SUPPLEMENTAL MATERIAL

Integrated Single Cell Atlas of Endothelial Cells of the Human Lung

Supplemental Methods:

Sample preparation, barcoding and library preparation for single-cell sequencing

While most data analyzed herein is derived from previously published datasets, we also subjected four additional control lung samples to single cell RNA sequencing (scRNAseq). These new samples were procured and processed similarly to the control samples from our previous publication[13]. Healthy lungs were rejected donor organs that underwent lung transplantation at the Brigham and Women's Hospital, or donor organs provided by the National Disease Research Interchange (NDRI). The study protocol was approved by the Partners Healthcare Institutional Board Review (IRB Protocol # 2011P002419) and the Yale University Institutional Review Board (IRB Protocol ID: 2000022618). Briefly, lung specimens were enzymatically dissociated. After several washing steps and red cell lysis, cell suspensions were frozen and stored in liquid nitrogen till further processing. After thawing, cells were filtered and washed again, then processed using the 10x Chromium single cell RNA seq platform (Single Cell 3′ Reagent Kits v2, 10× Genomics, USA). Single-cell barcoding and library preparation was performed according to the manufacturer's protocol (Single Cell 3′ Reagent Kits v2, 10× Genomics, USA) with a targeted cell output of 10,000 cells per library. cDNA and final libraries were analyzed on an Agilent Bioanalyzer High Sensitivity DNA chip for qualitative control purposes. cDNA libraries were sequenced with a HiSeq4000 Illumina aiming for 150 million reads per library with a sequencing configuration of 26 base pairs on read1 and 98 base pairs on read2. Basecalls were converted to reads using the software Cell Ranger (v3.0.2). For all downstream analysis, these four additional samples were combined with identically processed samples from previously published data sets GSE136831&GSE133747[13, 39] to form the cohort "Yale/Baylor". A schematic of the following computational methods is given in Supp. Figure XII.

Read processing

The dataset repositories GEO, dbGaP, and EMBL-EBI were searched for scRNAseq datasets of human lungs generated using the 10x Single Cell RNAseq technology (V2 chemistry). Control samples of the following human lung scRNAseq datasets used (for

overview of individual libraries, see supplemental table I): EGAD00001005064/5[10] ("WSI Groningen"), phs001750.v1.p1[11] ("Northwestern"), GSE135893[9] ("Vanderbilt TGen"), E-MTAB-6149/6653[12] ("Leuven VIB"), E-MTAB-6308[14] ("Leuven LKI") and GSE136831&GSE133747[13, 39] ("Yale Baylor", which includes the four additional samples now deposited at GEO under GSE164829). FASTQ files containing raw sequencing reads were either provided by the authors or downloaded from the appropriate repositories.

Furthermore, dataset repositories GEO and EMBL-EBI were searched for scRNAseq datasets of mouse experiments generated using the 10x Single Cell RNAseq technology (V2 chemistry) and were available without missing relevant sequencing data (i.e. without missing read1 or read2 data). Control samples of the following mouse lung scRNAseq datasets were identified and FASTQs downloaded from repositories (for overview of individual libraries, see supplemental table VII): E-MTAB-7458[14], E-MTAB-8077[16], GSE133747[39], GSE129605[45], GSE132901[46] and GSE133992[47].

Multiple FASTQs from the same library and read1/read2 were concatenated to single files. Adaptor contamination (AAGCAGTGGTATCAACGCAGAGTACATGGG 10x 3prime samples; CCCATATAAGAAA for 10x 5prime samples) and 20bps or longer poly(A) or poly(T) sequences (for samples from 10x 3prime or 5prime kits respectively) were removed using cutadapt (v2.9). After trimming, read pairs were removed if any of the reads was trimmed below 25 bp.

Subsequent read processing was conducted using the scRNAseq implementation STARsolo of the software STAR[54] (v2.7.3a). Reads were aligned to the human reference genome GRCh38 release 31 (GRCh38.p12) or the mouse reference genome GRCm38 release M22 (GRCm38.p6), both downloaded from GENCODE[55]. Collapsed unique molecular identifiers (UMIs) with reads that span both exonic and intronic sequences were retained as combined gene expression assays, as well as exonic-only derived UMIs gene expression assays. Valid cell barcodes representative of quality cells were distinguished from barcodes of dying cells or background RNA based on the following three thresholds, as described before[13]: at least 7.5% of transcripts arising from unspliced reads indicative of nascent mRNA; more than 1000 transcripts profiled; less than 20% of their transcriptome was of mitochondrial origin. Cell free mRNA background contamination was removed using the software SoupX (v1.2.2)[56]. Technical summaries related to sequencing and data processing can be found in supplemental table IV for human samples and X for mouse samples.

