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Supplemental information

Quantitative assays reveal cell fusion at minimal levels of SARS-CoV-2 spike protein and fusion from without

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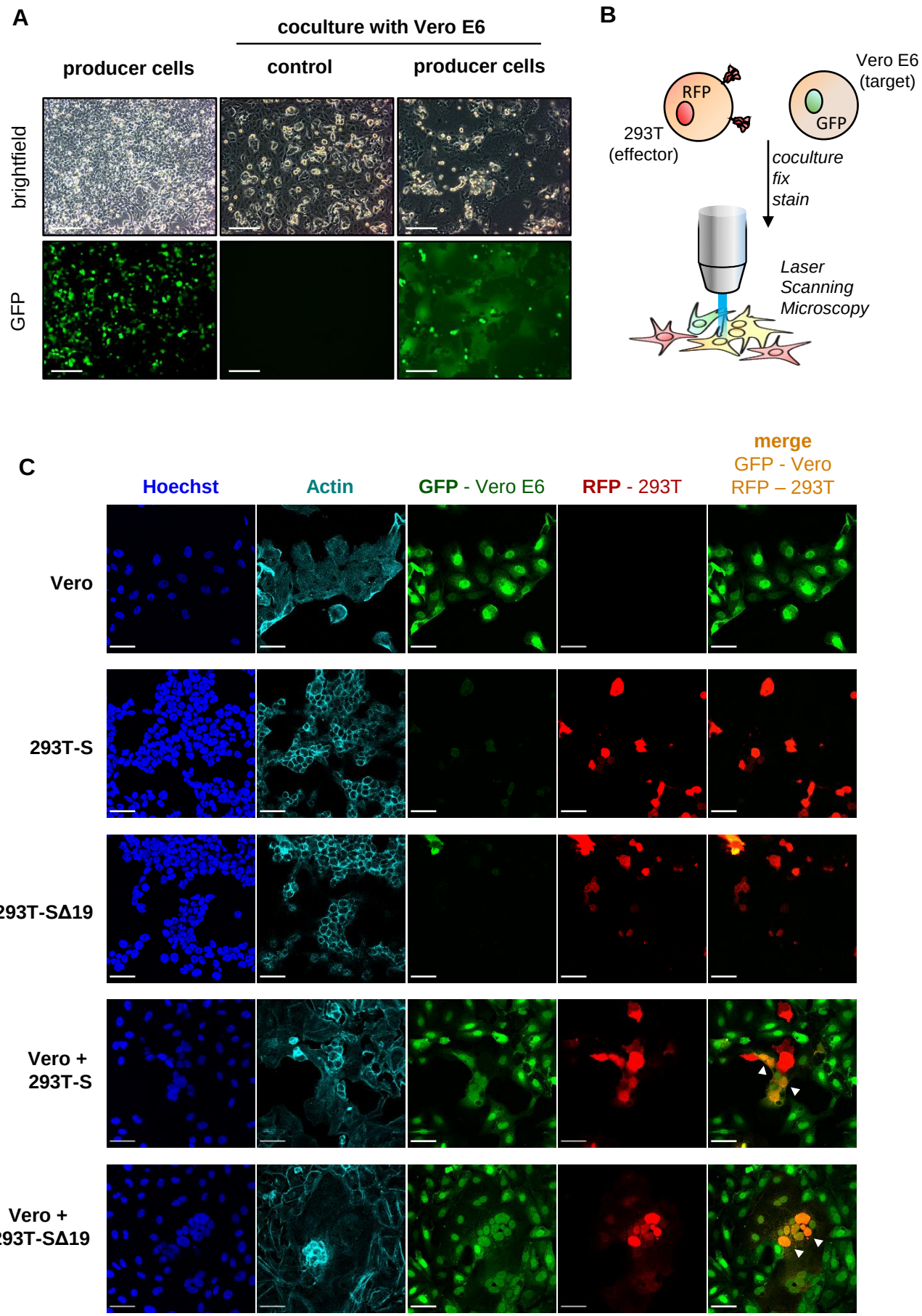
A
coculture with Vero E6
producer cells
control
producer cells
brightfield
GFP
B
Vero E6
(target)
RFP
GFP
293T
(effector)
coculture
fix
stain
Laser
Scanning
Microscopy
C
Hoechst
Actin
GFP - Vero E6
RFP - 293T
merge
GFP - Vero
RFP – 293T
Vero
293T-S
293T-SΔ19
Vero +
293T-S
Vero +
293T-SΔ19

