## MATERIALS AND METHODS

### iPSC directed differentiation into alveolar epithelial type 2 cells (iAT2s) and air-liquid interface (ALI) culture

All experiments involving the differentiation of human pluripotent stem cell (PSC) lines were performed with the approval of the Institutional Review Board of Boston University (protocol H33122). All PSC lines were maintained in feeder-free conditions, on growth factor reduced Matrigel (Corning) in 6-well tissue culture dishes (Corning), in mTeSR1 medium (StemCell Technologies) using gentle cell dissociation reagent for passaging. Further details of iPSC derivation, characterization, and culture are available for free download at http://www.bu.edu/dbin/stemcells/protocols.php.

The human induced pluripotent stem cell (iPSC) line, (clone SPC2-ST-B2), engineered to carry a tdTomato reporter targeted to one allele of the endogenous *SFTPC* locus (*9*), underwent directed differentiation to generate iPSC-derived alveolar epithelial type II like cells (iAT2s) in 3D Matrigel cultures using methods we have previously published . As previously described (*4*) we performed PSC directed differentiation via definitive endoderm into NKX2-1 lung progenitors as follows. In short, cells maintained in mTeSR1 media were differentiated into definitive endoderm using the STEMdiff Definitive Endoderm Kit (StemCell Technologies) and after the endoderm-induction stage, cells were dissociated with gentle cell dissociation reagent (GCDR) and passaged into 6 well plates pre-coated with growth factor reduced Matrigel in ‘‘DS/SB’’ anteriorization media, consisting of complete serum-free differentiation medium (cSFDM) base as previously described (*10*) supplemented with 10 μm SB431542 (‘‘SB’’; Tocris) and 2 μm Dorsomorphin (‘‘DS’’; Stemgent). For the first 24 hr after passaging, 10 μm Y-27632 was added to the media. After anteriorization in DS/SB media for 3 days (72 hr), cells were cultured in ‘‘CBRa’’ lung progenitor-induction media for 9-11 days. ‘‘CBRa’’ media consists of cSFDM containing 3 μm CHIR99021 (Tocris), 10 ng/mL recombinant human BMP4 (rhBMP4, R&D Systems), and 100nM retinoic acid (RA, Sigma), as previously described (Jacob et al., 2017). On day 15 of differentiation, live cells were sorted on a high-speed cell sorted (MoFlo Legacy or MoFlo Astrios EQ) to isolate NKX2-1+ lung progenitors based on $CD47^{hi}/CD26^{neg}$ gating (Hawkins et al., 2017). Sorted day 15 progenitors were then resuspended in undiluted growth factor-reduced Matrigel (Corning) and distal/alveolar differentiation of cells was performed in ‘‘**CK+DCI**’’ medium, consisting of cSFDM base, with 3 μm CHIR99021, 10 ng/mL rhKGF, and 50 nM dexamethasone (Sigma), 0.1mM8-Bromoadenosine 30,50-cyclic monophosphate sodium salt (Sigma) and 0.1mM3-Isobutyl-1-methylxanthine (IBMX; Sigma) (DCI) with a brief period of CHIR99021 withdrawal on days 31-35 to achieve iAT2 maturation. To establish pure cultures of iAT2s, cells were sorted by flow cytometry to isolate $SFTPC^{tdTomato+}$ cells on days 41 and 69 of differentiation. iAT2s were then maintained through serial passaging as self-renewing monolayered epithelial spheres (“alveolospheres”) by plating in Matrigel (Corning) droplets at a density of 400 cells/μl with refeeding every other day in CK+DCI medium according to our previously published protocol (Jacob et al., 2019). iAT2 culture quality and purity was monitored at each passage by flow cytometry, with >80% of cells expressing $SFTPC^{tdTomato}$ over time, as we have previously detailed (*4, 9*).

