

Isogenic induced-pluripotent-stem-cell-derived airway- and alveolus-on-chip models reveal specific innate immune responses

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Table S1. Top upregulated REACTOME pathways in SARS-CoV-2-infected airway chips

Term	Count	FDR
Interferon alpha/beta signaling	31	1.51E-38
Interferon signaling	41	2.95E-34
Cytokine Signaling in Immune system	55	2.02E-30
Immune system	70	6.21E-22
Interferon gamma signaling	19	1.99E-16
Antiviral mechanism by IFN-stimulated genes	14	2.40E-07
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	8	7.81E-07
Endosomal/ Vacuolar pathway	6	2.38E-06
ISG15 antiviral mechanism	10	2.37E-06
SARS-CoV-2 activates/modulates innate and adaptive immune responses	12	2.50E-06
DDX58/IFIH1-mediated induction of interferon-alpha/beta	10	5.43E-06
Negative regulators of DDX58/IFIH1 signaling	7	4.31E-05
OAS antiviral response	5	4.31E-05
ER-Phagosome pathway	9	0.0001
SARS-CoV Infections	17	0.0001
SARS-CoV-2-host interactions	12	0.0002
Antigen processing-cross presentation	9	0.0003
Viral infection pathways	23	0.0006
Class I MHC mediated antigen processing & presentation	15	0.0007
SARS-CoV-2 Infection	13	0.0011
Adaptive immune system	21	0.0018
Innate immune system	25	0.0030
Signaling by Interleukins	15	0.0059
TRAF3-dependent IRF activation pathway	4	0.0081
RSV-host interactions	7	0.0087
Infectious disease	23	0.0093

Table S2. Top upregulated REACTOME pathways in SARS-CoV-2-infected alveolus chips

Term	Count	FDR
Cell cycle	23	6.00E-12
Cell cycle, mitotic	21	8.13E-12
Resolution of sister chromatid cohesion	12	4.65E-10
Mitotic anaphase	14	9.33E-10
Mitotic metaphase and anaphase	14	9.33E-10
Mitotic spindle checkpoint	11	1.90E-09
Mitotic prometaphase	13	2.08E-09
M phase	16	5.21E-09
Cell cycle checkpoints	14	6.84E-09
Amplification of signal from the kinetochores	10	6.84E-09
Amplification of signal from unattached kinetochores via a MAD2 inhibitory signal	10	6.84E-09
Separation of sister chromatids	12	1.01E-08
EML4 and NUDC in mitotic spindle formation	10	3.43E-08
Rho GTPases activate formins	10	1.57E-07
APC/C-mediated degradation of cell cycle proteins	8	1.53E-06
Regulation of mitotic cell cycle	8	1.53E-06
Activation of APC/C and APC/C:CDC20 mediated degradation of mitotic proteins	7	1.42E-05
Rho GTPase effectors	11	1.81E-05
G2/M transition	9	2.84E-05
Mitotic G2-G2/M phases	9	2.91E-05
Cyclin A/B1/B2 associated events during G2/M transition	5	4.85E-05
Chemokine receptors bind chemokines	6	6.69E-05
APC/C:CDC20 mediated degradation of mitotic proteins	6	0.0002
Interferon alpha/beta signaling	6	0.0002
Signal transduction	26	0.0003
Polo-like kinase mediated events	4	0.0004
Interleukin-10 signaling	5	0.0005
Cytokine signaling in immune system	13	0.0011
Signaling by rho GTPases	12	0.0019
Signaling by rho GTPases, miro GTPases and rhobtb3	12	0.0021
Aurka activation by TPX2	5	0.0023
APC:CDC20 mediated degradation of cell cycle proteins prior to satisfaction of the cell cycle checkpoint	5	0.0023
Activation of nima kinases NEK9, NEK6, NEK7	3	0.0030
Regulation of APC/C activators between G1/S and early anaphase	5	0.0031
Interferon signaling	7	0.0083

Senescence-associated secretory phenotype (SASP)	5	0.0098
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Table S3. Selected gene ontology (GO) results for common genes upregulated in both airway and alveolus chips following IAV infection.

Term	Count	P value	FDR
Defense response to virus	75	9.48E-52	3.06E-48
Innate immune response	93	1.84E-40	2.97E-37
Inflammatory response	75	4.23E-33	4.55E-30
Negative regulation of viral genome replication	28	2.80E-29	2.26E-26
Response to virus	38	8.37E-28	5.40E-25
Immune response	65	6.19E-21	3.33E-18
Cellular response to lipopolysaccharide	40	3.126E-20	1.44E-17
Chemotaxis	29	1.31E-15	5.26E-13
Defense response to bacterium	32	5.25E-15	1.77E-12
Chemokine-mediated signaling pathway	22	5.50E-15	1.77E-12
Cellular response to interferon-gamma	24	2.97E-13	8.72E-11
Neutrophil chemotaxis	21	2.33E-12	6.26E-10
Cytokine-mediated signaling pathway	27	2.68E-12	6.65E-10
Positive regulation of I-kappa b kinase/NF-kappa b signaling	32	5.23E-12	1.21E-09
Apoptotic process	56	6.86E-12	1.48E-09
Positive regulation of inflammatory response	23	8.48E-12	1.71E-09
Response to interferon-gamma	13	1.81E-11	3.43E-09
Positive regulation of tumor necrosis factor production	22	9.17E-11	1.6E-08
Response to interferon-beta	9	2.15E-10	3.66E-08
Positive regulation of NF-kappa b transcription factor activity	24	2.66E-10	4.29E-08
Positive regulation of interferon-beta production	14	5.62E-10	8.64E-08
Cellular response to tumor necrosis factor	23	8.87E-10	1.30E-07
Defense response	18	1.30E-09	1.81E-07
Type I interferon signaling pathway	14	2.66E-09	3.58E-07

Table S4: Selected GO results for common genes downregulated in both airway and alveolus chips following IAV infection.

Term	Count	P value	FDR
Axon guidance	15	2.28E-07	0.0004
Cell adhesion	24	8.60E-07	0.0007
Extracellular matrix organization	10	0.0002	0.0792
Cell-cell adhesion	11	0.0002	0.0792
Long-chain fatty acid import	4	0.0002	0.0792

Table S5: Composition of the culture medium used during different phases of cell differentiation.

