 S, and CoV-2 S\(\Delta CT_{13}\), whereas S1 expression in CoV-2 S\(\Delta CT_{13}\) appeared higher than that in CoV-2 S, indicating the CT region may affect the spike processing.

We purified viral particles by ultracentrifugation, and analysis by western blot showed that p24 was generally comparable in all the groups, reflecting similar virus production. However, the S1+S2 and S1 demonstrated a sharp increase (Fig. 1D) for both CoV-1 S\(\Delta CT_{13}\) and CoV-2 S\(\Delta CT_{13}\). To confirm, we also collected the virion-containing supernatants and measured the concentration of HIV-1 p24 and CoV-2 spike by Enzyme-linked immunosorbent assay (ELISA). In line with Western blot data, more spike proteins as well as higher spike/p24 ratio were detected in CoV-2 S\(\Delta CT_{13}\) supernatants (Fig. 1E). The data support a model in which deletion of CT facilitates S incorporation into pseudovirions, thus leading to elevated viral entry efficiency.

HIV assembles on the plasma membrane, while coronaviruses bud predominantly through ER (30). We tested whether the enhanced S incorporation into pseudovirions with the \(\Delta CT\) constructs resulted from the higher expression of S on the plasma membrane. We performed cell surface staining of SARS-CoV-1 and SARS-CoV-2 spike proteins. Interestingly, the S and S\(\Delta CT_{13}\) showed similar expression levels, as reflected by the geometric mean of S intensity (Fig. 2A and 2B), suggesting that the
surface expression level unlikely explains the distinct spike incorporation efficiency. Taken together, the results show that deletion of the last 13 amino acids of S substantially facilitated S incorporation into pseudovirions and viral entry of SARS-CoV-2 in cells. Based on these data, we selected SARS-CoV-2 SΔCT13 for further study.

SARS-CoV-2 S and SΔCT demonstrated similar sensitivity to neutralization and inhibitor blocking

We next established a neutralization assay with this optimized lentivirus-based pseudovirus (see Materials and Methods for detailed protocol). To address the question whether the SΔCT13-based pseudovirus neutralization assay would correlate with the live virus neutralization assay we therefore performed a direct comparison between the pseudovirus-mediated and live virus-mediated neutralization assay of a total 67 rhesus monkeys and observed a strong correlation (R= 0.7470; Fig. 3A), reflecting that SΔCT13 maintained the key serologic features of the intact/authentic S.

Next, we examined the sensitivity of SARS-CoV2 S and SARS-CoV-2 SΔCT13 pseudovirus to neutralizing antibodies. To this end, we evaluated immunized rhesus monkey serum (N=16) (Fig. 3B) and purified convalescent IgG from SARS-CoV-2 infected rhesus monkey (Fig. 3C) to CoV-2 SΔCT13 or CoV-2 S pseudoviruses. Both the immunized serum and convalescent IgG demonstrated similar neutralization capacity against CoV-2 SΔCT13 and CoV-2 S pseudoviruses (Fig. 3B and 3C). In particular, we included two additional SARS-CoV-2 S variants, D614G and V367F, which are prevalent virus variants in the global pandemic (31, 32). All groups demonstrated similar 50% neutralization titers (NT50 titers) (Fig. 3B). Soluble ACE2 also similarly inhibited CoV-1 S, CoV-1 SΔCT13, CoV-2 S, and CoV-2 SΔCT13 pseudoviruses (Fig. 3D). Moreover, these data show the flexibility of this assay in evaluating neutralization of SARS-CoV-2 variants as they emerge.

