Cell, Volume *185*

Supplemental information

Evidence in favor of the essentiality of human cell membrane-bound ACE2 and against soluble ACE2 for SARS-CoV-2 infectivity

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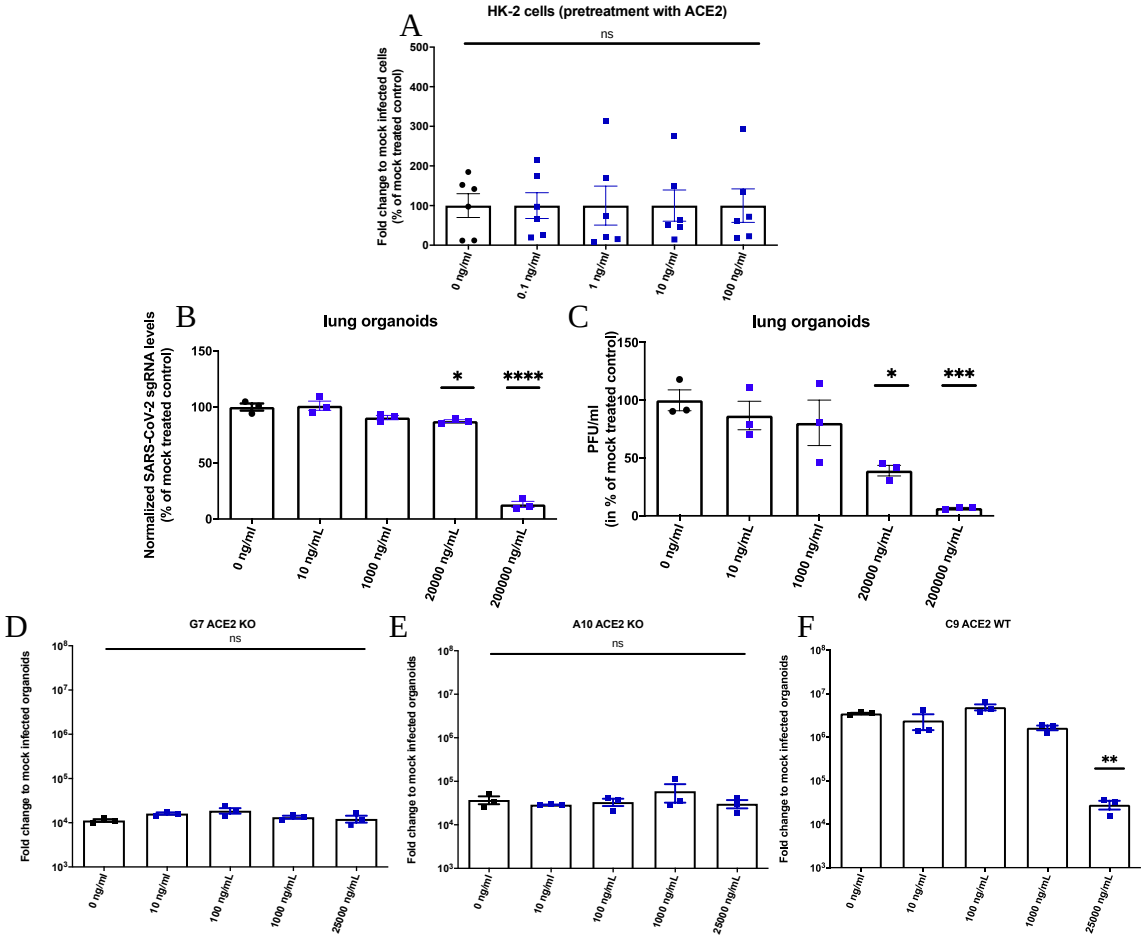
Figure S1. Very low concentrations of human soluble ACE2 have no significant effect on SARS-CoV-2 infectivity.



A) HK-2 cells were infected with SARS-CoV-2 after pretreatment with ACE2 for 24 hours prior to infection. Infectivity was determined 48 hours post infection for the detection of SARS-CoV-2 mRNA by qRT-PCR. The presence of very low concentrations (blue, 0.1, 1, 10 and 100 ng/ml soluble human ACE2) had variable but no significant effect on viral mRNA levels ($p>0.9999$, by one way ANOVA).

B, C) Human lung organoids were infected with SARS-CoV-2 in the presence of different concentrations of soluble human ACE2 (blue). PBS (black) was used as negative control. Infectivity was determined 24 hours later by measuring SARS-CoV-2 RNA by qRT-PCR (C) and infectious virus by plaque assay (D). The presence of low concentrations (10 and 1000ng/ml) of soluble ACE2 had no apparent effect on viral RNA levels nor levels of infectious virus. As expected, high concentrations of ACE2 20000 and 200000ng/ml), by contrast, lowered both RNA and plaque forming units (PFU). * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ vs. 0ng/ml.