Medium	Constituents	
Endodermization medium (Step 1)	Basal Medium	RPMI B27 supplement (2%), Penicillin/Streptomycin (50 U/ml), Activin A (100 ng/ml)
	Chemical and cytokines	CHIR99021 (1.0 μ M), Y27632 (Day 0; 10 μ M), Sodium butyrate (NaB) (Day 1; 0.25 mM, Days 2 and 4; 0.125 mM)
Anteriorization medium (Step 2)	Basal Medium	DMEM / F12, Glutamax, B27 supplement (2%), L-ascorbic acid (0.05 mg/ml), Monothioglycerol (0.4 mM), Penicillin/Streptomycin (50 U/ml)
	Chemical and cytokines	Human Noggin (100 ng/ml), SB431542 (10 μ M)
Ventralization medium (Step 3)	Basal Medium	DMEM / F12, Glutamax, B27 supplement (2%), L-ascorbic acid (0.05 mg/ml), Monothioglycerol (0.4 mM), Penicillin/Streptomycin (50 U/ml)
	Chemical and cytokines	ATRA 0.05 μ M, human BMP4 (20 ng/ml), CHIR99021 (3.0 μ M)
Step 4 medium	Basal Medium	DMEM / F12, Glutamax, B27 supplement (2%), L-ascorbic acid (0.05 mg/ml), Monothioglycerol (0.4 mM), Penicillin/Streptomycin (50 U/
	Chemical and cytokines	CHIR99021 (3.0 μ M), human FGF10 (10 ng/ml), human KGF (10 ng/ml), DAPT (20 μ M)

On-chip culture	Airway chip	Alveolus chip
Basal medium	PneumaCult™-ALI Medium	Ham's F12, dexamethasone (50 nM), 3-Isobutyl-1-methylxanthine (IBMX) (100 μ M), B27 supplement (1%), BSA (0.25%), HEPES (15 mM), CaCl ₂ (0.8 mM), ITS premix (0.1%), 8-BrcAMP (100 μ M)
Chemical and cytokines	Heparin (4 μ g/ml) Hydrocortisone (1.0 μ M) DAPT (10 μ M) Y-27632 (10 μ M) Penicillin/ Streptomycin (50 U/ml)	CHIR99021 (3 μ M), SB431542 (10 μ M), Y27632 (10 μ M), human KGF (10 ng/ml), and penicillin/streptomycin (50 U/ml),

Table S6. Primer sequences used for RT-qPCR.

Primer	Sequence
<i>SNTN</i>	GCTGCAAACCCAATTTAGGA TGCTCATCAAGTTCAGAAAGGA
<i>FOXJ1</i>	CCTGTCGGCCATCTACAAGT AGACAGGTTGTGGCGGATT
<i>SPDEF</i>	AAGTGCTCAAGGACATCGAGA AGGAGCCACTTCTGCACATT
<i>SCGB1A1(CCSP)</i>	CACCATGAAACTCGCTGTCAC AGTTCCATGGCAGCCTCATAAC
<i>P63</i>	ACTGCCAAATTGCAAAGACA TGACTAGGAGGGGCAATCTG
<i>ACTB(β-ACTIN)</i>	CAATGTGGCCGAGGACTTTG CATTCTCCTTAGAGAGAAGTGG
<i>NRP1</i>	AACAACGGCTCGGACTGGAAG A GGTAGATCCTGATGAATCGCGTG
<i>IFNB1</i>	CATTACCTGAAGGCCAAGGA CAGCATCTGCTGGTTGAAGA
<i>INFL1</i>	AACTGGGAAGGGCTGCCACATT GGAAGACAGGAGAGCTGCAACT
<i>ISG15</i>	GCAGATCAGCCAGAAGATCG GGCCCTTGTTATTCCTCACC
<i>ISG56</i>	CCTTGCTGAAGTGTGGAGGA CCAGGCGATAGGCAGAGA
<i>MxA</i>	CTTATCCGTTAGCCGTGGTG CAAGGTGGAGCGATTCTGAG
<i>SFTPB</i>	GAGCCGATGACCTATGCCAAG AGCAGCTTCAAGGGGAGGA
<i>SFTPC</i>	GCAAAGAGGTCTGATGGAG TGTTTCTGGCTCATGTGGAG
<i>PDPN</i>	TCCAGGAACCAGCGAAGAC CGTGGACTGTGCTTTCTGA
<i>AGER</i>	GCCACTGGTGCTGAAGTGTA TGGTCTCCTTTCCATTCTG
<i>SLC34A2</i>	TCGCCACTGTCATCAAGAAG CTCTGTACGATGAAGGTCATGC
<i>ACE2</i>	ACAGTCCACACTTGCCCAAAT TGAGAGCACTGAAGACCCATT
<i>TMPRSS2</i>	GTCCCCACTGTCTACGAGGT CAGACGACGGGGTTGGAAG
<i>N_Sarbeco (SARS-COV-2 nucleocapsid protein)</i>	CACATTGGCACCCGCAATC GAGGAACGAGAAGAGGCTTG
<i>Influenza A virus (M)</i>	CTTCTAACCGAGGTGAAACGTA GGTGACAGGATTGGTCTTGTCTTA

Table S7. List of antibodies used in immunofluorescence staining.

Antibody	Manufacturer	Catalog No.	Concentration
Anti-Acetylated Tubulin	Sigma-Aldrich	T7451	1:4000
Anti-MUC5B	Santa Cruz	sc-21768	1:100
Alexa Fluor® Cyt5-647	Abcam	ab193895	1:200
Anti-ZO-1	Thermo Fisher	33-9100	1:300
Anti-VE-Cadherin	Abcam	ab33168	1:300
Anti-GFP	Aveslabs	GFP-1020	1:500
Anti-CPM	Gotoh lab	43A1	1:100
Anti-SLC34A2	A kind gift from Dr. Ritter Gerd	MX35	1:100
Anti-HT1-56	Terrace Biotech	TB29 AHT1-56	1:150
Anti-IAV NP	Genetex	GTX125989	1:1000
Anti-FOXJ1	Abcam	ab235445	1:500
Alexa Fluor 488 Donkey Anti-mouse IgG (H+L)	Thermo Fisher Scientific	A21202	1:500
Alexa Fluor 568 Donkey Anti-Rabbit IgG (H+L)	Thermo Fisher Scientific	A10042	1:500
Alexa Fluor 647 Donkey Anti-mouse IgG (H+L)	Thermo Fisher Scientific	A31571	1:500
Alexa Fluor 568 Donkey Anti-mouse IgG (H+L)	Thermo Fisher Scientific	A31570	1:500
Microbeads-conjugated Anti-rat immunoglobulin kappa light chain antibody	Miltenyi Biotec	130-047-401	20µl microbeads in 30µl MACS buffer per 1x10 ⁷ cells