Finally, we measured the entry kinetics of CoV-1 S, CoV-1 SΔCT13, CoV-2 S, CoV-2 SΔCT13 with the inhibitor bafilomycin A1 (BafA1). BafA1 targets the vacuolar-type H+ -ATPase and disrupts the
low pH environment in intracellular organelles (33), which is essential for entry of several low-pH dependent viruses including coronavirus (Fig. 4A). Both SARS-CoV-1 and SARS-CoV-2 demonstrated a comparable half maximal inhibitory concentration (IC₅₀) to BafA1 (2-5 nM). To characterize the viral entry kinetics, viral entry was initially synchronized by cold arrest (spinoculation at 4 °C), which allows efficient virus binding but not virus-cell fusion. BafA1 was added at 0, 0.5, 1, 2, 3, 4, 6 hours post spinoculation at a final concentration of 5 nM, and virus titer was determined 48 hours post-infection. As shown in Fig. 4B, the VSVg-mediated viral entry reached plateau around 2 hours post infection and the half virus entry time (T₁/₂) was approximately 25 min, consistent with early reports (34). CoV-1 S and CoV-1 SΔCT13 pseudoviruses demonstrated slower kinetics, requiring three hours to accomplish maximal viral entry with T₁/₂ of about one hour (35). CoV-2 S and CoV-2 SΔCT13 pseudoviruses exhibited even slower entry, with T₁/₂ of about 2.5 hours, suggesting the intrinsic differences between the two coronavirus spikes but no difference between S and SΔCT13 for both viruses. Overall, SARS-CoV-2 S and SΔCT13 demonstrated similar sensitivity to neutralization and inhibition.

Qualification of the SARS-CoV-2 neutralization assay

As qualification experiments, we assessed the following parameters: linearity, limit of detection (LOD), specificity and reproducibility. Linearity between input virus and luciferase activity was analyzed with increasing amounts of pseudovirus. As shown in Fig. 5A, solid linearity was achieved between 3.125 and 50 µl virus inoculums (R=0.9894, P<0.0001). Of note, 1 µl of input virus was equivalent to 4 picograms of HIV-1 p24, as determined by HIV-1 p24 ELISA. To determine sample dilutional linearity combined with intermediate precision, a pooled, heat-inactivated, high titer COVID-19 human convalescent serum sample was tested undiluted (neat) and in two-fold serial dilutions ranging from 1:1 to 1:32. These assays were performed by three different operators over three different days. Linear regression of NT50 titer plotted as a function of the serum dilution factor suggested strong linearity when the serum concentration was between undiluted and 1:16 dilution (R=0.9984, P<0.0001; Figure 5B).
Intra- and intermediate assay precision was assessed using the dilutional linearity sample data. Using pre-specified acceptance criteria of \( \leq 25\% \) coefficient of variation (%CV) to define the upper limit of quantification (ULOQ), the assay ULOQ was established at an NT50 of 3,153 (Table 1). The limit of detection (LOD) was assessed by measuring the neutralizing activity of human serum at starting with a dilutions of 1:20. Serums from other three model species, rhesus macaque, hamster and mouse (naïve serum) were included for comparison. The LOD was established to 1:23, 1:28, 1:22, 1:20 for human, rhesus macaque, hamster and mouse, respectively (Fig. 5C). Due to limited availability of high volume COVID-19 human convalescent serum samples at the time of assay development, undiluted and 1:16 diluted samples were used to approximate intra-assay precision at the high and low ends of the assay range with samples meeting pre-defined criteria of CV\( \leq 25\% \) at each level for each operator (Table 2).

Development of discrete quality control samples that fall in the high (~80%), medium (~50%) and low (~20%) range for established assay range will subsequently be developed as high volume, high nAb titer reagents become available. The lentivirus-based neutralization assay demonstrated an overall assay precision of 15.7%.

