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Supplementary Materials for

SARS-CoV-2 disrupts respiratory vascular barriers by suppressing Claudin-5 expression

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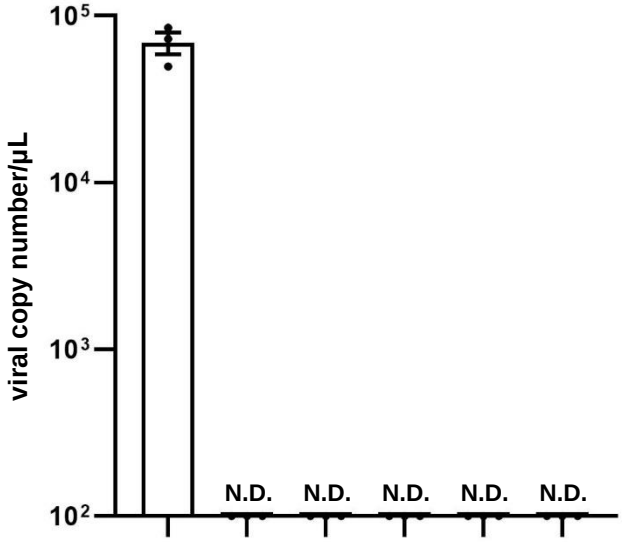
This PDF file includes:

Supplemental figures and figure legends



ACE2	+	-	-	-	-	-
SARS-CoV-2 (MOI)	0.1	0	0.1	1	0.1	1
exposure times	1	0	1	1	4	4

Figure S1. SARS-CoV-2 does not infect HMVEC-L in the absence of exogenous ACE2 expression.

HMVEC-L cells were transduced with ACE2-expressing adenovirus vector (Ad-ACE2) to overexpress ACE2 and cultured for 2 days before SARS-CoV-2 infection. Ad-ACE2 were generated according to our previous report (*43*). When exposing HMVEC-L to SARS-CoV-2 once, the cells were treated with 0.1 or 1 MOI SARS-CoV-2 for 120 min and then cultured with fresh medium for 4 days. When exposing HMVEC-L to SARS-CoV-2 4 times, the cells were cultured for 4 days with daily medium change containing 0.1 or 1 MOI SARS-CoV-2. The viral copy numbers in the cell culture supernatant of HMVEC-L in the presence or absence of exogenous ACE2 expression and SARS-CoV-2 are shown. Data are expressed as the mean ± s.e.m. (n=3). N.D., not detected.