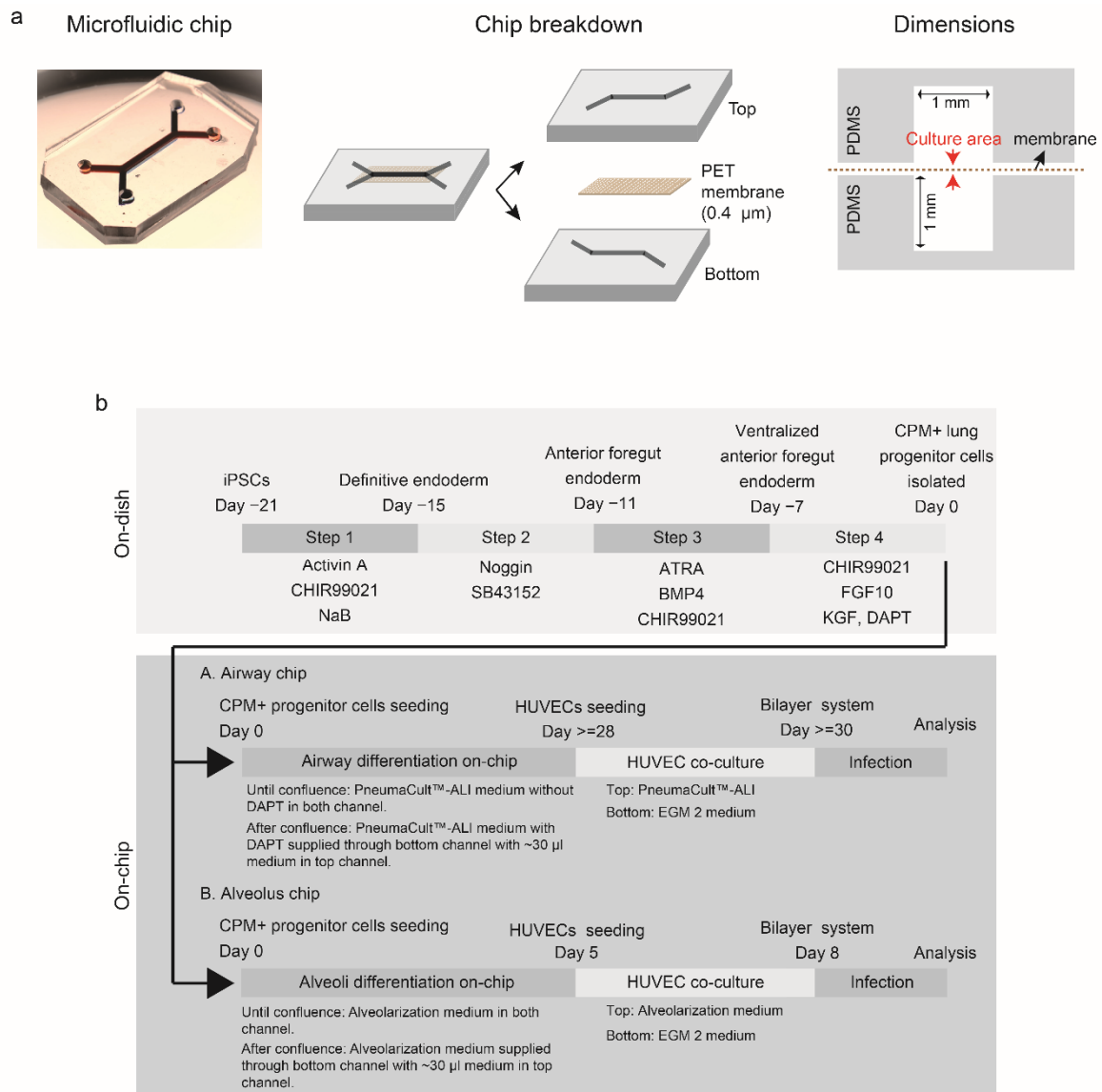


Figure S1: Differentiation of iPSCs into airway and alveolar cells within microfluidic chips. a, A detailed description of microfluidic chips used in the experiments. **b,** Detailed protocol explaining the development of airways and alveolar chips using hiPSC-derived CPM+ lung progenitor cells.

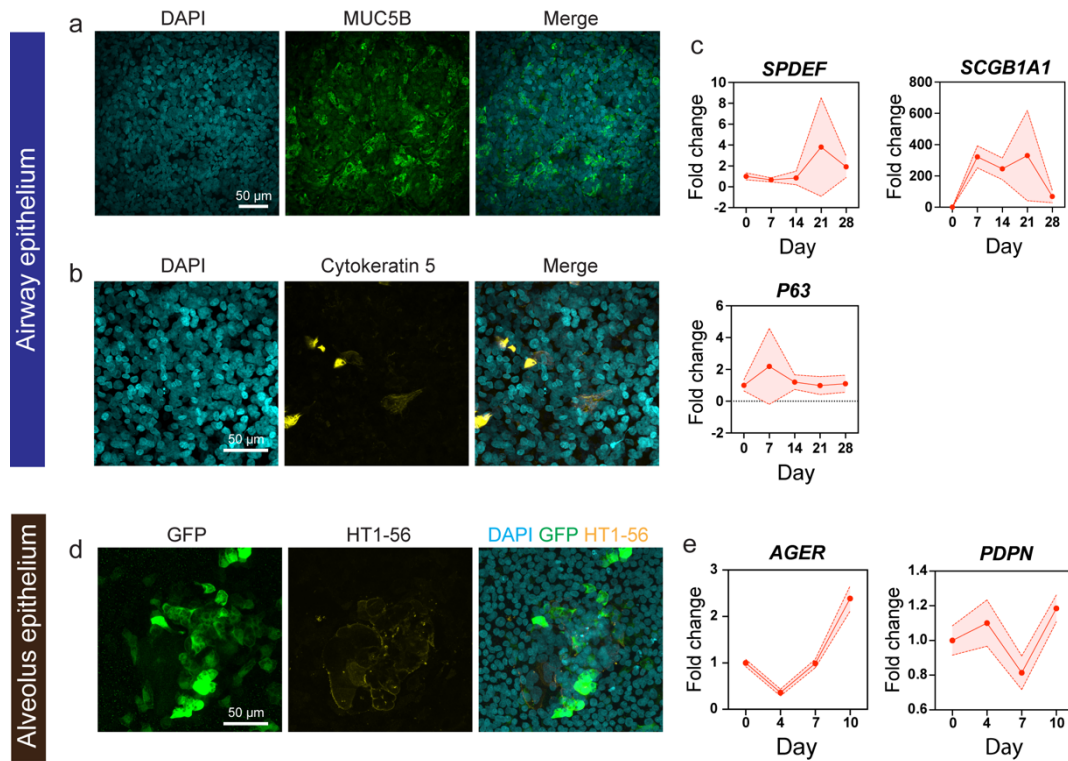


Figure S2. Mucus-secreting, basal, and club cell markers in airway epithelium and AT1 cell markers in alveolus epithelium. **a**, Representative immunofluorescence staining for DAPI and MUC5B in the airway epithelium (n = 2 chips). **b**, Representative immunofluorescence staining for DAPI and Cytokeratin 5 in airway epithelium (n = 1 chip). **c**, Gene expression levels of *SPDEF*, *SCGB1A1*, and *P63* in the airway epithelium (n = 3, except day 7, where n = 2 chips). **d**, Representative IFA images for DAPI, GFP (AT2 cells), and HT1-56 (n = 3 chips) in the alveolus epithelium on day 11 of on-chip culture. **e**, Gene expression levels of *AGER* and *PDPN* in the alveolar epithelium (n = 3 chips).