With this established SARS-CoV-2 neutralization assay, we tested human serum from pre-pandemic (n=28); early stage of COVID-19 (within 10 days since symptom onset, n=50); and late stage of COVID-19 (greater than 10 days since symptom onset, n=50). There was a clear enhanced neutralizing antibody titer upon infection, with median titers of 20, 218 and 776, respectively (Fig. 5D and 5E).
Pseudoviruses are versatile and valuable tools for both basic and applied virologic research and has particular advantages when live virus requires higher biosafety BL3 or BL4 facilities. High throughput assays using pseudoviruses in BL2 facilities can facilitate rapid testing and development of SARS-CoV-2 countermeasures such as vaccines. As such, SARS-CoV-2 pseudovirus neutralization assays are being established. In this manuscript, we describe the development and qualification of an optimized SARS-CoV-2 pseudovirus neutralization assay for preclinical studies and clinical trials. We found that the full length S of two closely related human coronaviruses, SARS-CoV-1 and MERS-CoV, were readily translatable into a standard pseudovirus system (15) (16). Full length of SARS-CoV-2 S mediated low level of infection but can be improved by deleting 13 amino acids in the CT.

We addressed several key issues in the development of this assay. First, expression of the virus-derived coding sequence in mammalian cells may be suboptimal due to potential codon usage bias (36). We therefore codon-optimized the entire spike DNA sequence by replacing the GC stretches and changing into Homo sapiens biased codons and validated its robust expression profile in HEK293T cells (Fig. 1C). Second, the viral entry efficiency of SARS-CoV-2 into target cells appear to be intrinsically inferior to SARS-CoV-1. SARS-CoV-1 exhibited a >100-fold higher entry efficiency than SARS-CoV-2 (Fig. 1A). This may be ascribed to the stronger binding affinity to the receptor hACE2 (37), greater spike incorporation, accelerated entry kinetics, or other factors. Regardless, we sought to improve SARS-CoV-2 pseudovirus entry into target cells to improve the assay performance. Because partial deletion of the SARS-CoV-1 CT was reported to increase the viral infectivity (29), we deleted the last 13 amino acids of the cytoplasmic tail for both SARS-CoV-1 and SARS-CoV-2. Both SARS-CoVs demonstrated greater viral entry, but this effect was more pronounced for SARS-CoV-2 (Fig. 1A) with a 20-fold enhancement (22). We also observed a marked increase in SACT incorporation into pseudovirions compared with S for both SARS-CoV-1 and SARS-CoV-2, which correlated with enhanced viral entry. The observed SARS-CoV S protein expressed on the cell surface (Fig. 2) may be due to leakiness of the ER retention upon overexpression (29). We hypothesize that the enhanced incorporation of SACT may reflect higher cell
surface expression as a result of the removal of a reported ER retention motif KxHxx in the last 13 amino acids (38) (26). However, we observed the same cell surface expression level for both SΔCT13 and S. In addition, the SΔCT6 deleted the KxHXX motif while maintained a similar viral entry profile to the S, arguing against the hypothesis that the spike cell surface expression makes a difference. Interestingly, when we performed cell-cell fusion assay, where the CoV-2 S and CoV-2 SΔCT13 had comparable expression level (Fig. 1C and 2), cell-cell fusion events were similarly induced (<2-fold difference, Fig. 1B), suggesting that the functioning of the spike may not be altered by CT deletion. A direct measurement of viral entry kinetics (Fig. 4B) similarly implicated that truncation of the 13 amino acids unlikely altered the viral entry properties.

One question regarding the use of SΔCT is whether the C-terminal deletion may have changed the conformation of the protein, thus potentially altering neutralization sensitivity. There is currently a lack of direct structural evidence for the CT region of SARS-CoV-2 (39). We observed a strong positive correlation between the pseudovirus- and live virus-based neutralization assay (Fig. 3A), suggesting the utility of the pseudovirus neutralization assay. Interestingly, we noticed that a subset of samples with modest NT50 titers were detected with pseudovirus assay but not under live virus assay, suggesting different assay sensitivities. Additional studies are warranted to test this hypothesis. Moreover, immunologic and virologic comparisons between SARS-CoV-2 pseudoviruses with S and S ΔCT13 suggested similar sensitivity to polyclonal antibody, hACE2-mIg and BafA1. Therefore, it is probably quantitative rather than qualitative changes of the spike in the virion that determine the increased viral infection rate. Further investigation including detailed structural insight may be warranted. Currently, VSV-based and lentiviral vector-based pseudovirus systems stand for the primary assays for neutralizing antibody titer determination (17-22, 26). The intrinsic properties of rapid replication of infectious VSV accelerate the data generation. While the lentiviral vectors morphologically resemble to the SARS-CoV-2 and may more genuinely reflect the entry process of SARS-CoV-2.