To establish air-liquid interface (ALI) cultures, single cell suspensions of iAT2s were prepared as we have recently detailed (*6*). Briefly, Matrigel droplets containing iAT2s as 3D sphere cultures were dissolved in 2 mg/ml dispase (Sigma) and alveolospheres were dissociated in 0.05% trypsin

(Gibco) to generate a single-cell suspension. 6.5mm Transwell inserts (Corning) were coated with dilute Matrigel (Corning) according to the manufacturer's instructions. Single-cell iAT2s were plated on Transwells at a density of 520,000 live cells/$cm^2$ in 100µl of CK+DCI with 10uM Rho-associated kinase inhibitor ("Y"; Sigma Y-27632). 600ul of CK+DCI+Y was added to the basolateral compartment. 24 hours after plating, basolateral media was refreshed to CK+DCI+Y. 48 hours after plating, apical media was aspirated to initiate air-liquid interface culture. 72 hours after plating, basolateral media was changed to CK+DCI to remove the rho-associated kinase inhibitor. Basolateral media was changed 3 times per week thereafter.

**SARS-CoV-2 propagation and titration**

SARS-CoV-2 stocks (isolate USA_WA1/2020, kindly provided by CDC's Principal Investigator Natalie Thornburg and the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA)) were grown in Vero E6 cells (ATCC CRL-1586) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 mg/ml). To remove confounding cytokines and other factors, viral stocks were purified by ultracentrifugation through a 20% sucrose cushion at 80,000xg for 2 hours at 4°C (*25*). SARS-CoV-2 titers were determined in Vero E6 cells by tissue culture infectious dose 50 ($TCID_{50}$) assay. All work with SARS-CoV-2 was performed in the biosafety level 4 (BSL4) facility of the National Emerging Infectious Diseases Laboratories at Boston University, Boston, MA following approved SOPs.

**SARS-CoV-2 infection of iAT2s**

iAT2 cells plated in ALI culture were infected with purified SARS-CoV-2 stock at the indicated multiplicity of infection (MOI). 100 µl inoculum was prepared in CK+DCI media (or mock-infected with medium-only). Inoculum was added to the apical chamber of each Transwell and incubated for 1 hour at 37°C and 5% $CO_2$. After the adsorption period, the inoculum was removed and cells were incubated at 37°C for 1 or 4 days. At the time of harvest, basolateral media was collected for further analysis and apical washes were performed by adding 100 µl CK+DCI media to the apical chamber, incubated for 15 mins at room temperature before collection for further analysis. Both the apical washes and basolateral media were used for viral titration and Luminex assays as described below. Infected and mock-infected iAT2 cells were fixed in 10% formalin and used for immunofluorescence analysis or electron microscopy as described below. For flow cytometry, infected and mock-infected iAT2 cells were first detached by adding 0.2mL Accutase (A6964, Sigma) apically and incubated at room temperature for 15 minutes. Detached cells were pelleted by low speed centrifugation, resuspended in 10% formalin, and used for flow cytometry as described below. Cells were lysed in TRIzol for RNAseq and RT-qPCR analysis.

**Immunofluorescence microscopy of iAT2s**

For nucleoprotein immunofluorescence, infected or control iAT2s on Transwell inserts were fixed in 10% formalin for 6 hours, washed twice in PBS (10 min, room temperature), permeabilized with PBS containing 0.25% Triton X-100 and 2.5% normal donkey serum (30 min, room temperature), and blocked with PBS containing 2.5% normal donkey serum (20 min, room temperature). Subsequently, cells were incubated with primary antibody diluted in anti-SARS-CoV nucleoprotein (N) antibody (rabbit polyclonal, 1:2500, Rockland Immunochemicals, Cat #200-401-A50) diluted in 4% normal donkey serum overnight at 4°C. This antibody cross-reacts with the SARS-CoV-2 nucleoprotein (*26*). Next, cells were washed with PBS three times (5 min, room temperature), and incubated with secondary antibody (AlexaFluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L), 1:500, Jackson ImmunoResearch 711-545-252) for 2 h at room

temperature. Cells were washed with PBS three times (5 min, room temperature), incubated with Hoechst (1:500, Life Technologies) for 30 min, and washed again. Transwell inserts were then cut out with a scalpel and mounted with Prolong Diamond Mounting Reagent (Life Technologies). Slides were imaged with a confocal microscope (Zeiss LSM 700).

For ACE2 immunofluorescence, never-infected iAT2s at ALI were fixed for 10 min at RT in 4% PFA. After fixation, the same protocol was followed as above, using an anti-ACE2 primary antibody (R&D, AF933, 1:100) or pre-immune serum for overnight incubation, and an appropriate secondary (AlexaFluor 647 Donkey Anti-Goat IgG (H+L), 1:500, Invitrogen A21447).