Figure S1: Microscopic evaluation of S protein mediated cell fusion, Related to Figure 2.
(**A**) HEK-293T packaging cells releasing SΔ19-LV having the GFP reporter gene packaged or untransfected control cells were detached by trypsinization and cocultured with Vero E6 cells. After overnight reattachment, presence of syncytia in the cocultures was determined by brightfield (top panel) and epifluorescense microscopy (bottom panel). Scale bars are 500 µm. (**B**) Workflow for the microscopical assessment of syncytia formation induced by SARS-CoV-2 S. 293T cotransfected with RFP and SARS-CoV-2 S (FL or Δ19) were cocultured with Vero E6 cells stably expressing GFP. Cocultures were fixed and stained for nuclei (Hoechst) and actin (phalloidin) before being imaged by confocal laser scanning microscopy. (**C**) Confocal laser scanning micrographs of cocultures described in (B). Scale bars are 50 µm. Where necessary, micrographs underwent differential histogram stretching to ease qualitative analysis of cell morphology. Arrows point to signal colocalization resulting from fusion of both cell populations.

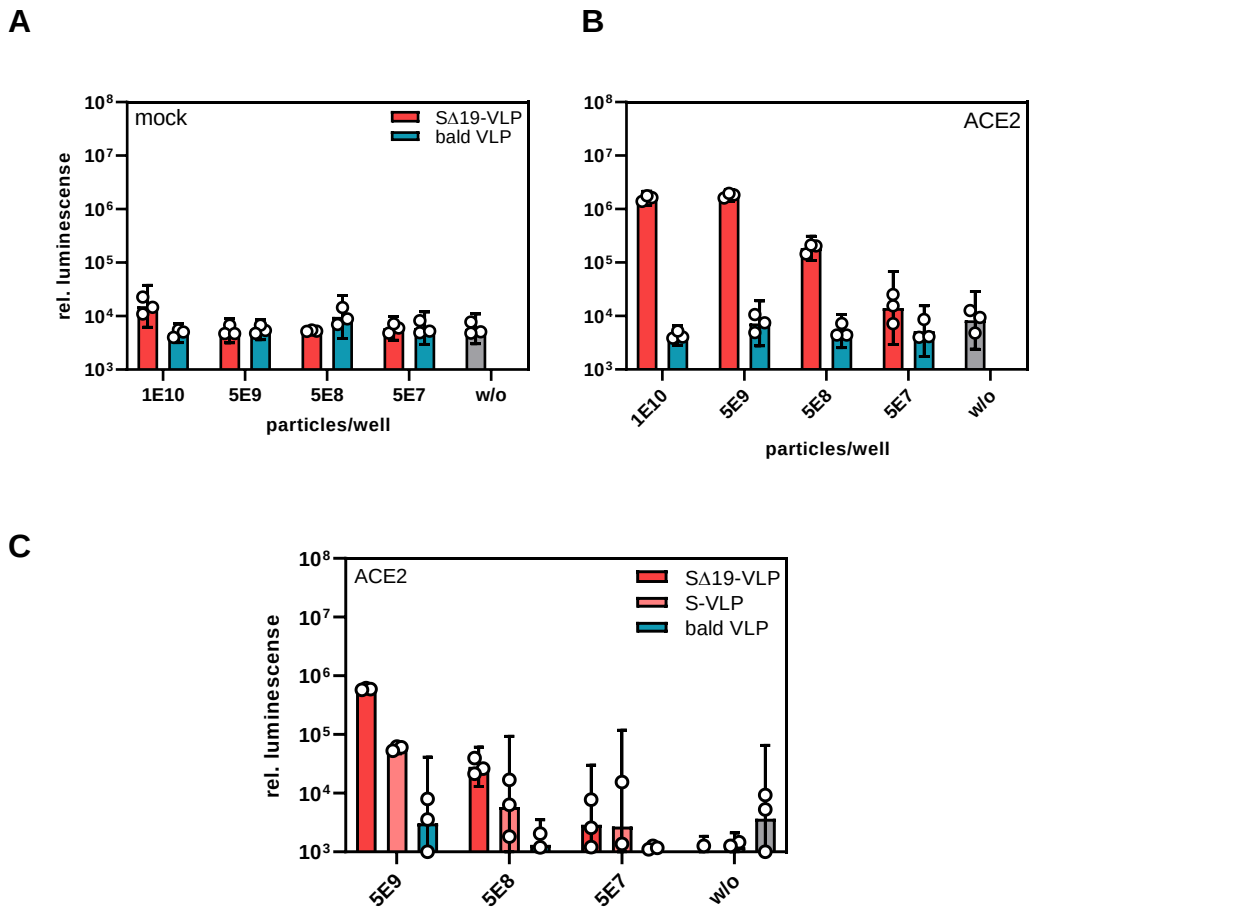

Figure S2: Fusion-from-without mediated by S protein is enhanced by ACE2, Related to Figures 1 and 3.

The indicated numbers of SΔ19-VLPs (**A, B**), full-length S-VLPs (**C**) or bald VLPs were added to cocultures of HEK-293T target cells expressing the α- and ω-fragments of β-galactosidase. In addition, cells were transfected with a mock plasmid (**A**) or the ACE2 encoding plasmid (**B**). Reporter complementation was quantified in luminescence reactions after overnight incubation. Bars and error bars represent geometric means of technical triplicates and 95 % confidence intervals, respectively.