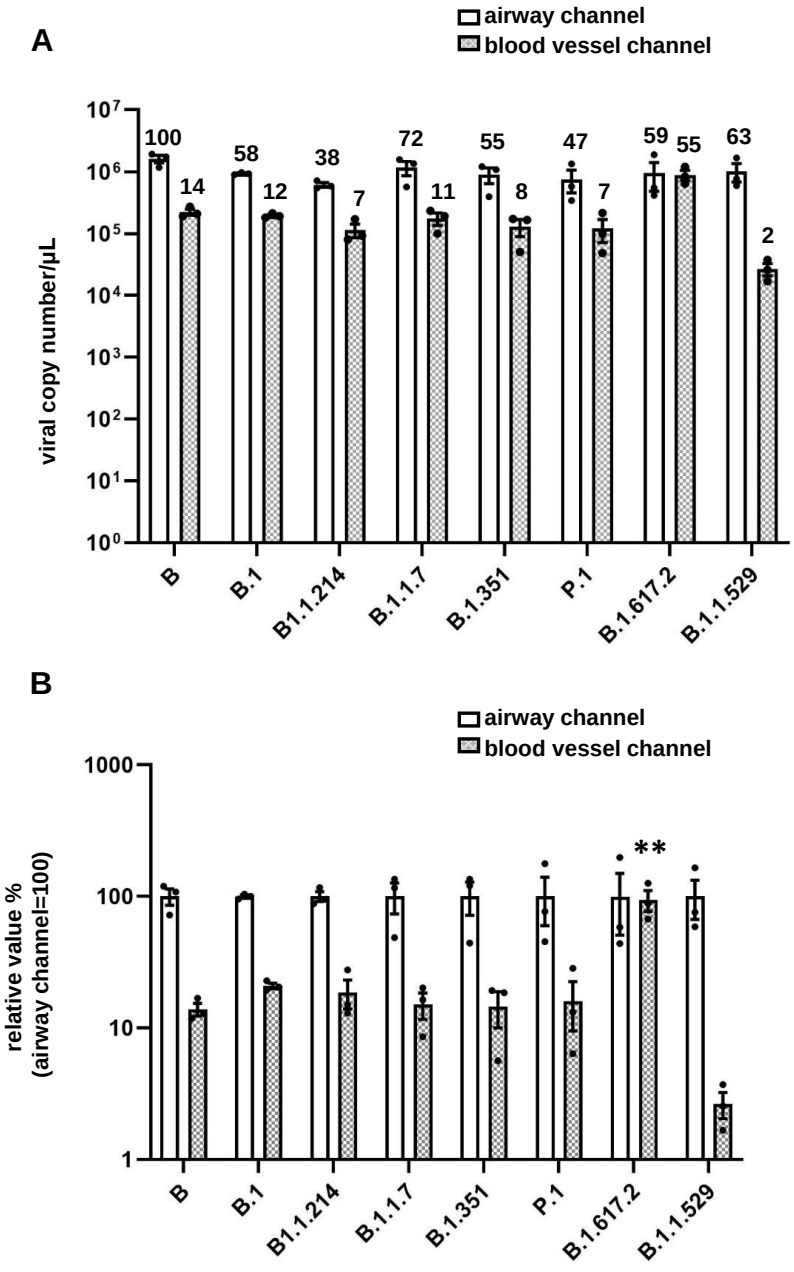


Figure S2. Viral copy number in the blood vessel channel of airway-on-a-chip exposed to various SARS-CoV-2 variants.

Medium containing 0.1 MOI SARS-CoV-2 (B, B.1, B.1.1.214, B.1.1.7, B.1.351, P.1, B.1.617.2, and B.1.1.529) was injected into the airway channel of the airway-on-a-chip, which was then cultured for 8 days. (**A**) Viral copy numbers in the cell culture supernatant of

the airway and blood vessel channels. The numbers above the bars are normalized x100 to the viral copy numbers in the cell culture supernatant of the airway channels of SARS-CoV-2 B-infected airway-on-a-chip. (**B**) Relative viral copy numbers in the cell culture supernatant of the airway and blood vessel channels relative to SARS-CoV-2 B in the airway channel. The viral copy numbers in the cell culture supernatant of blood vessel channels were compared between SARS-CoV-2 variants by performing one-way ANOVA followed by Tukey's post hoc test (**p<0.01). Data are expressed as the mean ± s.e.m. (n=3).