**Pseudotyped lentiviral entry experiments**

Pseudotyped particles carrying the pHAGE-EF1αL-GFP lentiviral vector were packaged using a 5-plasmid transfection protocol (*27*). In brief, 293T cells were transfected using Trans-IT (Mirus) with a plasmid carrying the lentiviral backbone (pHAGE-EF1αL-GFP; plasmid map downloadable from www.kottonlab.com), 3 helper plasmids encoding Rev, tat, and gag/pol genes, in addition to plasmids encoding either the VSV-G or the SARS-CoV-2 Spike envelope (plasmid HDM-IDTSpike-FixK, cloned by Jesse Bloom and a kind gift from Alex Balazs, (*18*)). Supernatants carrying packaged lentivirus were collected at 48, 60 and 72 hours and then concentrated by ultracentrifugation. Lentiviral titers were determined by infecting FG293 with VSV-G pseudotype or 293T cells overexpressing ACE2 (created by Michael Farzan and a kind gift from Alex Balazs, (*18*)) with Spike pseudotype and then quantifying GFP+ cells by flow cytometry. To transduce iAT2s growing in ALI cultures, VSV-G (MOI 50) or Spike (MOI 30) pseudotyped lentiviruses, diluted in CK+DCI medium supplemented with Polybrene (5 µg/mL; EMD Millipore), were applied to the apical surface for 4 hours. GFP expression was assessed 48-72 hours after transduction by microscopy (Keyence) or flow cytometry.

**Flow cytometry**

For post infection flow cytometry, fixed iAT2s were either stained for cell surface expression of ACE2 (R&D, #AF933, 4-8µg/2.5x$10^6$ cells) followed by donkey anti-goat IgG-AF647 (Invitrogen, #A21447) or were permeabilized with saponin buffer (Biolegend) then stained with SARS-CoV nucleoprotein (N) antibody (Rockland, #200-401-A50, 1:1000), followed by donkey anti-rabbit IgG-AF488 (Jackson ImmunoResearch, #711-545-152). Gating was based on either mock infected stained controls or infected, isotype-stained controls. Flow cytometry staining was quantified using a Stratedigm S1000EXI and analysed with FlowJo v10.6.2 (FlowJo, Tree Star Inc). FACS plots shown represent single-cells based on forward-scatter/side-scatter gating.

**Reverse Transcriptase Quantitative PCR (RT-qPCR)**

iAT2 cells were collected in Qiazol (Qiagen) or TRIzol (ThermoFisher) then RNA was extracted using the RNAeasy mini kit (Qiagen) or following the manufacturer's protocol, respectively. Complementary DNA (cDNA) was generated by reverse transcription using MultiScribe Reverse Transcriptase (Applied Biosystems). PCR was then run for 40 cycles using an Applied Biosystems QuantStudio 384-well system. Predesigned TaqMan probes were from Applied Biosystems or IDT (see table below). Relative gene expression was calculated based on the average Ct value for technical triplicates, normalized to 18S control, and fold change over mock-infected cells was calculated using $2^{-\Delta\Delta Ct}$. If probes were undetected, they were assigned a Ct value of 40 to allow for fold change calculations, and biological replicates, as indicated in each figure legend, were run for statistical analyses.

| Target | Catalogue number | Supplier |
|---|---|---|
| *CXCL2* | Hs00601975_m1 | Applied Biosystems |
| *CXCL3* | Hs00171061_m1 | Applied Biosystems |
| *CXCL8* | Hs00174103_m1 | Applied Biosystems |
| *CXCL10* | Hs00171042_m1 | Applied Biosystems |
| *CXCL11* | Hs00171138_m1 | Applied Biosystems |
| *IFNB1* | Hs01077958_s1 | Applied Biosystems |
| *IFNL1 (IL29)* | Hs00601677_g1 | Applied Biosystems |
| *IFNL2 (IL28A)* | Hs00820125_g1 | Applied Biosystems |
| *IL6* | Hs00174131_m1 | Applied Biosystems |
| *IFIT1* | Hs00356631_g1 | Applied Biosystems |
| *ISG15* | Hs00192713_m1 | Applied Biosystems |
| *LAMP3* | Hs01111316_m1 | Applied Biosystems |
| *MX1* | Hs00895608_m1 | Applied Biosystems |
| *NFKB1* | Hs00765730_m1 | Applied Biosystems |
| *NFKB2* | Hs01028890_g1 | Applied Biosystems |
| *RELA* | Hs00153294_m1 | Applied Biosystems |
| *RELB* | Hs00232399_m1 | Applied Biosystems |
| *SARS-CoV2 N* | #10006606 | IDT |
| *SFTPC* | Hs00161628_m1 | Applied Biosystems |
| *SFTPA1* | Hs00831305_s1 | Applied Biosystems |
| *TNF* | Hs00174128_m1 | Applied Biosystems |
| *18S* | 4318839 | Applied Biosystems |