Dataset integration

UMIs from each valid cell barcode, irrespective of whether they derived from spliced or unspliced mRNA, were retained for all downstream analyses and analyzed using the R package Seurat (version 3.1.4)[57]. UMI counts were normalized with a scale factor of 10,000 UMIs per cell and then natural log transformed using a pseudocount of 1. In order to identify and isolate endothelial cells from cells that belong to other lineages, cells from all datasets were subject to a recursive process of integration, graph-

embedding and cluster analysis. For each iteration, integration of datasets and clustering was performed as recommended in Seurat (https://satijalab.org/seurat/v3.0/integration.html). Briefly, the top variable genes within each dataset were selected using the Seurat implementation *FindVariableGenes* using the "vst" parameter. Shared patterns of variance in these genes within each dataset where then used to integrate the datasets using Seurat's *FindIntegrationAnchors* and *IntegrateData*[57], then the resulting integrated expression matrix was scaled using Seurat's *ScaleData*.

Dimension reduction, graph embedding, clustering and visualization

Dimension reduction for visualizing and clustering cells in global transcriptome space was performed in a stepwise manner. First, scaled values from the afore mentioned integration approach were subject to principal component analysis (PCA), and these principal components were then subject to feature selection based on ranked contribution to variance. Selected principal components were used to estimate Euclidean distances between cells in feature-space, and cells are graph embedded with edges connecting the nearest neighbors for each cell. This network of connected cells is subject to Louvain clustering, which seeks to optimize the modularity of clusters in the network – or the number of within-cluster edges when compared to a null model of random connections. For visualization, cell distances and their graph embeddings are subject to uniform manifold approximation and projection (UMAP), where cells are presented in an abstract, two-dimensional feature-space, such that cells with similar transcriptional profiles will appear closer to one another.

Endothelial cell identification

Clusters without noteworthy gene expression differences between them were collapsed. Clusters comprised of heterogeneous cell populations were subsetted alongside phenotypically similar clusters, then subject to another iteration of data integration and cluster analysis. This process continued until all cells from all datasets could be assigned to a discrete cell type, whose distinguishing features are consistently represented across different subjects and datasets. After comprehensively categorizing all cells into distinct cell-type clusters, we then determined each cluster's respective lineage assignment based on its expression profile of classical lineage markers. Five clusters were found to co-express classical endothelial markers PECAM1, CLDN5, ERG and CDH5; notably these clusters were the only clusters collectively missing expression of classical markers of epithelial (EPCAM, CDH1), mesenchymal (PDGFRA, PDGFRB) and immune cells (PTPRC). Mesothelial cells were grouped with mesenchymal cell types for reasons of simplicity. As lymphatic ECs were substantially different from vascular ECs, establishing marker genes that enable differentiation of subvarieties within vascular ECs deemed most important. Therefore, a final subset consisting of only vascular ECs was created for downstream analyses. This final vascular EC subset also included a sixth

dataset (“Leuven LKI”) containing sorted pulmonary EC cells. As two samples of the total of 75 subjects did not contain any ECs, the vascular ECs is derived from 73 subjects only. Sample “NEC50” of the cohort “Leuven LKI” was found to contain relevant cluster of cancer cells (n=789) and therefore not included in this study.

Identification of multiplets

Multiplets and other cell barcodes of low quality were identified using a multilayer approach: First, the software DoubletFinder[58] was used to predict multiplet clusters in an automated and unbiased fashion using an estimated multiplet rate of 0.8% per 1000 cells, according to 10x Genomics Single Cell 3′ Reagent Kits v2 protocol. DoubletFinder was applied per sample due to very different cell numbers per sample and, by that, different expected multiplet rates. However, DoubletFinder was not able to identify several multiplet clusters, especially of cell types with low frequencies. Therefore, multiplet clusters were additionally identified manually as having a transcriptomic signature that resembled the combination of two or more different cell type signatures that already existed in the data set. This approach was applied to the full dataset as well as all lineage subsets. Barcodes identified as multiplets or being of low quality were not included in any downstream analyses.