To define performance characteristics of this SARS-CoV-2 pseudovirus neutralization assay, we performed a series of qualification experiments. The limit of detection for four species appears to be
similar (approximately 1:20). Intra- and intermediate precision demonstrated precision levels <25%.

Overall, this optimized and qualified assay has been developed to evaluate SARS-CoV-2 neutralizing antibody responses in both preclinical and clinical studies. Moreover, this pseudovirus neutralization assay is readily adaptable to incorporating S sequences from SARS-CoV-2 variants, such as currently circulating UK and South African variants.
MATERIALS AND METHODS

Plasmids, cells, and reagents

Sequences of full-length spike (S) or 13 amino acids in cytoplasmic tail deleted S (S ΔCT) of SARS-CoV-1 (Tor2, GenBank: AAP41037.1) and SARS-CoV-2 (Wuhan/WIV04/2019) were codon optimized and commercially synthesized (Integrated DNA Technologies, NJ, USA). Synthetic genes were cloned into the mammalian expression plasmid pcDNA3.1+ (Invitrogen, CA, USA). SARS-CoV-2 S D614G and V367F variants were generated by site-directed mutagenesis based on SARS-CoV-2 S ΔCT. Human ACE2 genes were commercially synthesized (Integrated DNA Technologies, NJ, USA) and further cloned into pQCXIP retroviral vector (Cat# 631516, Takara). pHEF-VSVG expressing Vesicular Stomatitis Virus (VSV) glycoprotein was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 4693). PsPAX2 (Cat# 12260), pLenti-CMV-Puro-Luc (Cat#17447), and pBS-CMV-gag-pol (Cat#35614) were obtained from Addgene (Boston, MA).

HEK293T (ATCC® CRL-11268™), Vero-E6 (ATCC® CRL-1586™) and 786-O (ATCC® CRL-1932™), TZM-bl (AIDS Reagent, Cat# 8129) were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 0.5% penicillin/streptomycin and 5% FBS. HEK293T-hACE2 were maintained in DMEM, supplemented with 0.5% penicillin/streptomycin, 5% FBS and 1 μg/ml puromycin (Sigma).

Rabbit polyclonal antibody against SARS-CoV-2 RBD and hACE2 conjugated with mouse IgG Fc (hACE2-mFc, Cat#10108) was purchased from Sino Biological. Bafilomycin A1 was purchased from Sigma. HRP-Conjugated Beta Actin Antibody (Cat# HRP-60008) was purchased from Proteintech. HIV p24 ELISA kit was ordered from Abcam (Cat# ab218268).

Rhesus and human serum samples
Rhesus macaque anti-SARS-CoV-2 serum was obtained after an adenoviral vector-based vaccination (3).

Serums from 16 animals were collected 4 weeks post single-shot vaccination. The convalescent serum IgG from our early study (40) was purified and pooled. 12 de-identified SARS-CoV-2 pre-pandemic human serum samples from Boston, MA were obtained. All human studies were conducted in compliance with all relevant local, state, and federal regulations and were approved by the Partners Institutional Review Board (IRB).

**Generation of HEK293T-hACE2 stable cell**

The retroviral pseudotypes expressing human ACE2 (hACE2) were generated by co-transfecting HEK293T cells with pHEF-VSVG, pBS-CMV-gag-pol, and pQCXIP-hACE2 at a ratio of 0.5:1:1. Viruses in the supernatants were collected every 12 h. The collected viruses were used to transduce HEK293T cells, and positive cell populations were selected by using 1 μg/ml puromycin.