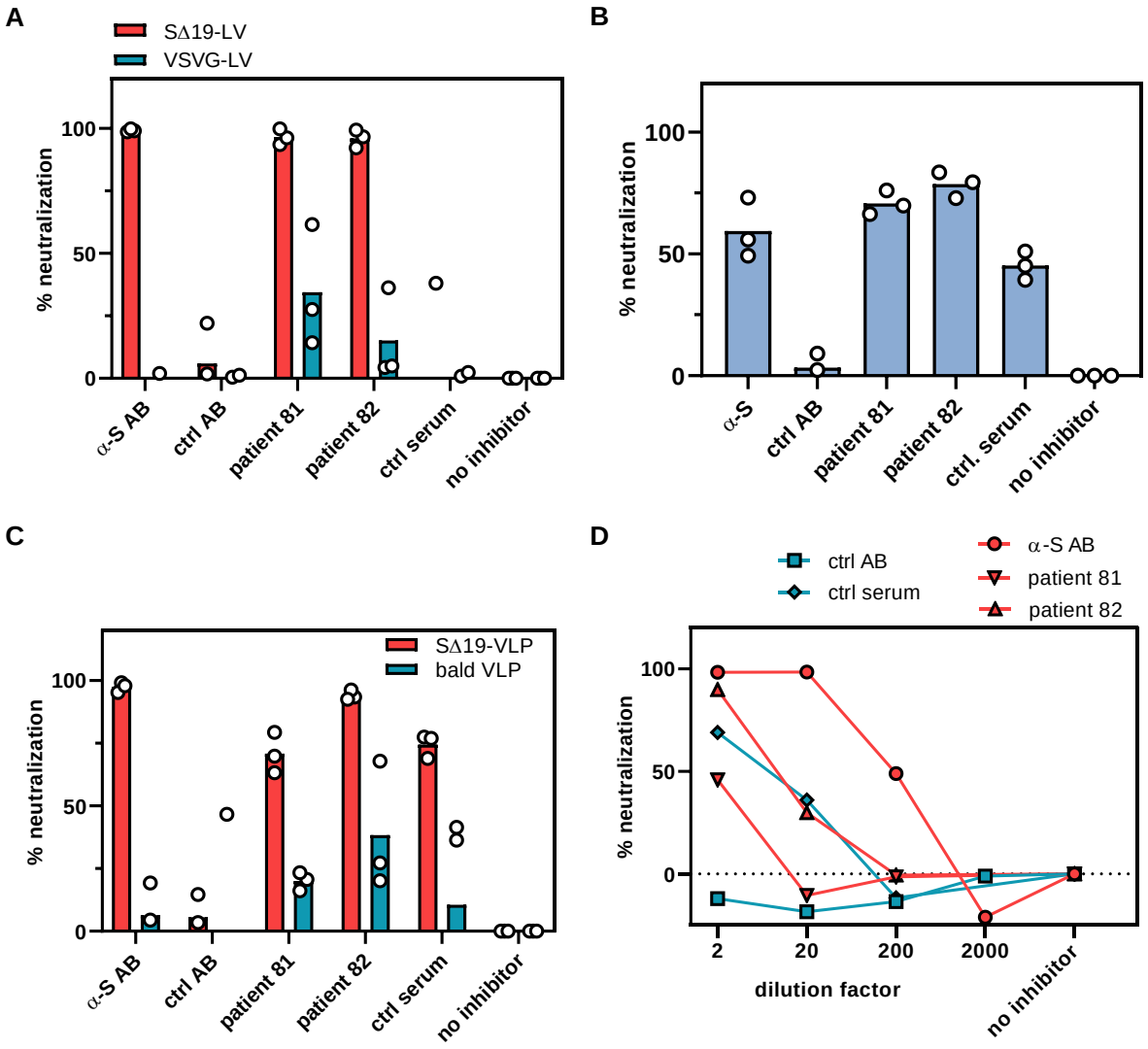

Figure S3: Antibody mediated neutralization of membrane fusion, Related to Figure 4.
Alternative representation of the data represented in Fig. 4A-D. The neutralizing activities of the S-protein specific antibody and the sera from two convalescent Covid-19 patients were determined against S-protein mediated particle entry (**A**), cell-cell fusion (**B**) and FFWO (**C-D**). Data is represented as percent neutralization relative to the control without any inhibitor, with each symbol representing the neutralization determined in a separate run. Bars represent means of neutralization activities, n=3.

Transparent Methods

Expression plasmids

An expression plasmid for codon-optimized SARS-CoV-2 S with a C-terminal HA tag (pCG-SARS-CoV-2-S-HA) was kindly provided by Karl-Klaus Conzelmann (Hennrich et al., 2020).The Plasmid pCG-SARS-CoV-2-SΔ19 encoding the SΔ19 variant was generated by PCR amplification of the truncated S sequence, inserting PacI and SpeI restriction sites as well as a stop codon by PCR with primers 5'-TTATTAATTAAATGTTCGTGTTTCTGGTG-3' and 5'-TATACTAGTTCTAGCAGCAGCTGCC-3'. The PCR fragment was inserted into the pCG backbone by restriction cloning. The lentiviral transfer vector plasmid pCMV-LacZ was generated by amplifying the lacZ coding sequence under CMV promotor control, simultaneously