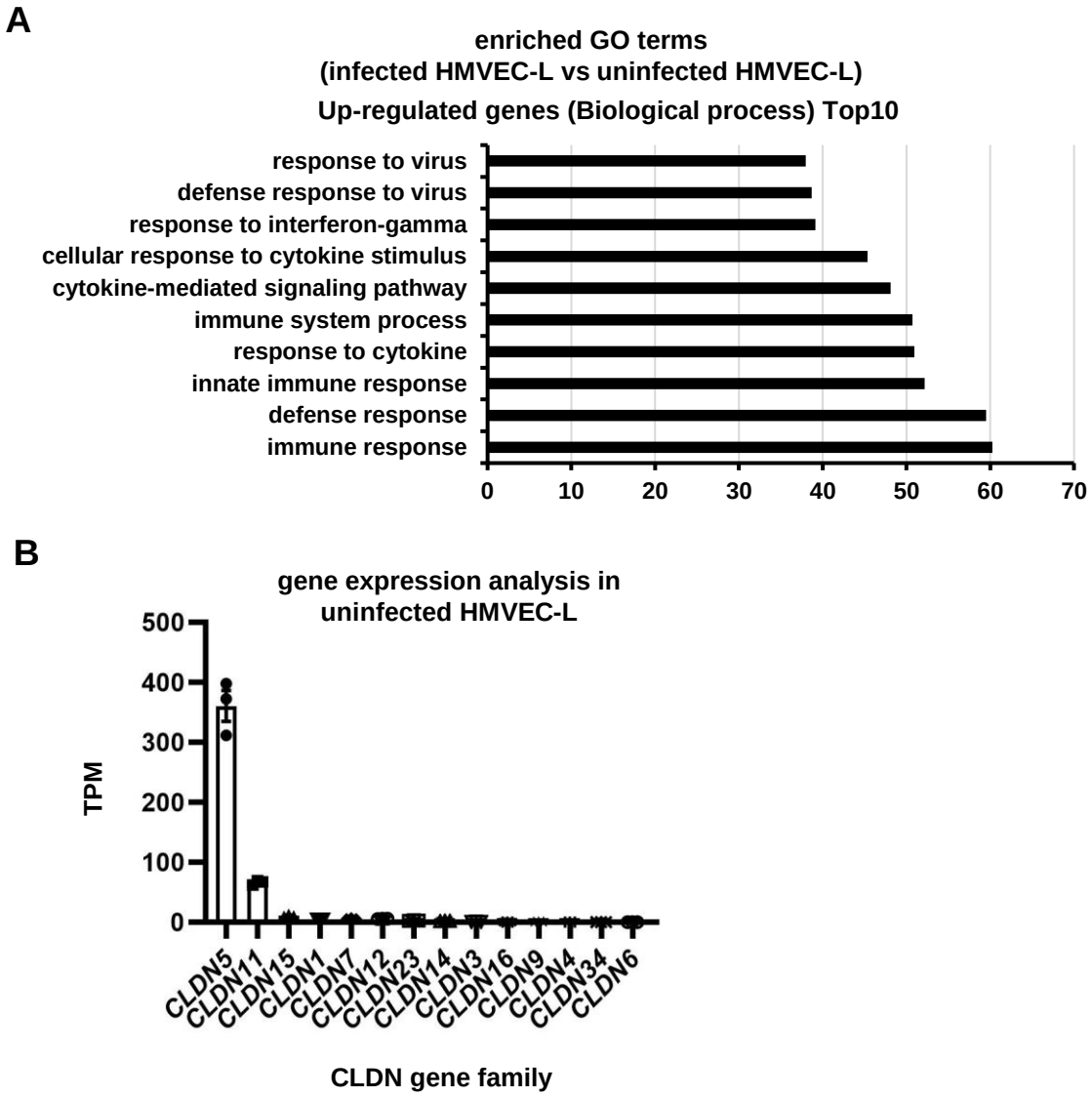


Figure S3. Gene expression analysis of HMVEC-L in infected airway-on-a-chip.

Medium containing 0.1 MOI SARS-CoV-2 was injected into the airway channel of the airway-on-a-chip, which was then cultured for 8 days. (**A**) A GO enrichment analysis of uninfected versus infected HMVEC-L in airway-on-a-chip. (**B**) The endogenous gene expression levels of the *CLDN* family in uninfected HMVEC-L in airway-on-a-chip. Data are expressed as the mean ± s.e.m. (n=3).

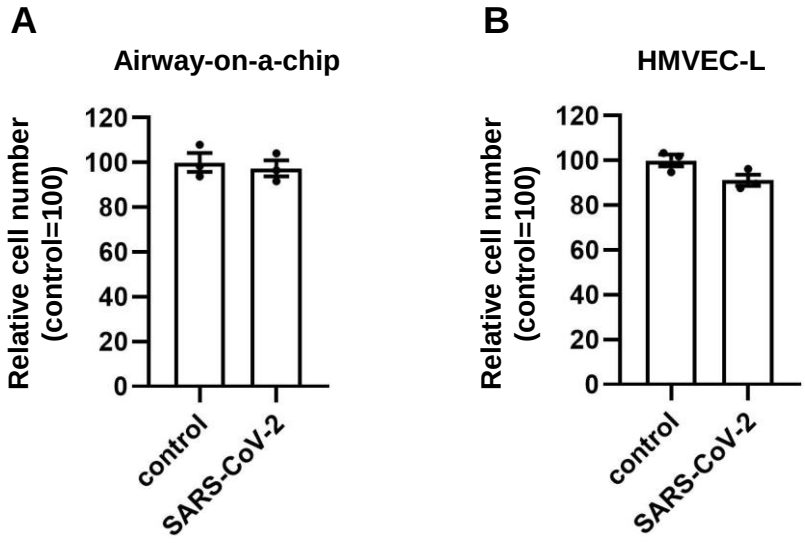


Figure S4. Cell number of HMVEC-L was not changed by SARS-CoV-2 infection.