**Production of pseudotyped lentiviral particles**

The SARS-CoV-2 pseudoviruses expressing a luciferase reporter gene were generated in an approach similar to as described previously (2, 40, 41). 10 μg packaging construct psPAX2, 10 μg luciferase reporter plasmid pLenti-CMV Puro-Luc, and 5 μg spike protein expressing pcDNA3.1-SARS CoV-2 SΔCT were co-transfected into 5 x 10^6 HEK293T cells in 10-cm dish with lipofectamine 2,000 (Sigma). Six hours post transfection, the supernatants were replaced with fresh DMEM (plus 5% FBS). The supernatants containing the pseudotype viruses were collected 48 hours post-transfection; pseudotype viruses were purified by filtration with 0.45 μm filter.

**Lentiviral luciferase-based neutralization assay**

The SARS-CoV-2 pseudoviruses neutralization assay was generated in an approach similar to as described previously (2, 40, 41). To determine the neutralization activity of the serum, plasma, or IgG samples from cohorts, HEK293T-hACE2 cells were seeded in 96-well tissue culture plates at a density of 1.75 x 10^4 cells/well overnight. Three-fold serial dilutions of heat inactivated plasma samples were
prepared and mixed with 50 µL of pseudovirus. The mixture was incubated at 37 °C for 1 h before adding
to HEK293T-hACE2 cells. 48 h after infection, cells were lysed in Steady-Glo Luciferase Assay
(Promega) according to the manufacturer’s instructions. SARS-CoV-2 neutralization titers were defined
as the sample dilution at which a 50% reduction in relative light unit (RLU) was observed relative to the
average of the virus control wells.

Live virus neutralization assay

A full-length SARS-CoV-2 virus based on the Seattle Washington isolate was designed to express
luciferase and GFP and was recovered via reverse genetics. The virus was titered in Vero E6 USAMRID
cells to obtain a relative light units (RLU) signal of at least 10X the cell only control background. Vero
E6 USAMRID cells were plated at 20,000 cells per well the day prior in clear bottom black walled 96-
well plates. Neutralizing antibody serum samples were tested at a starting dilution of 1:40 and were
serially diluted 4-fold up to eight dilution spots. Antibody-virus complexes were incubated at 37 °C with
5% CO2 for 1 hour. Following incubation, growth media was removed and virus-antibody dilution
complexes were added to the cells in duplicate. Virus-only controls and cell-only controls were included
in each neutralization assay plate. Following infection, plates were incubated at 37 °C with 5% CO2 for
48 hours. After the 48 h incubation, cells were lysed and luciferase activity was measured via Nano-Glo
Luciferase Assay System (Promega) according to the manufacturer specifications. SARS-CoV-2
neutralization titers were defined as the sample dilution at which a 50% reduction in RLU was observed
relative to the average of the virus control wells.

Cell-cell fusion assay

HEK293T cells were transfected with spike-encoding plasmids, along with HIV-1 Tat optimized
eexpression vector (AIDS Reagent, Cat# 827). Meanwhile, TZM-bl cells were transfected with human
ACE2 plasmid. Twenty-four hours after transfection, cells were cocultured with TZM-bl at 1:1 ratio for
12 h, lysed, and measured for firefly luciferase activity. All samples were tested in duplicates, and the results were averaged.

**Virus entry kinetics**

1.5 x 10^4 HEK293T-ACE2 cells/well were seeded in flat 96-well plate at a density of 1.5 x 10^5 cells per ml. The second day, 50 µl virus were added into each well, and spun at 1,680g, 30 min, 4 °C. The cell culture plates were transferred into 37 °C incubator immediately after spin, to initiate viral internalization and infection. 15 µl DMEM medium containing 50 nM BafA1 were added into each well immediately after spinoculation (0 hour) and 0.5, 1, 2, 3, 4, 6-hour post-temperature shift. 12 hours after culturing, cells were replenished with fresh DMEM. 48 hours post spinoculation, cells were lysed for luciferase activity quantification.