**Transmission electron microscopy**

iAT2 ALI cultures on Transwell inserts were infected with SARS-CoV-2 at an MOI of 140 or mock-infected. At 1 day post infection, cells were fixed and inactivated in 10% formalin for 6 hours at 4°C and removed from the BSL-4 laboratory. The cells were washed with PBS and then post-fixed in 1.5% osmium tetroxide (Polysciences) overnight at 4°C. The membrane was excised

from the insert, block stained in 1.5% uranyl acetate (Electron Microscopy Sciences, EMS) for 1 hour at room temperature (RT). The samples were dehydrated quickly through acetone on ice, from 70% to 80% to 90%. The samples were then incubated 2 times in 100% acetone at RT for 10 min each, and in propylene oxide at RT for 15 min each. Finally, the samples were changed into EMbed 812 (EMS), left for 2 hours at RT, changed into fresh EMbed 812 and left overnight at RT, after which they were embedded in fresh EMbed 812 and polymerized overnight at 60°C. Embedded samples were thin sectioned (70 nm) and grids were stained in 4% aqueous uranyl acetate for 10 min at RT followed by lead citrate for 10 min at RT. Electron microscopy was performed on a Philips CM12 EM operated at 100kV, and images were recorded on a TVIPS F216 CMOS camera with a pixel size of 0.85-3.80 nm per pixel.

**Entry inhibitors treatment of iAT2s**

iAT2s plated in ALI culture were pre-treated apically (100 µL) and basolaterally (600 µL) with 100 µM camostat mesylate (Tocris #59721-29-5), 50 µM E-64D (Selleckchem, #S7393), both, or DMSO control for 30 min at 37°C. After 30 min, all apical media were aspirated and SARS-CoV-2 (MOI 1) was added for 1 hour without any drugs apically, after which the inoculum was removed. iAT2s were exposed to the compounds basolaterally for the entire duration of the experiment. Cells were harvested in TRIzol after 2 dpi and processed for RT-qPCR.

**Luminex analysis**

Apical washes and basolateral media samples were clarified by centrifugation and analyzed using the Magnetic Luminex® Performance Assay Human HighSensitivity Cytokine Base Kit A (R&D Systems, Inc). Apical washes were diluted 1:2, basolateral media was undiluted, except to detect IL-8 and VEGF, where it was diluted 1:10. Limit of detection: GM-CSF = 0.13 pg/mL, IL-6 = 0.31 pg/mL, IL-8 = 0.07 pg/mL, TNF-α = 0.54 pg/mL and VEGF = 1.35 pg/mL. Mean fluorescence intensity was measured to calculate final concentration in pg/mL using Bioplex200 and Bioplex Manager 5 software (Biorad).

**RNA sequencing and bioinformatic analyses**

For bulk RNA sequencing (RNA-Seq), biological triplicate (n=3) samples of purified RNA extracts were harvested from each group of samples prepared as follows. After 208 days of total time in culture, iAT2s cultured as serially passaged 3D spheres were single-cell passaged onto Transwell inserts. Apical media was removed on day 210 to initiate air-liquid interface (ALI) culture. On day 218, 6 replicate wells of iAT2s were exposed to SARS-CoV-2 in an apical inoculum and 3 replicate wells were exposed to mock. On day 219, 3 mock and 3 post-infection samples (1 dpi) were collected. Three additional post-infection samples (4 dpi) were collected on day 222. mRNA was isolated from each sample using magnetic bead-based poly(A) selection, followed by synthesis of cDNA fragments. The products were end-paired and PCR-amplified to create each final cDNA library. Sequencing of pooled libraries was done using a NextSeq 500 (Illumina). The quality of the raw sequencing data was assessed using FastQC v.0.11.7. Sequence reads were aligned to a combination of the human and SARS-CoV-2 genome reference (GRCh38 and Wuhan Hu-1 isolate) and the TdTomato reporter sequence, using STAR v.2.5.2b (*28*). Counts per gene were summarized using the featureCounts function from the subread package v.1.6.2. The edgeR package v.3.25.10 was used to import, organize, filter and normalize the data. Genes that were not expressed in at least one of the experimental groups were filtered out (keeping only genes that had at least 10 reads in at least 3 libraries, that is, a worthwhile number of samples as determined by the replicate number in the design matrix). The TMM method was used for normalization. Principal Component Analysis (PCA) and Multidimensional Scaling (MDS) were