(**A**) Medium containing 0.1 MOI SARS-CoV-2 was injected into the airway channel of the airway-on-a-chip, which was then cultured for 8 days. At 8 dpi, the cell number of HMVEC-L was calculated. (**B**) HMVEC-L were cultured on a chamber slide in the presence or absence of 1 MOI SARS-CoV-2 for 4 days. At 4 dpi, the cell number of HMVEC-L was calculated. Data are expressed as the mean ± s.e.m. (n=3).

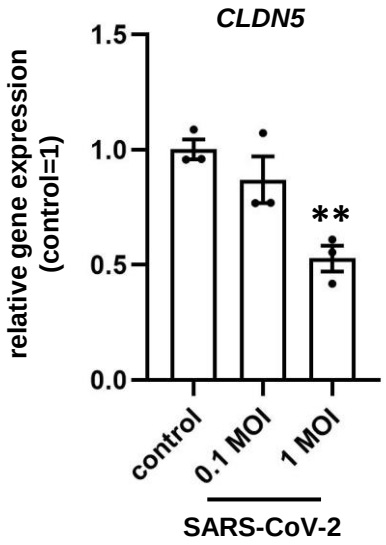


Figure S5. *CLDN5* expression level in HMVEC-L is decreased by exposure to high-titer SARS-CoV-2.

The gene expression levels of *CLDN5* in HMVEC-L cultured for 4 days with daily medium change containing 0.1 or 1 MOI SARS-CoV-2. One-way ANOVA followed by Tukey's post hoc test (**p<0.01). Data are expressed as the mean ± s.e.m. (n=3).

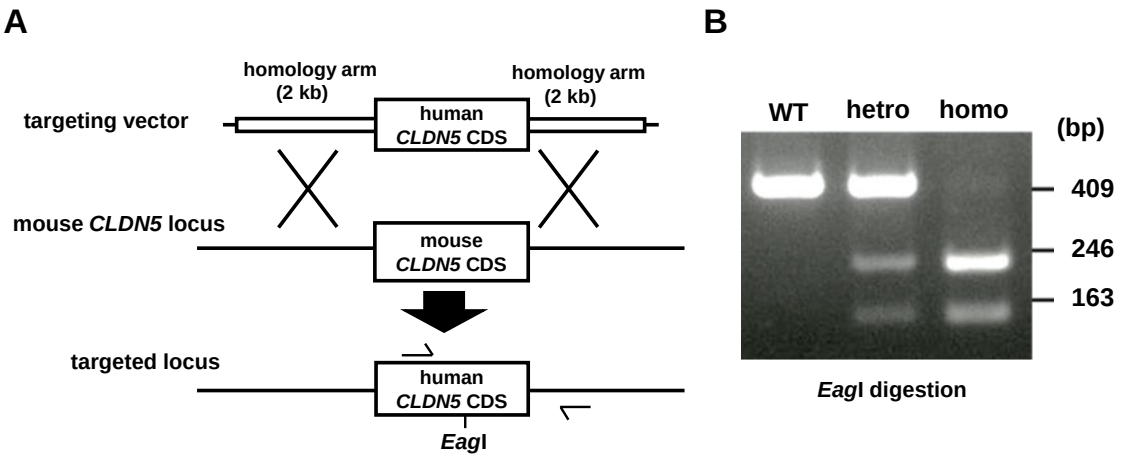


Figure S6. Generation of human CLDN5 knock-in mouse

(**A**) Schematic illustration of the homologous recombination. Human and mouse *CLDN5* coding sequences (CDS) are the same length and included in the single exon. Mouse *CLDN5* CDS was precisely replaced with human CDS without any alteration of other DNA sequences. (**B**) Genotyping results for homo and hetero human CLDN5 knock-in mice. Genomic fragments were amplified by PCR using the primers indicated in **figure S6A** and digested with *Eag*I, which is a unique restriction site included in human *CLDN5* CDS.