**Western Blot**

Western blot was performed as previously described (42). Briefly, transfected HEK293T cells were collected, washed once with icy 1 x DPBS (Sigma), and lysed in Radioimmunoprecipitation assay buffer (RIPA, Thermo Fisher, MA) for 20 min on ice. The pseudoviral particles were purified from DMEM culture medium by ultracentrifugation (32,000 rpm, 2 h, 4 °C) and resuspended in 1 x DPBS. The cell lysates and viral particles were dissolved in sample buffer (4x Thermo Fisher, MA), separated on 4-15% gradient gel (Bio-Rad), and detected by anti-HIV-1 p24, anti-SARS-CoV-2 RBD, anti-β-actin antibodies.

**Cell surface staining**

Cells were washed twice with cold 1x DPBS plus 2% FBS, detached with 1x DPBS containing 5 mM EDTA, and incubated on ice with the appropriate primary antibodies for 1 h. After three washes with PBS plus 2% FBS, cells were further incubated with FITC-conjugated secondary antibodies for 45 min. After two washes, cells were fixed with 2% formaldehyde and analyzed in BD LSRII flow cytometer.

**Intermediate precision and dilutional linearity**
A pooled, heat-inactivated, high titered COVID-19 convalescent serum was undiluted or serially pre-
diluted in two-fold dilutions (i.e. neat, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32). Each of these diluted samples was
measured with a starting dilution of 1:20 and further 1:2 serial dilution. These assays were performed by	hree independent operators over three different days.

**Intra Assay Precision**

In the absence of pre-defined, pre-calibrated quality control samples, two dilutions within the linear
range established in Intermediate precision and dilutional linearity assay were selected to approximate
high and low levels of the range. These values subsequently were used to determine intra-assay precision.

**Limit of Detection (LOD)**

The LOD was determined by testing multiple COVID-19 pre-pandemic negative serums. To establish the
LOD, 12 pre-pandemic negative human serums, 28 naïve rhesus monkey serums, 30 naïve hamster
serums and 30 naïve mouse serums were utilized to set the LOD. The LOD of the assay was determined
as the mean of the negative serum samples plus the standard deviation (SD) on the assay nominal scale.

**Statistical Analyses**

All statistical analyses were carried out in GraphPad Prism 8 with Student's t-tests or one-way analysis of
variance unless otherwise noted. Typically, data from at least three independent experiments were used
for analyses.
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AUTHOR CONTRIBUTIONS

J.Y. and D.H.B. designed the study. J.Y., Z.L., X.H., M.S.G., E.B.A., C.J.-D. H.W., D.R.M., and R.S.B. performed the immunologic and virologic assays. J.P.N. analyzed the quality control data. J.Y., J.P.N., and D.H.B. wrote the paper with all the co-authors.

COMPETING INTERESTS

The authors declare no competing financial interests.
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**FIGURE LEGENDS**