D-F) Human kidney organoids generated from wild type (WT; clone C9) or ACE2 knock out (KO; clones G7 and A10) human pluripotent stem cell lines were infected with SARS-CoV-2 in the absence (black) or presence of soluble ACE2 (blue). Infectivity was determined 72 hours post infection for the detection of SARS-CoV-2 mRNA by qRT-PCR, as described previously (Monteil et al., 2020). ACE2 KO kidney organoids from either ACE2 KO line (clones G7 and A10) were completely resistant to SARS-CoV-2 infections and SARS-CoV-2 mRNA expression levels were not increased by the presence of soluble ACE2 (G7: $p=0.0660$, A10: $p= 0.5072$, by one-way ANOVA) (D and E). In WT kidney organoids, the presence of low concentrations (10, 100 and 1000 ng/ml) of soluble human ACE2 had no effect on viral mRNA levels in WT ACE2 kidney organoids, whereas a high concentration (25000ng/ml) of soluble ACE2 decreased mRNA levels significantly (F). n=3 organoids/group per experiment. ** $p<0.01$ vs. 0ng/ml. Data are mean ± SE. One-way ANOVA was used for analysis and when significant a post hoc Dunnett's multiple comparisons test was used.

Methods

SARS-CoV-2 viruses

SARS-CoV-2, isolate USA-WA1/2020 (NR-52281) was deposited by the Center for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH. SARS-CoV-2 was propagated in Vero E6 cells in DMEM supplemented with 2% FBS, 4.5 g/L D-glucose, 4 mM L-glutamine, 10 mM Non-Essential Amino Acids, 1 mM Sodium Pyruvate and 10 mM HEPES as described previously (Blanco-Melo et al., 2020). All work involving live SARS-CoV-2 was performed in the CDC/USDA-approved BSL-3 facility of the Global Health and Emerging Pathogens Institute at the Icahn School of Medicine at Mount Sinai in accordance with institutional biosafety requirements.

SARS-CoV-2, isolate Sweden/01/2020, was isolated on Vero-E6 cells, from a nasopharyngeal sample of a patient in Sweden. Virus was tittered using a plaque assay as previously described (Blanco-Melo et al., 2020) with fixation of cells 72 hours post infection. The SARS-CoV-2 isolate was sequenced by Next-Generation Sequencing (GenBank accession number MT093571). All work involving SARS-CoV-2 for kidney organoid infections was performed in BSL-3 facility of the Public Health Agency of Sweden in accordance with institutional biosafety requirements. SARS-CoV-2, isolate USA-WA1/2020 (NR-52281), was used to infect lung organoids. SARS-CoV-2, isolate Sweden/01/2020 (MT093571) was used to infect kidney organoids and HK-2 cells.

Soluble ACE2 protein

The human soluble ACE2 used by us consists of the amino acids 18-740, without his-tag (Poglitsch et al., 2012). The first 17 amino acids are the signal peptide. This protein was commercialized by Apeiron under the name APN01. Due to the collectrin like domain APN01 dimerizes (Poglitsch et al., 2012). Yeung et al (Yeung et al., 2021) indicated that the source of their protein was the Sigma product Cat#SAE0064 which is also human ACE2 18-740 with a 10xHis tag. Under denaturing conditions (SDS-PAGE) the soluble 18-740 ACE2 from Sigma used by Yeung et al. behaves as a monomer (https://www.sigmaaldrich.com/US/en/product/sigma/sae0064?gclid=EAIaIQobChMI8uWL1OmI9gIVTOh3Ch3bXwv8EAAYAiAAEgKIRvD_BwE Accessed 05-11-22). As shown previously by Poglitsch et al (Poglitsch et al., 2012) the soluble ACE2 used by us here can behave as a monomer in SDS-Page. This is likely because the intermolecular non-covalent bounds are disrupted by SDS. However, as shown in the same paper side-by side, the dimerization of human 18-740 ACE2 can only be seen in native PAGE, when those bounds are intact under native conditions (Poglitsch et al., 2012). Therefore, the soluble protein used by us and Yeung et al can dimerize and it is essentially the same except for the presence of a his-tag in the protein from Sigma-Aldrich as noted above.

Infectivity studies in HK-2 cells

We used a protocol designed to replicate Yeung et al (Yeung et al., 2021), based on information provided by the editors from Cell. HK-2 cells ($5x10^4$ cells per well, ATCC- CRL-2190) were seeded in a 48 well plate in DMEM/F12 containing 10%FBS. 24 hours post-seeding, cells were treated with defined concentrations of human soluble ACE2 (APN01) (0, 0.1, 1, 10 and 100ng/ml) in DMEM/F12 containing 10%FBS. 24 hours post-treatment, cells were infected with SARS-CoV-2 Wuhan strain isolated in Sweden (Genbank number MT093571) at a MOI 0.01 in DMEM/F12 0%FBS in presence of human soluble ACE2. 1-hour post-infection, cells were washed with PBS and incubated in DMEM/F12 10% FBS for 48 hours at 37˚C 5% CO_2. 48-hours post-infection, cells were washed 3 times with PBS and lysed using Trizol (ThermoFisher) before analysis by qRT-PCR for viral RNA detection as previously described (Monteil et al., 2020).