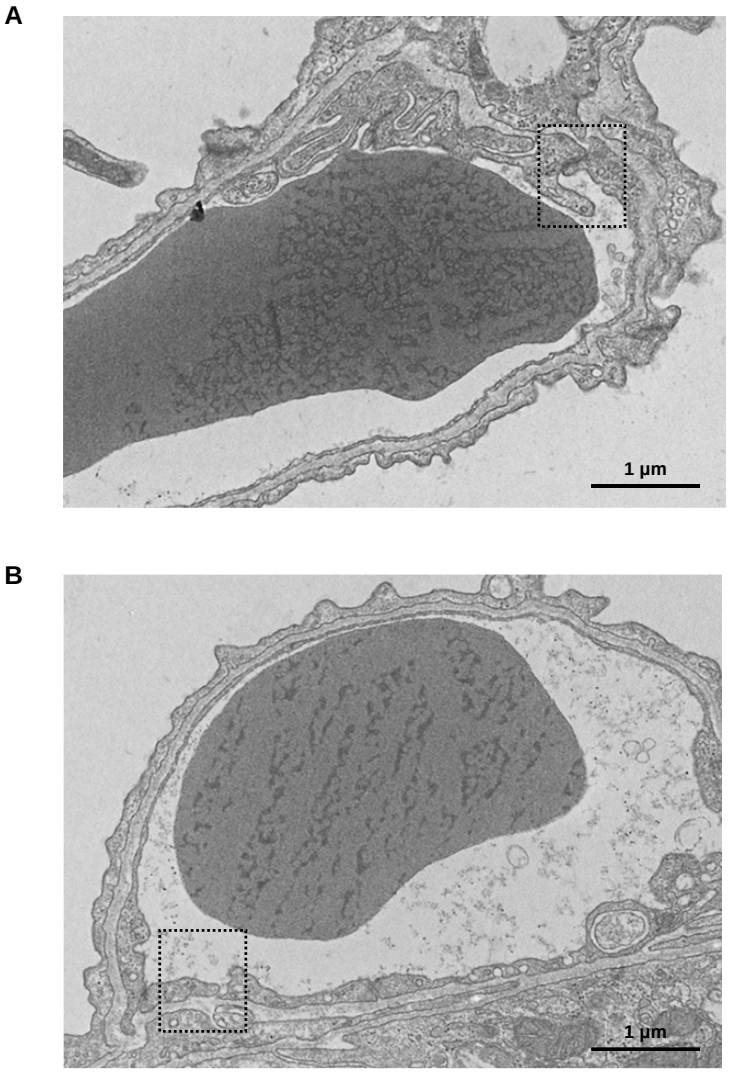


Figure S7. TEM images of lung endothelial cells from CLDN5 antibody-injected mice. TEM images of vasculatures were obtained using lungs from hCLDN5-KI mice injected with anti-CLDN5 antibody (**A**) or control IgG (**B**). The high-magnification TEM images shown in **Figure 3G** are the areas surrounded by the dotted lines.

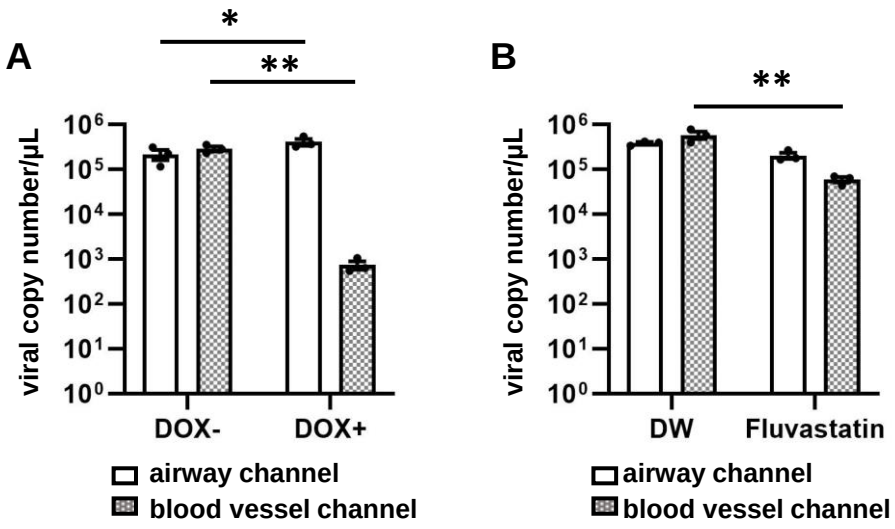


Figure S8. CLDN5 overexpression and Fluvastatin treatment inhibit SARS-CoV-2-induced respiratory endothelial barrier disruption.