inserting a SbfI restriction site for subcloning into pSEW via EcoRI/Bsu36I (Funke et al., 2008). Expression plasmids pCMV-α and pCMV-ω encoding the α and ω parts of β-galactosidase have been described previously (Holland et al., 2004). The expression plasmid for N-terminally myc-tagged hACE2 (pCMV3-SP-Myc-ACE2) was purchased from Sino Biological (HG10108-NM).

Cell culture and transfection

HEK-293T (Lenti-X 293T, Takara Bio) and Vero E6 (ATCC) cells were cultured in Dulbecco's Minimal Essential Medium High Glucose (Sigma, D6546) supplemented with 10% FBS (Sigma, F7524, lot BCCB7222) and 1x L-glutamine (Sigma, G7513) (i.e. DMEM complete). HEK-293T cells were subcultured twice a week at ratios between 1:8 and 1:10 using 0.25% trypsin in 1 mM EDTA - PBS without Ca^{2+} or Mg^{2+}. MRC-5 cells (ATCC) were cultured in Minimal Essential Medium Eagle (Sigma, M2414) supplemented with 10% FBS, 1x L-glutamine and 1x non-essential amino acids (Gibco, 11140-035). Calu-3 cells (AddexBio, C0016001, lot 0179286) were cultured in Minimal Essential Medium Eagle (Sigma, M2414) supplemented with 10% FBS, 1x L-glutamine, 1x non-essential amino acids (Gibco, 11140-035) and 1x sodium pyruvate (Gibco, 11360-070). MRC-5 and Calu-3 were subcultured with trypsin in EDTA-PBS every two weeks at ratios between 1:2 and 1:3, medium was exchanged twice weekly.