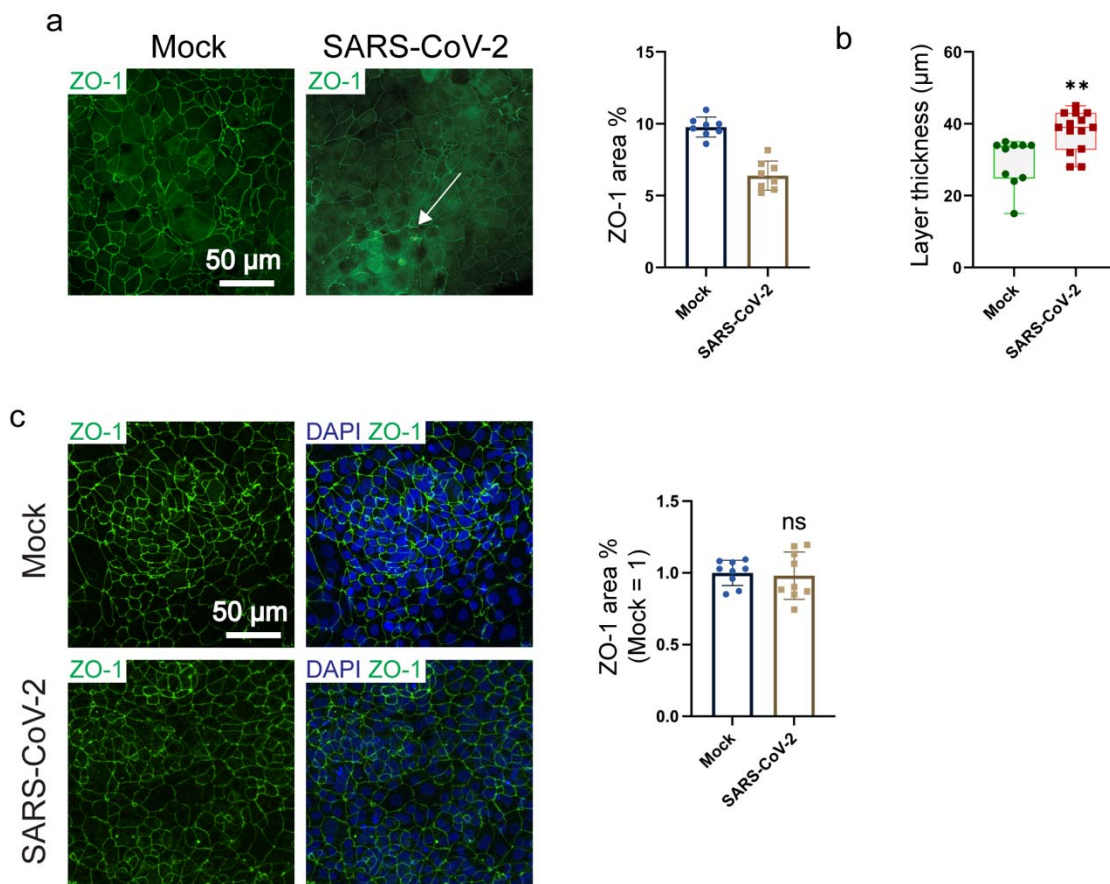


Figure S3. Quantification of ZO-1 perimeter in SARS-CoV-2-infected airway and alveolus epithelium at 3 dpi. **a**, Representative IFA images for ZO-1 in mock and SARS-CoV-2 infected airway epithelium at 3 dpi (arrows indicate locally disrupted ZO-1) with quantification of total area percentage covered by ZO-1 ($n = 8$ fields of view from 1 chip; different laser intensities were applied during image acquisition to ensure accurate ZO-1 identification). **b**, Image analysis of immunofluorescence staining of mock and SARS-CoV-2 infected samples at 3 dpi to assess cell layer thickness ($p = 0.0049$) ($n = 3$ or more fields of view from 3-4 chips). **c**, Representative immunofluorescence staining for DAPI and ZO-1 in mock and SARS-CoV-2 infected alveolus epithelium at 3 dpi, with quantification of ZO-1 area coverage normalized to control samples ($n = 3$ fields of view from 3 chips each). Data represent mean $\pm$ s.d. Mann-Whitney non-parametric test (c) was conducted using GraphPad Prism (** $p < 0.01$).

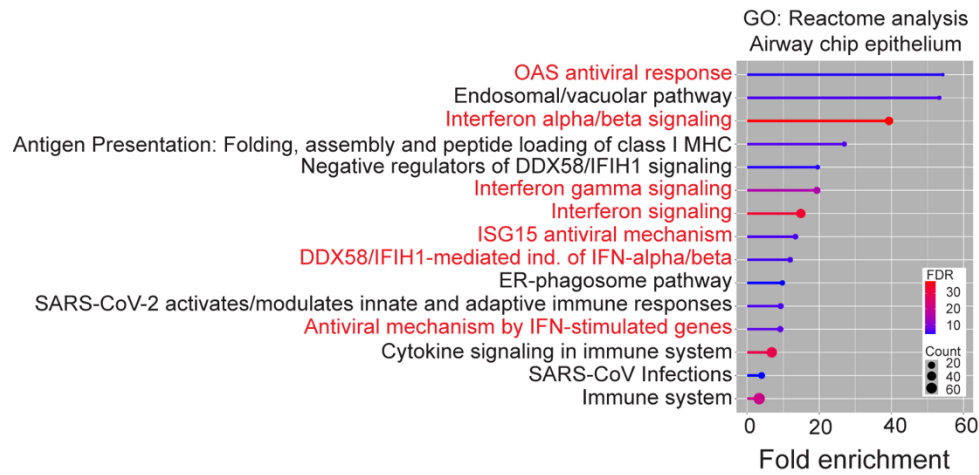
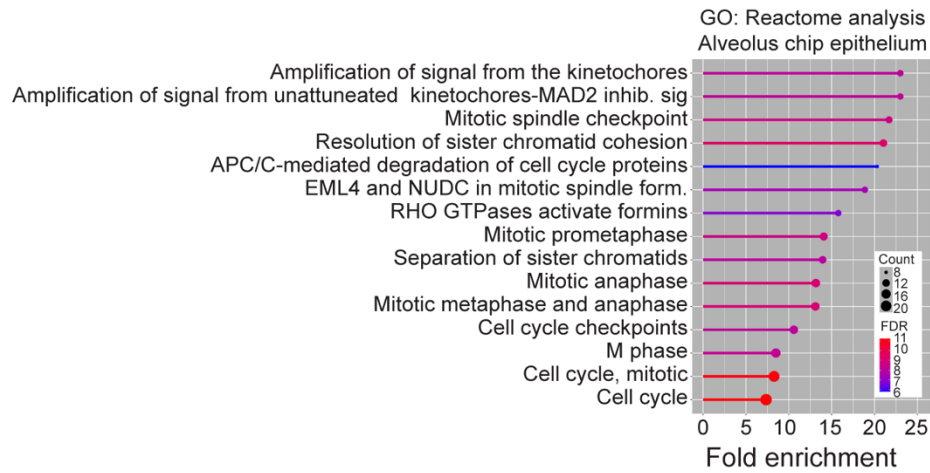