(**A**) Viral copy numbers in the cell culture supernatant of the airway and blood vessel channels in the presence or absence of 1 μM DOX. Two-way ANOVA followed by Sidak post hoc test (*p<0.05, **p<0.01). DW=vehicle (distilled water)-treated cells. (**B**) Viral copy numbers in the cell culture supernatant of the airway and blood vessel channels in the presence or absence of 10 μM Fluvastatin. Two-way ANOVA with Sidak post hoc test (**p<0.01). Data are expressed as the mean ± s.e.m. (n=3).

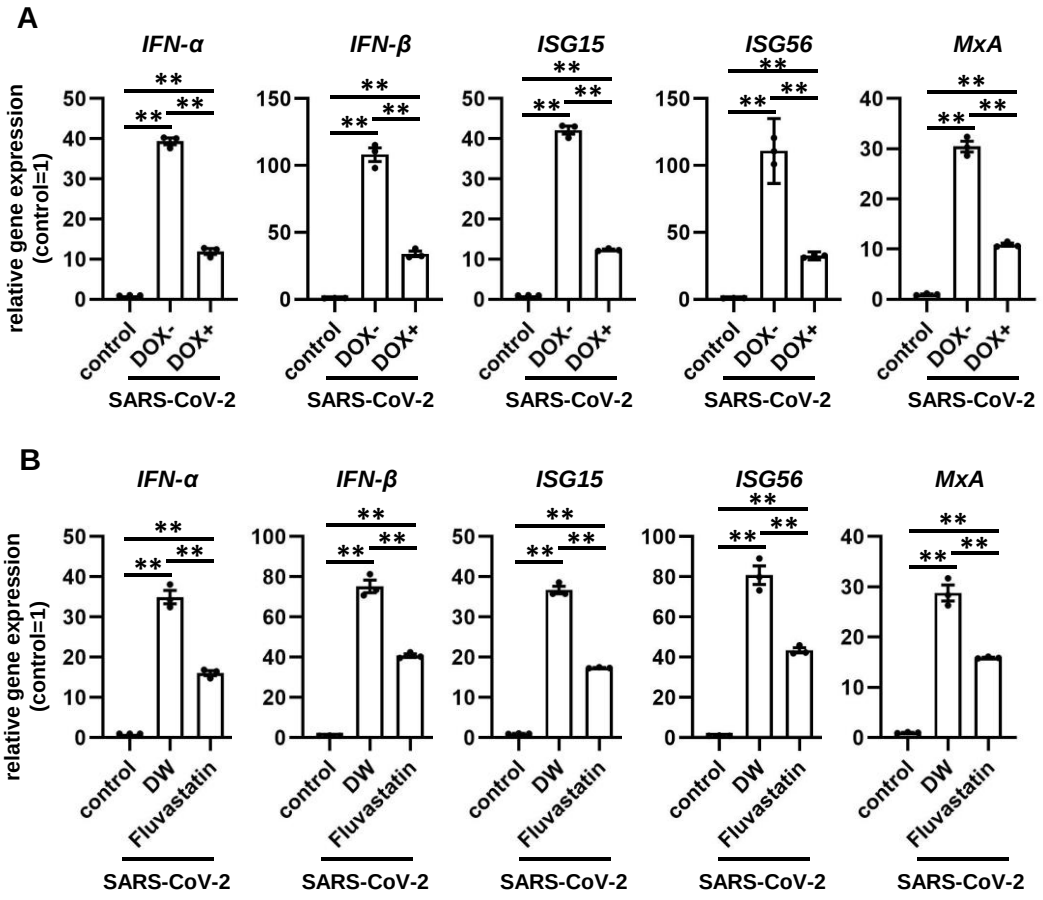


Figure S9. CLDN5 overexpression and Fluvastatin treatment inhibit SARS-CoV-2-induced expression levels of innate immune response-related genes.