For transfection of HEK-293T in T75 flasks, two mixes were prepared, each in 1 mL plain DMEM High Glucose, one containing 60 µL of 18 mM TA-Trans (polyethyleneimine) and the other 15 µg plasmid DNA. When the amount of S protein encoding plasmid was varied, pCDNA3.1(+) was added to compensate for the overall plasmid quantity. The mixtures were combined and incubated together for at least 20 min. Medium on cells was replaced by 4.3 mL of 1.5 x growth medium (15% FBS, 1.5 x L-Glutamine) and 2 mL transfection mix were added. Cells were incubated for 4-8 h before medium was replaced by 10 mL of fresh growth medium.

Production of LVs and VLPs

For the production of LV particles and VLPs, we adapted our established protocol for the generation of LVs pseudotyped with paramyxoviral glycoproteins (Bender et al., 2016). In brief, HEK-293T cells were transfected with the envelope plasmid pCG-SARS-CoV-2-SΔ19, the transfer vector plasmid pCMV-LacZ and the packaging plasmid pCMVd8.9 in a 35:100:65 ratio. For VLPs, the transfer vector plasmid was omitted, but the total amount of plasmid and the ratio of envelope to packaging plasmid retained. Supernatants harvested 48 hours after transfection were clarified by 0.45 µm filtration. Particles were purified and concentrated 333-fold into PBS from the clarified supernatant by overnight centrifugation over a 20% sucrose cushion at 4500 x g and 4°C. Concentrated stocks were frozen to -80°C before use, and aliquots used once after thawing. Particle numbers were determined by nanoparticle tracking analysis using the NanoSight300 system (Malvern).

Transduction and neutralization

$2x10^4$ cells/ well were seeded into flat-bottom 96-well plates in complete growth medium. On the next day, 0.2 µL/well of SΔ19-LV or VSV-LV stock was added in complete growth medium. For neutralization, antibody and sera were incubated with the vector stock at concentrations of 40 µg/ mL and 50% v/v, respectively, in a final volume of 50 µL/well for 30-60 min at 37°C. Medium was aspirated from the seeded cells and replaced by 50 µL/well of the transduction mix. The next day, medium was exchanged for 100 µL/well of fresh complete growth medium. Transduction efficiencies were determined three days after vector addition by luminescence readout of cellular galactosidase activity.

Sera & S-Neutralizing Antibodies

Sera donation with informed consent was approved by an ethics vote from the local committee at Frankfurt

University Hospital. Sera were from two convalescent patients who had been diagnosed with SARS-CoV-2 by PCR from throat swabs approximately 4 months prior to donation. Both had experienced mild symptoms. A commercially available pool of human off-the-clot sera (PAN Biotech, P30-2701) served as a negative control. A commercially available neutralizing antibody against SARS-CoV-2 (Sino Biological, 40592-R001) and the corresponding normal control (Sino Biological, CR1) served as a positive control.

Fusion assays and neutralization

HEK-293T cells were transfected as described above, in the T75 format. Two days after transfection, cells were detached by incubation in 0.25% trypsin - 1 mM EDTA – PBS for 10-15 min. Cells were characterized with regards to count and viability using the Luna-Fl cell counter and acridine orange/propidium iodide dye (Logos Bio). Cells were pelleted at 300 x g for 5 min and resuspended in complete growth medium to yield a cell density of $5x10^4$ cells/20 µL. Cocultures were set up in V-bottom plates at 10^5 cells/well. Cells were pelleted (300 x g, 30 s at start) just before transfer to the incubator. For FFWO, VLPs were diluted to desired concentrations in complete growth medium and added to cocultures at 20 µL/well by thorough pipetting prior to the 30 s centrifugation. For neutralization of FFWO, $5x10^8$ particles/well were incubated with antibodies or sera for 30 min in the incubator before addition to the coculture. For the neutralization of particle-free cell fusion, effector cells were pre-incubated with antibodies or sera in a volume of 40 µL/well (i.e. $5x10^4$ cells/well) for 30 min in the incubator, retaining the inhibitor concentrations specified above. After pre-incubation, effector cells were mixed with target cells in a total culture volume of 60 µL/well.