Figure S4. SARS-CoV-2 activates IFN pathways in airway epithelium at 3 dpi. GO analysis showing the most significantly upregulated REACTOME pathways (IFN-related pathways shown in red) in the airway and alveolus chips epithelium (FDR shows $-\log_{10}(\text{FDR})$, $n = 3$ chips).

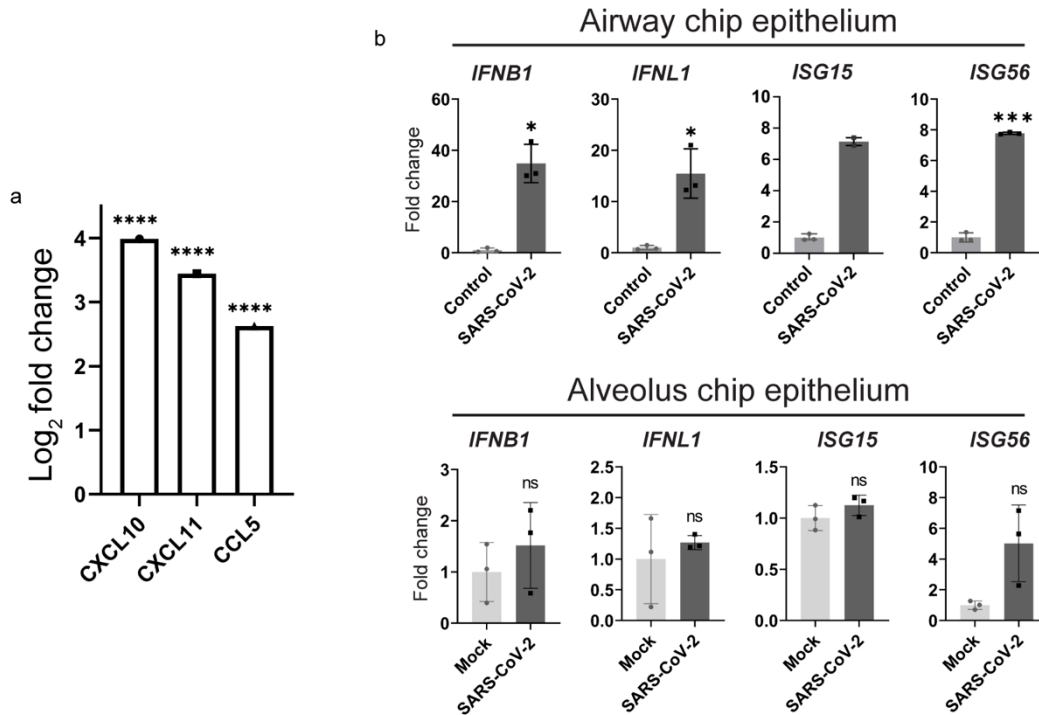


Figure S5. RT-qPCR confirms low IFN-related gene expression in SARS-CoV-2-infected alveolus epithelium at 3 dpi. **a**, Log₂ fold change in key chemokine-related gene expression post-SARS-CoV-2 infection in airway epithelium obtained from RNA-seq data (n = 3 chips, ****p < 0.0001). **b**, Expression of the key IFN genes *IFNB1* (airway, p = 0.0340), *IFNL1* (airway, p = 0.0148), *ISG15*, and *ISG56* (airway, p = 0.0003) in the mock and infected airway and alveolus epithelium in respective chips (n = 3 chips except *ISG15* in airway chips, where n = 2 chips). Data represent mean ± s.d, except for airway-*ISG15* in **b**, where data represent the mean with range. An unpaired two-tailed Student's t-test with Welch's correction (except *ISG15* in airway chips) was conducted using GraphPad Prism (***p < 0.001, **p < 0.01, *p < 0.05).

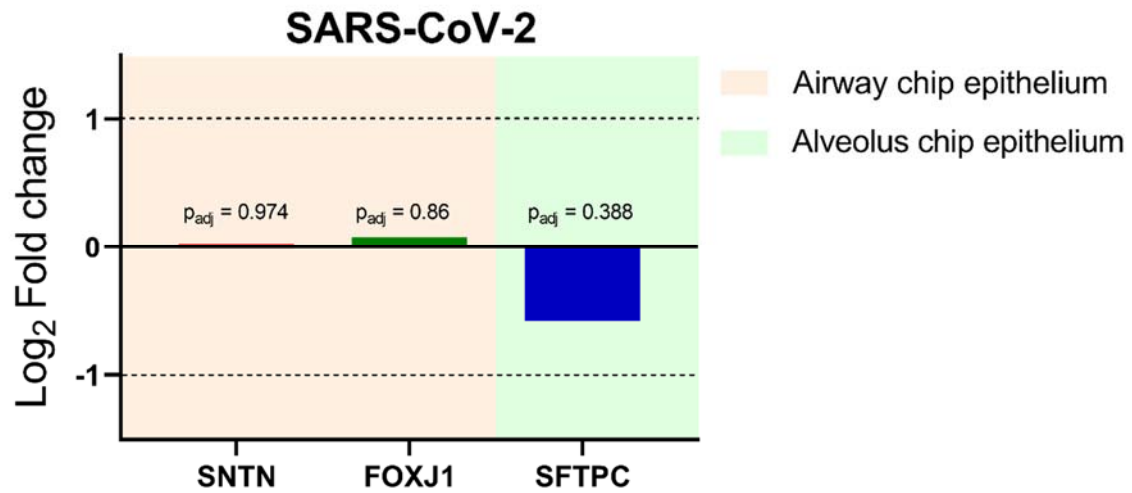


Figure S6. Changes in multiciliated cells and AT2 cell markers 3 days post-SARS-CoV-2 infection. Log₂ fold change in gene expression from RNA sequencing of mock and SARS-CoV-2 infected airway epithelium, with adjusted p-value (n = 3 chips). The p-values attained by the Wald test are corrected for multiple testing using the Benjamini and Hochberg methods to obtain P_{adj} values