(**A**) Effect of CLDN5 overexpression on human CLDN5-expressing HMVEC-L cells of infected airway-on-a-chip. The gene expression levels of *IFN-α*, *IFN-β*, *ISG15*, *ISG56*, and *MxA* in human CLDN5-expressing HMVEC-L cells treated with or without 1 μM DOX. One-way ANOVA followed by Tukey's post hoc test (**p<0.01). (**B**) Effect of Fluvastatin treatment on HMVEC-L in infected airway-on-a-chip. The gene expression levels of *IFN-α*, *IFN-β*, *ISG15*, *ISG56*, and *MxA* in HMVEC-L treated with or without 10 μM Fluvastatin. DW=vehicle (distilled water)-treated cells. One-way ANOVA followed by Tukey's post hoc test (**p<0.01). Data are expressed as the mean ± s.e.m. (n=3).

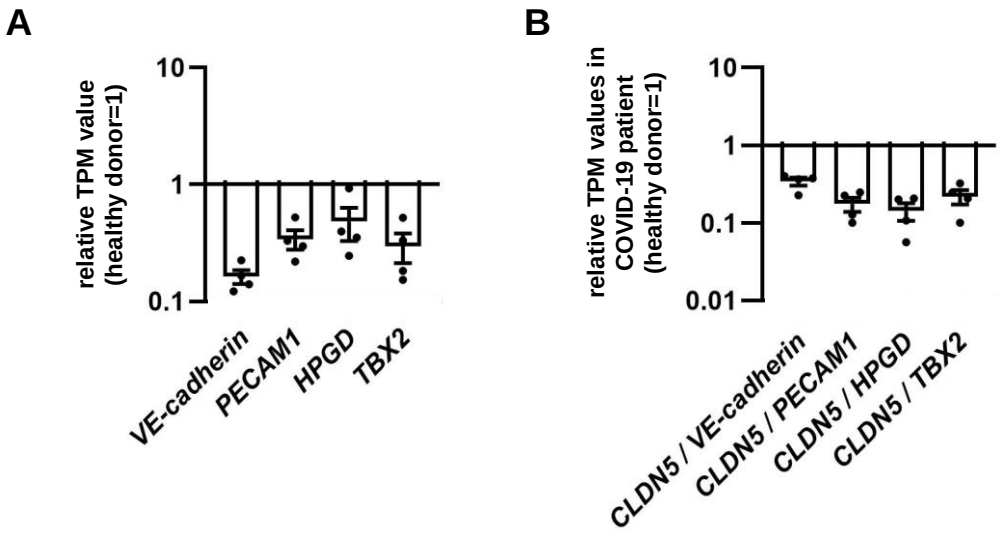


Figure S10. Gene expression analysis of CLDN5, conventional EC markers, and aerocyte-specific markers.

(**A**) The gene expression levels of conventional EC markers (*VE-cadherin* and *PECAM1*) and aerocyte-specific markers (*HPGD* and *TBX2*) in the lungs of patients with or without COVID-19. (**B**) The gene expression levels of CLDN5 normalized to conventional EC markers or aerocyte-specific markers in the lungs of patients with or without COVID-19. Data are expressed as the mean ± s.e.m (*n*=4).

Supplemental tables

Table S1. Primers used in this study.