Identification of cell type specific marker genes

Cell type specific marker genes were identified using the Wilcoxon rank sum test by comparing all cells within a specific cluster to all cells outside said cluster. All p-values were adjusted for multiple comparisons using the conservative Bonferroni correction, and an adjusted p-value below 0.05 was considered significant. Pan-endothelial marker genes were defined as genes significantly expressed in all bona fide pulmonary EC populations (arterial, pulmonary-venous, aerocytes, general capillary ECs, incl. lymphatic ECs) and a logFC of greater than 0.25, when compared to all other cell types. Pan-vascular marker genes were defined as genes significantly expressed in all the bona fide pulmonary vascular EC populations (arterial, pulmonary-venous, aerocytes, general capillary ECs) and a logFC of greater than 0.25, when compared to all other cell types, but not in lymphatic ECs.

As described before[13], we used an additional approach of a binary classifier system to assess the utility of detecting a given gene for classifying a cell. For each specific cell type, we selected all genes whose expression was log fold change $\geqslant 0.25$ greater in all other cells in the data. Of those genes, we calculated the diagnostics odds ratio (DOR), where we binarize the expression values by treating any detection of a gene (normalized expression value > 0) as a positive value and zero expression detection as negative. To avoid undefined values, we included a pseudocount of 0.5 as follows:

DOR= ((TruePositives+0.5)/(FalsePositives+0.5))/
((FalseNegatives+0.5)/(TrueNegatives+0.5))

where TruePositives represents the number of cells within a cluster detected expressing the gene (value > 0), FalsePositives represents the number of cells outside of the cluster detected expressing the gene, FalseNegatives represents the number of cells within the cluster with no detected expression, and TrueNegatives represents the number of cells outside of the cluster with no detected expression of the gene. Log-transformed DOR values are given in all supplemental tables on marker genes.

Connectome Analysis:

To study cell-cell signaling, the data was mapped to the NicheNet ligand-receptor interaction database[59] using the R software Connectome (v0.2.2) (https://msraredon.github.io/Connectome/)[39, 60]. In brief, each cell type was treated as a single node for network creation. Average expression values, for all data slots, were calculated for all ligand and receptor genes on a per-cell-type basis, and an unfiltered edgelist ("connectome") was created linking all producers of a ligand to all producers of a receptor, with associated quantitative edge attributes. This mapping leveraged only well-annotated and literature supported ligand-receptor interactions (i.e., "kegg_cytokines", "kegg_cams", "kegg_neuroactive", "kegg_ecm", "pharmacology", "ramilowski_known"). Selected ligand-receptor interactions were visualized as Circos plots using the R package *circlize*[61] after filtering the connectome based on the following criteria: Ligands being significantly expressed in EC subpopulations and receptors significantly expressed in non-EC populations or vice versa (using an arbitrarily chosen, more stringent Bonferroni adjusted p-value threshold of p_adjust < 1e-5 based on a system-wide Wilcoxon Rank Sum test); ligands and receptors being expressed in at least 20% of the cells of their respective cell types with ligand and receptor mean scaled expression values > 0; omitting all integrin receptors due to their promiscuity; and finally, selecting the top 75 interactions for both Circos Plots in Figure 4, when ranked based on the scaled weight, defined as the mean of the scaled expression values of the ligand and receptor.

Analysis of mouse datasets

Integration, clustering, cell type annotation and multiplet identification of control mouse datasets was performed as described above for the human samples. When using the Seurat implementation *FindIntegrationAnchors* to integrate the mouse dataset, we reduced the default parameter for neighbors for filtering from 200 to 150, in order to accommodate a dataset with less than 200 cells, with otherwise default settings. Cell were assigned to lineages based on canonical marker genes as follows: lymphoid (Ptprc+ and Cd79a+ or Cd2+), myeloid (Ptprc+ and Lyz2+), endothelial (Cldn5+, Erg+, Cdh5+ and Pecam1+), epithelial (Epcam+) and stromal (Pdgfra+ or Pdgfrb+) lineages. Cell type annotations were performed to a similar granularity compared to humans, e.g. cDC2a and cDC2b were kept as cDC2. Barcodes identified as either

multiplet or low quality were not included in any downstream analyses. Identification of cell type marker genes were performed as described above for the human samples.