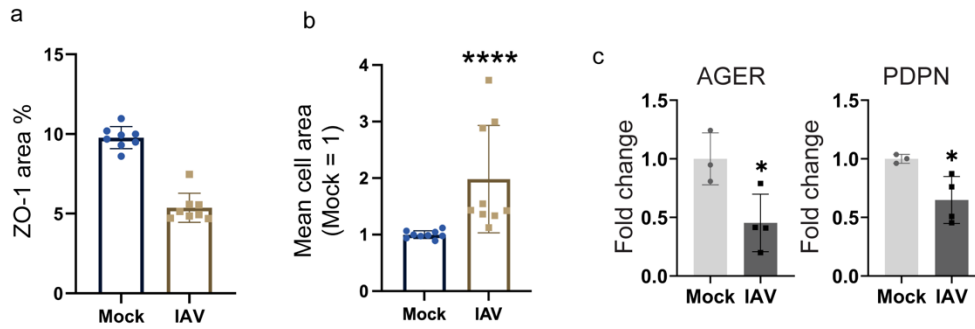


Figure S7. IAV infection causes tight junction disruption in airway epithelium and increased cellular size in alveolus epithelium at 3 dpi. **a**, Percentage area covered by ZO-1 in mock and IAV-infected airway epithelium at 3 dpi (n = 8 fields of view from 1 chip). **b**, Area covered by each cell in IAV-infected alveolus epithelial cells normalized to control samples at 3 dpi (n = 3 field of view from 3 chips). **c**, Expression of the AT1-like cell markers, *AGER* (p = 0.0297), and *PDPN* (p = 0.0363) in mock and IAV-infected alveolar epithelium (n = 3 or 4 chips). Data represent mean $\pm$ s.d. A two-tailed Mann-Whitney non-parametric test (**b**) and unpaired two-tailed student t-test with Welch's correction (**c**) were conducted using GraphPad Prism (****p < 0.0001, *p < 0.05).

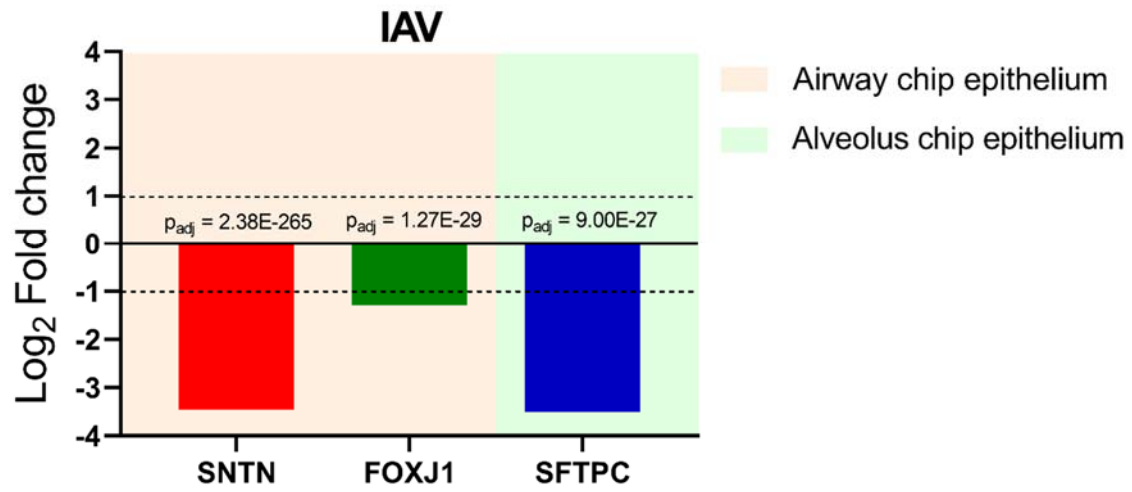


Figure S8. IAV significantly decreases multiciliated and AT2 cell markers at 3 dpi. Log₂ fold change in gene expression from RNA sequencing of mock and IAV-infected airway epithelium, along with adjusted p-value (n = 3 chips).

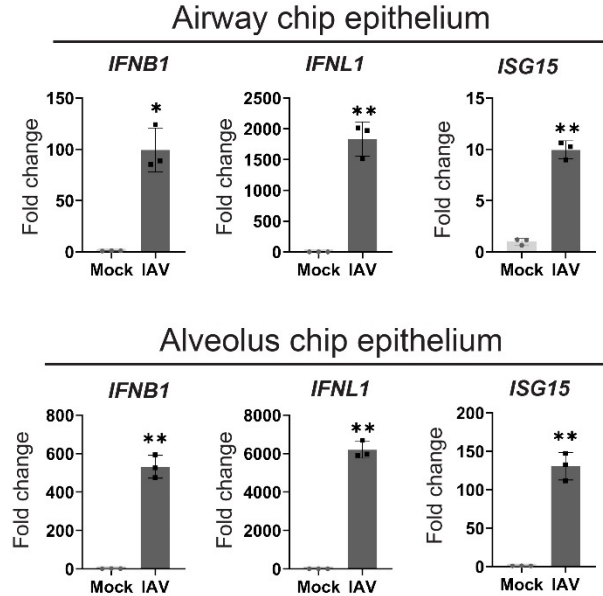


Figure S9. RT-qPCR analysis confirms increased IFN-related gene expression in IAV-infected airway and alveolus epithelium at 3 dpi. Expression of the key IFN genes *IFNB1* (airway chip ($p = 0.0154$), alveolus chip ($p = 0.0042$)), *IFNL1* (airway chip ($p = 0.0077$), alveolus chip ($p = 0.0017$)), *ISG15* (airway chip ($p = 0.0013$), alveolus chip ($p = 0.0063$)) in mock and IAV-infected airway and alveolus epithelium ($n = 3$ chips). Data represent mean $\pm$ s.d. An unpaired two-tailed Student's t-test with Welch's correction was conducted using GraphPad Prism (** $p < 0.01$, * $p < 0.05$).