**Fig. 1 | Deletion of the last 13 amino acids in spike protein enhances SARS-CoV-2 pseudovirus infection.**

(A) Lentiviral particles pseudotyped with CoV-1 S, CoV-1 SΔCT13, CoV-2 S, CoV-2 SΔCT13, CoV-2 SΔCT6, VSVg or empty (pcDNA3.1) were used to infect HEK293T-hACE2 cells, the firefly luciferase activity was quantified 48 hours post infection. Data are represented as Mean ± SD of 6-12 replicates. (B) S, CoV-1 S, CoV-1 SΔCT13, CoV-2 S, CoV-2 SΔCT13, CoV-2 SΔCT6, VSVg or empty (pcDNA3.1) were co-transfected with HIV-1 Tat-expressing plasmid into HEK293T cells; meanwhile, pQCXIP-hACE2 was transfected into TZM-bl cells. Cells were mixed in a 1:1 ratio and the firefly luciferase activity was quantified 12 hours post co-culture. Data are represented as Mean ± SD of 6 replicates. (C) The cell lysates from virus producer cells in (A) were analyzed by western blot. Meanwhile, the viral particles in supernatants were purified, concentrated and analyzed by western blot (D). Internal control β-actin, HIV-1 p24 and coronavirus spike precursor (S1+S2) and S1 proteins were examined in both cell lysates and viral particles. (E) The concentration of HIV p24 and SARS-CoV-2 spike of indicated pseudoviruses were assayed by HIV p24 ELISA and SARS-CoV-2 S ELISA. Data of CoV-2 S ΔCT13 was set as 100%. The spike density was quantified as Spike/p24. NS, not significant, **⁎⁎⁎ p < 0.001.

**Fig. 2 | Cell surface staining of SARS-CoV spike expression.**

The same batch of cells was detached by 5 mM EDTA/DPBS digestion buffer and stained with anti-SARS-CoV-2 RBD antibody. The surface expression of spike proteins was analyzed by flow cytometry. (A) representative flow plot was analyzed by FlowJo software. (B) spike expression was quantified as geometric mean fluorescence intensity. Data are represented as Mean ± SD of 2 replicates. NS, not significant.

**Fig. 3 | SARS-CoV-2 ΔCT13 maintains the same sensitivity to neutralization and inhibition.**
(A) Correlation of pseudovirus NAb titers and live virus NAb assays in vaccinated macaques (N=67). Red line reflects the best-fit relationship between these variables. P and R values reflect two-sided Spearman rank-correlation tests. (B) NT50 titer of vaccinated macaque serums (N=16) against SARS-CoV-2 S, S ΔCT, S ΔCT D614G and S ΔCT V367F pseudovirus. Red bar indicates the median response.

Inhibition curve of pooled purified IgG from convalescent macaques (C), hACE2-mFc (D) against SARS-CoV-1 S, SARS-CoV-1 S ΔCT13, SARS-CoV-2 S, SARS-CoV-2 S ΔCT13 or VSVg pseudovirus were generated by pre-incubating indicated antibody or inhibitors for one hour and performing virus infection in HEK293T-hACE2 cells. Dotted lines interpolate 50% inhibition or neutralization. Data are represented as mean ± standard deviation of technical triplicates.

**Fig. 4 | SARS-CoV-2 S ΔCT13 maintain the same entry kinetics as the SARS-CoV-2 S.**

(A) Inhibition curve of BafA1 against CoV-1 S, CoV-1 S ΔCT13, CoV-2 S, CoV-2 S ΔCT13 or VSVg pseudovirus were generated by pre-incubating indicated antibody or inhibitors for one hour and performing virus infection in HEK293T-hACE2 cells. Dotted lines interpolate 50% inhibition or neutralization. (B) The viral entry kinetics of the five pseudoviruses were analyzed by BafA1 block assay. The viruses were bound to target cells at 4 °C by spinoculation and then placed at 37 °C allowing virus endocytosis and fusion. The entry inhibitor BafA1 (final concentration 5 nM) was added as indicated time points. 12 hours post treatment, the cells were replenished with fresh DMEM. Viral entry was normalized to mock (DMSO) treated infection group. Data are represented as mean ± standard deviation of technical triplicates.