qPCR for viral copy number		**5'-3'**
SARS-CoV-2 RNA	Fw	AGCCTCTTCTCGTTCCTCATCAC
	Rv	CCGCCATTGCCAGCCATTC
qPCR for airway-on-a-chip		**5'-3'**
human CLDN5	Fw	CTCTGCTGGTTCGCCAACAT
	Rv	CAGCTCGTACTTCTGCGACA
human VE-cadherin	Fw	TTGGAACCAGATGCACATTGAT
	Rv	TCTTGCGACTCACGCTTGAC
human IL-6	Fw	CCTGAACCTTCCAAAGATGGC
	Rv	TTCACCAGGCAAGTCTCCTCA
human VCAM-1	Fw	GGGAAGATGGTCGTGATCCTT
	Rv	TCTGGGGTGGTCTCGATTTTA
human ICAM-1	Fw	ATGCCCAGACATCTGTGTCC
	Rv	GGGGTCTCTATGCCCAACAA
human IFN-α	Fw	GCCTCGCCCTTTGCTTTACT
	Rv	CTGTGGGTCTCAGGGAGATCA
human IFN-β	Fw	ATGACCAACAAGTGTCTCCTCC
	Rv	GGAATCCAAGCAAGTTGTAGCTC
human ISG15	Fw	GCAGATCACCCAGAAGATCG
	Rv	GGCCCTTGTTATTCCTCACC
human ISG56	Fw	CCTTGCTGAAGTGTGGAGGA
	Rv	CCAGGCGATAGGCAGAGA
human GAPDH	Fw	GGAGCGAGATCCCTCCAAAAT
	Rv	GGCTGTTGTCATACTTCTCATGG
qPCR for HMVEC-L monolayer		**5'-3'**
human CLDN5	Fw	TGCGAGGCGTTGGATAAGCC
	Rv	TTCATTCCGTCTGTTAAGGGCAGG

human VE-cadherin	Fw	GCGACTACCAGGACGCTTTCA
	Rv	CATGTATCGGAGGTCGATGGTG
human IL-6	Fw	GGTACATCCTCGACGGCATCT
	Rv	GTGCCTCTTTGCTGCTTTCAC
human VCAM-1	Fw	GAATGGGAGCTCTGTCACTGTAAG
	Rv	CTTGCACACAGTGCCAAACAC
human ICAM-1	Fw	CTCCAATGTGCCAGGCTTG
	Rv	CAGTGGGAAAGTGCCATCCT
human GAPDH	Fw	TGGAGTCCACTGGCGTCTTC
	Rv	GGCTGTTGTCATACTTCTCATGGT
Generation of CLDN5 knock-in mouse		**5'-3'**
genotyping primer	Fw	TCTGCTGGTTCGCCAACAT
	Rv	ATGGTCAACGGACTCTGAG
qPCR for CLDN5 knock-in mouse		**5'-3'**
mouse CLDN5	Fw	CTGGACCACAACATCGTGAC
	Rv	AGTGCTACCCGTGCCTTAAC
mouse GAPDH	Fw	AAATGGTGAAGGTCGGTGTGAACG
	Rv	ATCTCCACTTTGCCACTGC

Table S2. Primary antibodies used in this study.

antigen	catalogue	clone	host	company	application
Claudin 5	35-2500	4C3C2	mouse	Thermo Fisher Scientific	WB, IF
FoxO1	2880	C29H4	rabbit	Cell signaling	IF
GAPDH	MAB374	6C5	mouse	Sigma-Aldrich	WB
VE-cadherin	sc-9989	F-8	mouse	Santa Cruz Biotechnology	WB, IF
VE-cadherin	AF1002	polyclonal	goat	R&D Systems	WB
β-catenin	sc-7963	E-5	mouse	Santa Cruz Biotechnology	IF

Table S3. COVID-19 patient information.

patient number	age	severity of COVID-19	sex	anamnesis	blood sampling (days after onset)
Patient 1	85	severe	male	epilepsy	5, 6, 7
Patient 2	83	severe	female	high blood pressure, dyslipidemia, asthma	5, 6
Patient 3	87	critical	female	-	4, 5, 6, 7
Patient 4	89	severe	male	high blood pressure, reflux esophagitis	6, 7
Patient 5	86	severe	male	sick sinus syndrome, malignant tumor	1, 2, 5
Patient 6	85	severe	male	hepatitis C, bullous pemphigoid, diabetes	5, 6
Patient 7	55	moderate	female	brain tumor	6
Patient 8	58	asymptomatic	male	-	3, 6
Patient 9	27	mild	female	missed abortion	4
Patient 10	55	moderate	male	dyslipidemia, sleep apnea syndrome	6
Patient 11	58	moderate	male	asthma	4, 6
Patient 12	47	moderate	male	alveolar proteinosis, hepatitis	3, 4, 5
Patient 13	67	mild	female	interstitial pneumonia	2, 3, 6
Patient 14	64	mild	male	diabetes	2