Infectivity studies in human lung organoids

Human induced pluripotent stem cells generated from human fibroblasts were cultured in mTeSR media as previously described (Ludwig and J, 2007) and differentiated into hPSC derived lung organoids as previously reported (Han et al., 2021). These organoids express not only the receptor ACE2 but also the protease TMPRSS2, needed for activation and subsequent internalization (Han et al., 2021, Hoffmann et al., 2020). Lung organoids were then infected with a combination of infectious SARS-CoV-2 particles (1000 PFU) and soluble human soluble ACE2 (0, 10, 1000, 20000, 200000ng/ml) (n=3 each) for 1 hour (Wysocki et al., 2021). PBS was used as

a negative control. 24 hours post infection, human pluripotent stem cell derived lung organoids were washed three times in PBS and lysed in TRIzol for RNA analysis.

qRT-PCR for SARS-CoV-2 RNA after infection of human lung organoids
Total RNA samples were prepared from organoids using TRIzol and Direct-zol RNA Miniprep Plus kit (Zymo Research) according to the manufacturer's instructions. To quantify viral replication, measured by the accumulation of subgenomic N transcripts, one-step quantitative real-time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) with primers specific for the TRS-L and TRS-B sites for the N gene as well as GAPDH as an internal reference as described previously (Yang et al., 2020). Quantitative real-time PCR reactions were performed on a LightCycler 480 Instrument II (Roche). Delta-delta-cycle threshold (ΔΔCT) was determined relative to the GAPDH and mock infected/treated samples. Error bars indicate the SEM of the mean from three biological replicates.

Plaque assay for infectious virus after infection of human lung organoids
After infections of lung organoids were completed, supernatant was collected and spun at 1000 RPM to remove debris and then infectious titers of SARS-CoV-2 were determined by plaque assay in Vero E6 cells in Minimum Essential Media supplemented with 2% FBS, 4 mM L-glutamine, 0.2% BSA, 10 mM HEPES and 0.12% $NaHCO_3$ and 0.7% agar.

Kidney organoid differentiation. Wild type (WT) and ACE2 knock out (KO) human embryonic stem cells were used for the generation of kidney organoids as previously described (Garreta et al., 2021). Kidney organoid differentiation was performed as previously described elsewhere (Monteil et al., 2020, Garreta et al., 2021). Briefly, 100,000 cells/well were plated on a 24 multi-well plate coated with 5µl/ml vitronectin and further incubated with supplemented Essential 8 Basal medium at 37ºC overnight. The day after cells were treated for 3 subsequent days in Advanced RPMI 1640 basal medium (ThermoFisher) supplemented with 8µM CHIR (Merck) and 1% Penicillin-Streptomycin and 1% of GlutaMAX TM (ThermoFisher). The medium was changed every day. From day 3 to 4, media were changed to Advanced RPMI supplemented with 200ng/ml FGF9 (Peprotech), 1µg/ml heparin (Merck) and 10ng/ml activin A (Vitro). On day 4, cultures were rinsed twice with PBS, and resuspended in Advanced RPMI supplemented with 5µM CHIR, 200ng/ml FGF9 and 1µg/ml Heparin. Cellular suspensions were seeded in V-shape 96 multi-well plate at a final concentration of 100,000 cells/well and centrifugated at 2000 rpm for 3 minutes. The resulting spheroids were incubated during 1h at 37ºC. Culture media was changed to Advanced RPMI supplemented with 200ng/ml FGF9 and 1µg/ml Heparin for 7 additional days. From that moment the media was changed every second day until day 16 of differentiation. From that moment kidney organoids were further used for SARS-CoV-2 infections.

SARS-CoV-2 infections in human kidney organoids. Kidney organoids were infected with 10^3 SARS-CoV-2 infectious particles in advanced RPMI medium (Thermofisher) in 100µl per well for 3 days. On day 3 post-infection, organoids were washed 3 times with PBS before being lysed with Trizol™ (Thermofisher) for ulterior qPCR analysis.

Treatment of human kidney organoids with hrsACE2. Different concentrations of human soluble ACE2 (APN01) (0, 10, 100, 1000, 25000ng/ml) were mixed with 10^3 particles of SARS-CoV-2 for 30min at 37°C in a final volume of 100µl per well in advanced RPMI medium (Thermofisher). Organoids were then infected with the mixes for 3 days. After three days, organoids were washed 3 times with PBS, pooled (3 organoids/condition for kidneys) and lysed using Trizol™ (Thermofisher) to detect intracellular viral RNA.

qRT-PCR for SARS-CoV-2 RNA after infection of human kidney organoids. Samples were extracted using Direct-zol RNA MiniPrep kit (Zymo Research). qRT-PCR was performed using E-gene SARS-CoV-2 primers/probe following guidelines by the World Health Organization (https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf). RNase P was used as an endogenous gene control to normalize the levels of intracellular viral RNA as previously described (Monteil et

al., 2020, Garreta et al., 2021). Delta-delta-cycle threshold (ΔΔCT) was determined relative to the RNase P and mock infected samples. Error bars indicate the SD of the mean from three biological replicates.

Statistics. Shapiro-Wilk-test was used to test normality. Normally distributed data was analyzed by one-way ANOVA and when significant followed by post hoc Dunnett's multiple comparisons test. The number of each experiment is given in the figure. Data are mean ± SE.

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