**Fig. 5 | Qualification of the SARS-CoV-2 neutralization assay.**

(A) The linearity between the virus input volume and firefly luciferase activity was analyzed (N=5). (B) The linearity between the human serum dilution and NT50 titer was demonstrated (combined data of three operators, with three repeats of each, N=9). P and R values reflect two-sided Pearson rank-correlation tests. (C) Limit of detection of established neutralization assay was validated by examining
serums from COVID-19 pre-pandemic human donors (N=12), SARS-CoV-2 negative rhesus macaques (N=28), naïve hamsters (N=30) and naïve Balb/C mice (N=30). Serums were 1:20 diluted and then undergone 1:2 serial dilution, data demonstrated 50% neutralization titer. (D) human serums from pre-pandemic (n=28); early stage of COVID-19 (within 10 days since symptom onset, n=50); late stage of COVID-19 (greater than 10 days since symptom onset, n=50) were applied into established SARS-CoV-2 neutralization assay. Data demonstrated as 50% neutralization titer and red bar reflects the median titer. (E) Representative data from each group was plotted as inhibition curve.
| Serum dilution | Operator 1 | Operator 2 | Operator 3 | GeoMean | Std Dev | % CV |
|---------------|------------|------------|------------|---------|---------|------|
|               | Run 1 | Run 2 | Run 3 | Run 1 | Run 2 | Run 3 | Run 1 | Run 2 | Run 3 |        |       |
| Undiluted     | 2370 | 3290 | 3411 | 3047 | 2788 | 3714 | 4024 | 2774 | 3289 | 3153.1 | 508.2 | 16.1 |
| 1:1           | 1488 | 1410 | 1710 | 1567 | 1498 | 1907 | 2447 | 1516 | 2086 | 1708.5 | 346.3 | 20.3 |
| 1:2           | 763  | 912  | 983  | 704  | 846  | 983  | 1039 | 1042 | 1054 | 916.4  | 128.4 | 14.0 |
| 1:4           | 391  | 464  | 445  | 364  | 527  | 481  | 462  | 503  | 434  | 449.6  | 51.3  | 11.4 |
| 1:8           | 193  | 210  | 166  | 168  | 224  | 254  | 251  | 168  | 177  | 198.3  | 35.4  | 17.8 |
| 1:16          | 111  | 101  | 111  | 75   | 97   | 99   | 98   | 83   | 87   | 95.1   | 12.2  | 12.8 |
| 1:32          | 46   | 28   | 21   | 31   | 43   | 38   | 52   | 26   | 31   | 33.9   | 10.2  | 30.0 |
Table 2 | Intra-assay precision with pooled convalescent human serum

| Operator 1       | NT50 | Serum dilution | Run 1 | Run 2 | Run 3 | GeoMean | Std Dev | %CV |
|------------------|------|----------------|-------|-------|-------|---------|---------|------|
|                  |      | Undiluted      | 2370  | 3290  | 3411  | 2985    | 569.3   | 19.1 |
|                  |      | 1:16           | 111   | 101   | 111   | 108     | 5.8     | 5.4  |
| Operator 2       | NT50 | Serum dilution | Run 1 | Run 2 | Run 3 | GeoMean | Std Dev | %CV |
|                  |      | Undiluted      | 3047  | 2788  | 3714  | 3160    | 477.7   | 15.1 |
|                  |      | 1:16           | 75    | 97    | 99    | 90      | 13.3    | 14.9 |
| Operator 3       | NT50 | Serum dilution | Run 1 | Run 2 | Run 3 | GeoMean | Std Dev | %CV |
|                  |      | Undiluted      | 4024  | 2774  | 3289  | 3324    | 628.2   | 18.9 |
|                  |      | 1:16           | 98    | 83    | 87    | 89      | 7.8     | 8.7  |
Fig. 1

A

B

C

D

E

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C

D

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Fig. 2
Fig. 3
Fig. 4

A

B

BalA1

SARS-CoV-1 S
SARS-CoV-1 S ΔCT
SARS-CoV-2 S
SARS-CoV-2 S ΔCT
VS Vg

Relative pseudovirus infection (% to mock treatment)

BalA1 (nM)

Time of BalA1 addition (hour)

Relative pseudovirus infection (% to mock treatment)
Fig. 5

A

B

C

D

E

Fig. 5