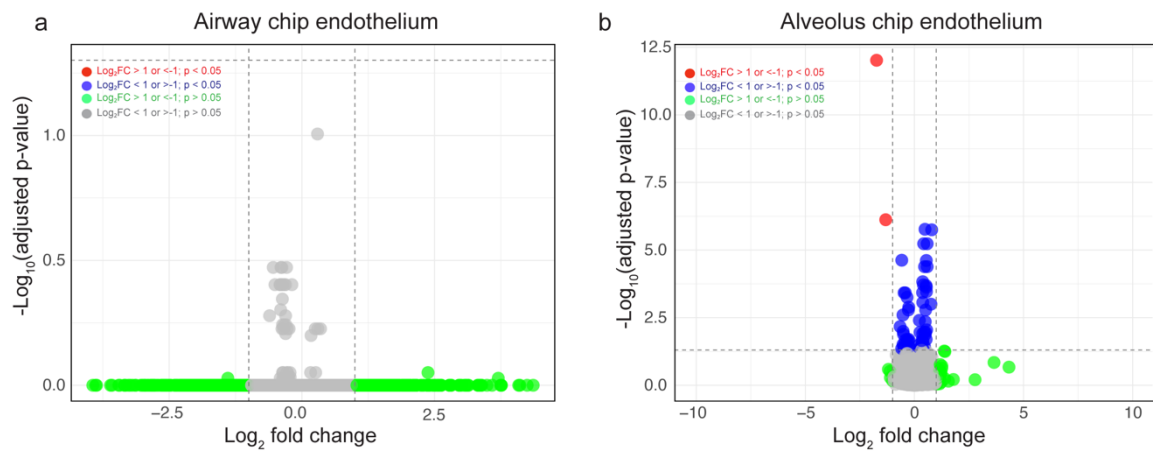


Figure S10. No immune response was observed in the endothelium of both chips following SARS-CoV-2 infection at 3 dpi. a, Volcano plot of differentially expressed genes between mock and infected airway endothelium ($\log_2$ fold change > 1 and ($P_{\text{adj}} < 0.05$, $n = 3$ chips). **b,** Volcano plot of differentially expressed genes between mock and infected alveolus endothelium ($\log_2$ fold change > 1 and ($P_{\text{adj}} < 0.05$, $n = 3$ chips). Differentially expressed genes in SARS-CoV-2 infected alveolus chips include *MGP* and *MMP10*.

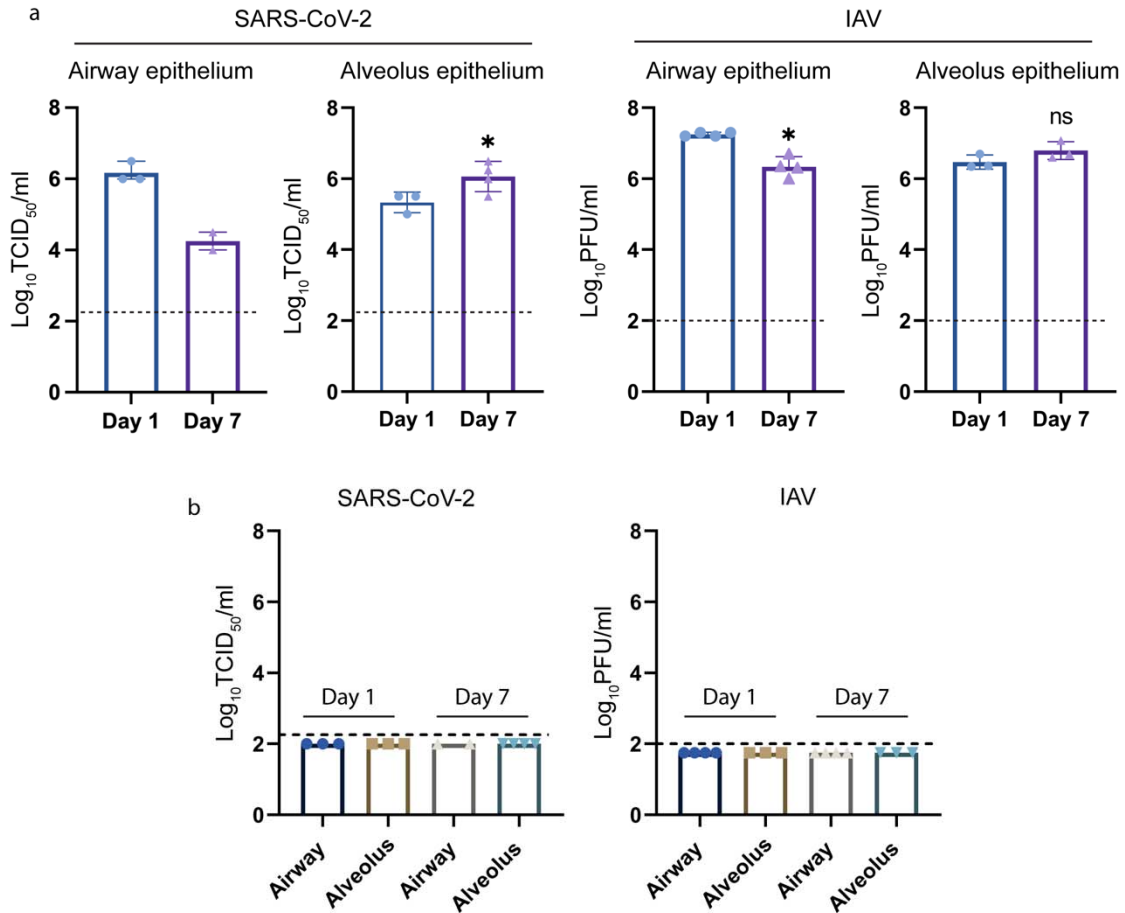


Figure S11: SARS-CoV-2 and IAV viral titers observed at days 1 and 7 post-infection in the epithelial channel of both chips. a, SARS-CoV-2 and IAV viral titers in the airway ($p_{IAV} = 0.0286$) and alveolus ($p_{SARS-CoV-2} = 0.0433$) chips were measured on day 1 and day 7 post-infection using the TCID₅₀ and PFU assays, respectively, from samples collected by the top channel washes (n of at least 3 chips except for SARS-CoV-2 day 7 airway, where n = 2 chips). **b,** Viral titers measured from bottom channel washes at day 1 and day 7 post-infection using the TCID₅₀ and PFU assays (n = 3 or 4 chips except for SARS-CoV-2 day 7 airway, where n = 2 chips). Data represent the mean $\pm$ s.d, except for airway-SARS in **a**, where data represents the mean with range. An unpaired two-tailed Student's t-test with Welch's correction (**a**) was conducted using GraphPad Prism except for the airway chip IAV experiment, where the two-tailed Mann-Whitney non-parametric test was conducted (* $p < 0.05$).