Luminescense readout

Activity of the β-galactosidase reporter was quantified using the Galactostar assay kit (Thermo, T1012). At the assay endpoint (3 days post transduction or 20 h after coculture setup), cultures were lysed: Cocultures in V-bottom plate were pelleted at 300 x g, 5 min. Supernatant was removed completely, 50 µL/well of lysis buffer was added and plates were agitated on an orbital shaker at 450 rpm at room temperature for 10 min. Plates were then frozen to -80°C. For the luminescence readout, samples were equilibrated to room temperature and mixed by orbital shaking at 750 rpm for 2 min. 10 µL/well of lysate was added to 50 µL/well of substrate working dilution (prepared according to manufacturer's instructions) in a white flat-bottom plate and mixed by orbital shaking at 750 rpm for 2 min. After 30-60 min incubation at room temperature in the dark, luminescence was measured on an Orion II plate luminometer (Berthold Systems) with an exposure time of 0.1 s/well.

Immunofluorescence staining and laser scanning microscopy

Vero E6 cells constitutively expressing GFP were generated by LV mediated transduction and subsequent puromycin selection. HEK-293T cells were cotransfected with RFP and the SΔ19 or full-length S protein. $5x10^4$ transfected HEK-293T cells were seeded in chamber slides (Thermo, 177402) and left to attach overnight. On the next day $5x10^4$ Vero-GFP cells were added and cocultured for 7 h. Cells were fixed in 4% PFA, permeabilized with 0.5% Triton X-100 in PBS and blocked with 1% BSA/PBS for 15 min. Subsequently, cells were stained with Phalloidin-Atto633 (1:500, Sigma 68825) and HOECHST3342 (1:10,000, Sigma B2261) for 1h at RT before being imaged on an SP8 Lightning laser scanning microscope (Leica) with a HC PL APO CS2 40x/1.30 lens.

Western Blot

Cells were detached by trypsin treatment, counted with a hemocytometer and lyzed in RIPA buffer (50 mM Tris/HCL pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with a protease inhibitor cocktail (Roche, 05892970001). Cell lysates were incubated for 10 min at 95°C in 4x sample buffer (240 mM Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 0.2% bromphenol blue, 20% β-mercaptoethanol). Vector particles were denatured for 10 min at 95°C in 2x Urea buffer (200 mM TRIS/HCl pH 8.0, 5% SDS, 8 M Urea, 0.1 mM EDTA, 2.5% DTT, 0.03% bromphenol blue) (Münch et al., 2011). Samples were electrophoretically separated on a 10% polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham, 10600004). The lower part of the membranes were incubated with mouse anti-p24 (Clone 38/8.7.47, 1:1000, Gentaur) and the upper part with mouse anti-SARS-CoV-2 spike (Clone: 1A9, 1:1000, GeneTex) overnight at 4°C. Subsequently, the membranes were incubated with the secondary antibody rabbit anti-mouse conjugated to horseradish peroxidase (Dako, 1:2000) for 90 min at RT. Luminescence signals were detected on the chemiluminescence reader MicroChemi (DNR) after adding ECL Western Blotting Substrate (Thermo, 32106).

Flow cytometry

10^5 HEK-293T effector cells used for fusion assays were stained for surface expression of S protein. Cell suspensions were washed twice in wash buffer (2% FCS, 0.1% sodium azide, 1 mM EDTA in PBS). S protein was specifically stained with the mouse IgG1 anti-SARS-CoV-2 Spike (Clone: 1A9, GeneTex, 1 µL/10^5 cells in 100 µL) antibody for 45 min at 4°C followed by the incubation with the secondary antibody anti-IgG1-PE (REA1017, Miltenyi Biotec, 1 µL/10^5 cells in 100 µL) for 30 min at 4°C. Viability of the cells was assessed using the fixable viability dye eFluor780 (eBioscience, 1:1000). Finally, cells were fixed in 1% PFA and analyzed by flow cytometry using the MACSQuant Analyzer 10x (Miltenyi Biotec).

Statistical Analysis

All statistical analyses were carried out in GraphPad Prism version 8.4.2. Luminescense data and flow cytometry MFIs were assumed to be lognormally distributed. Accordingly, tests were performed on log-transformed data which were assumed to be normally distributed. Data generated from the same batch of transfected cells (i.e. from the same biological replicate) were handled as matched data. For all repeated measures tests, sphericity was assumed. For particle and inhibitor titrations, five-parameter asymmetric sigmoidal curves were fitted to the data. Differences of population means were quantified by repeated measures 2-way ANOVA (one-way ANOVA, where indicated) on log-transformed data and Tukey's multiple comparisons test. Select multiplicity-adjusted p-values are reported.

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