used for exploratory analysis, to assess sample similarities and potential batch effects. Subsequently, the limma package v3.39.19 (*29*) with its voom method, namely, linear modelling and empirical Bayes moderation was used to test differential expression (moderate t-tests). P-values were adjusted for multiple testing using Benjamini-Hochberg correction (false discovery rate-adjusted p-value; FDR). Differentially expressed genes between the groups in each experiment were visualized using Glimma v 1.11.1, and FDR<0.05 was set as the threshold for determining significant differential gene expression. Gene set analysis was performed with Hallmark gene sets using the Camera package (*30*).

**Human COVID-19 autopsy specimens and lung tissue sections**

This study was reviewed by the IRB of Boston University and found not to constitute human subjects research. With consent from next-of-kin, human lung tissues from decedents with COVID-19 were collected at the time of autopsies performed at Boston Medical Center. Samples from individuals with shorter duration between symptom onset and death were prioritized for analysis. Samples were fixed in formalin and embedded in paraffin. Healthy tissue adjacent to a lung tumor resected prior to the emergence of COVID-19 was collected with IRB approval under protocol H-37859 and utilized as a control.

| Patient | Symptom onset to death (Days) | Hospitalization to death (Days) | $O_2$ requirement at death |
|---|---|---|---|
| COVID 1 | 8 | 7 | 7L nasal cannula |
| COVID 2 | 9 | 4 | 15L nasal cannula |

Immunohistochemistry on autopsy lung tissue was performed using freshly cut 5 μm thick FFPE tissue sections and stained with AE1/AE3 (Ventana, 760-2135) on a Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, AZ, USA) after mild protease 3 (Ventana, 760-2020) digestion and heat induced epitope retrieval with alkaline CC1 buffer (Ventana, 950-124). Slides were visualized with DAB using Optiview detection (Ventana, 760-700). Parallel sections stained with H&E and AE1/AE3 were performed to identify sloughed pneumocytes from admixed inflammatory cells. Immunofluorescent staining was performed on additional FFPE sections from each patient. After deparaffinization in xylene, hydration, and antigen retrieval in citrate-based unmasking solution (Vector, H-3300), sections were blocked and permeabilized in 4% normal donkey serum and 0.1% Triton X-100 (Sigma) for 1 hr and incubated overnight with anti-pro-SFTPC antibody (Santa Cruz, sc-518029) in blocking solution. Sections were washed with 0.1% Triton X-100 in PBS and incubated with secondary antibody (anti-mouse IgG AlexaFluor647, Invitrogen A32787, 1:500) for 2 hrs at RT. Nuclei were counterstained with Hoechst and sections were mounted with Prolong Diamond Anti-Fade Mounting Reagent (ThermoFisher) and coverslipped. Immunofluorescent imaging was performed on a Zeiss LSM 700 confocal microscope.

**Statistical methods**

Statistical methods relevant to each figure are outlined in the figure legend. In short, unpaired, two-tailed Student's t tests were used to compare quantitative analyses comprising two groups of n = 3 or more samples, or one-way ANOVAs with multiple comparisons were used to compare three or more groups. Further specifics about the replicates used in each experiment are available

in the figure legends. In these cases, a Gaussian distribution and equal variance between samples was assumed as the experiments represent random samples of the measured variable. The p value threshold to determine significance was set at $p = 0.05$. p value annotations on graphs are as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data for quantitative experiments is typically represented as the mean with error bars representing the standard deviation or standard error of the mean, as specified in the figure legends.