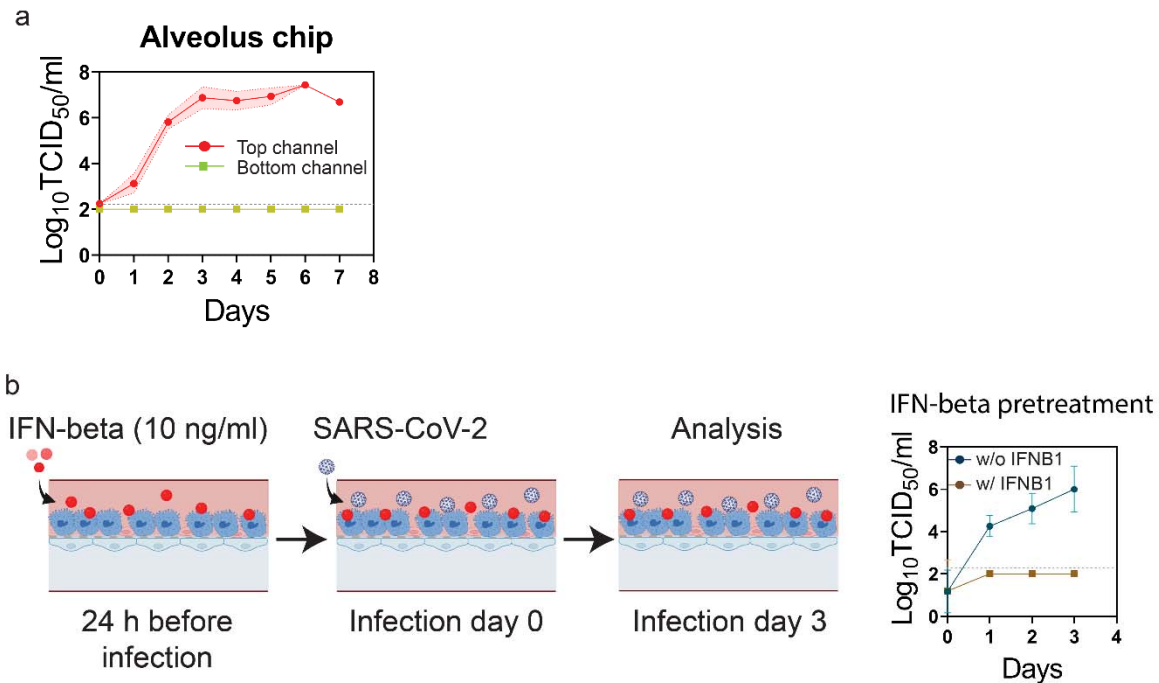


Figure S12. IFN-beta inhibits SARS-CoV-2 replication in alveolus chips. **a**, SARS-CoV-2 viral titer in the alveolus chips was measured until 7 dpi using the TCID₅₀ method from samples collected by top and bottom channel washes (n = 4 chips). **b**, SARS-CoV-2 viral titer in the alveolus chips with and without IFN-beta pretreatment measured until 3 dpi using the TCID₅₀ method from samples collected by top channel washes (one chip without IFN-beta pretreatment is not plotted due to failure in viral titer increase, n = 3 or 4 chips). Data represent the mean $\pm$ s.d (**a,b**). The illustration in (**b**) is created with BioRender.com (<https://BioRender.com/c6yl5pt>).

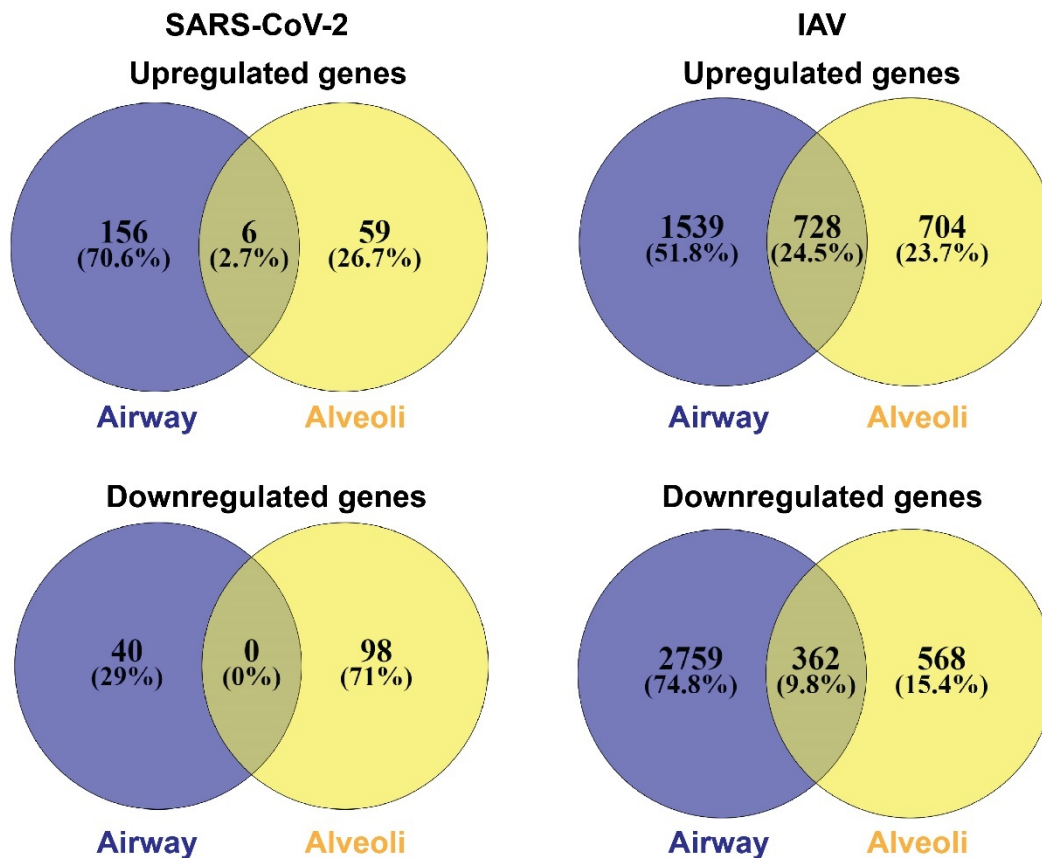


Figure S13. IAV infection shares ~25% common genes between proximal and distal regions of the respiratory tract at 3 dpi. Differentially expressed genes from SARS-CoV-2 and IAV experiments at 3 dpi were used to generate a Venn diagram with Venny⁶² (n=3 chips).