Cell type annotation in a pulmonary arterial hypertension scRNAseq dataset

A scRNAseq data set consisting of cells from 6 controls and 3 patients with pulmonary hypertension[44] was re-analyzed regarding EC subpopulations. The raw cell-gene matrix was filtered for cells in which more than 1000 transcripts profiled and less the 20% of the transcriptome originated from mitochondrial genome. Normalization, selection of variable features, scaling of the data (regressing out the number of profiled transcripts and percent of transcript originating from the mitochondrial genome), dimensionality reduction by principal component analysis, graph embedding in UMAP space and clustering using the Louvain algorithm was performed as described above. Vascular endothelial cells were subsetted and integrated on the disease status level as described above. The vascular EC subset was scaled, subjected to a PC analysis, graph embedded and clustered, with cell type and multiplet identification as described above.

Single cell RNA sequencing of primary lung endothelial cells

Human primary pulmonary arterial (Lonza), venous (Cell Biologics), and microvascular (Lonza) endothelial cells were cultured on gelatin-coated surfaces at a density of 5000 cells/cm2 in EGM2 endothelial growth medium (Lonza) for 3 days with daily medium change. Cells were trypsinized, washed in PBS, counted, then barcoded using the 10x Chromium single cell RNA seq platform (Single Cell 3′ Reagent Kits v3, 10× Genomics, USA) according to the manufacturer's instruction and aiming for 10,000 cells. Sequencing, data processing and graph embedding was performed as described above.

Comparison of mouse and human ECs

For comparison of EC marker gene expression between human and mouse, mice gene names were translated using the R package biomaRt[62] if there was a one-to-one homologue available. As a proof-of-principle, we performed an integration of mouse and human control ECs. To this end, both barcode-gene-matrices were subsetted, keeping only genes for which a one-to-one homologue in the respective other species was available. All datasets were integrated following the same integration workflow described for the separate human and mouse analyses above. The only parameter change made was a change from 200 to 150 neighbors for anchor filtering with the Seurat package's *FindIntegrationAnchors* implementation, for the same reasons described in the mouse analysis methods, above.

To identify conserved pan-endothelial, pan-vascular or specific marker genes of the bona fide pulmonary vascular ECs (aerocytes, general capillary, arterial, pulmonary-venous) and lymphatic ECs, datasets were randomly downsampled to 500 cells per cell type in both species, to ensure balanced proportions. Identification of cell type specific

marker genes of this balanced dataset was performed using the Wilcoxon Rank sum test with adjustment for multiple comparisons as described above. Conserved marker genes are defined as being uniquely found differentially expressed in the same cell type of both species. Conserved lymphatic marker genes were defined as genes being significantly expressed in lymphatic ECs compared to all other lung cells in both species. Conserved pan-endothelial marker genes were defined as genes being significantly expressed in all four bona fide pulmonary vascular and lymphatic ECs compared to all other lung cells in both species. Conserved pan-vascular marker genes were defined as genes being significantly expressed in all four bona fide pulmonary vascular ECs when compared to all other lung cells in both species, but not significantly expressed in lymphatic ECs compared to all other lung cells in both species. For results of this comparison between mouse and human ECs, please refer to Supp. Table XI.

Availability of sequencing data

The processed raw data of the four additional sample from the "Yale/Baylor" cohort as well as scRNAseq data of primary lung endothelial cells has been deposited on GEO under the accession number GSE164829. Additional human single cell RNA sequencing raw data had already been published and is available at GEO (Vanderbilt/TGen[9]: GSE135893; Yale/Baylor[13, 39]: GSE136831 and GSE133747), dbGaP (Northwestern[11]: phs001750.v1.p1), EGA (WSI/Groeningen[10]: EGAD00001005064 and EGAD00001005065), and EMBL-EBI (Leuven VIB[12]: E-MTAB-6149 and E-MTAB-6653). Murine single cell RNA sequencing raw data had been published and is available at GEO (GSE133747[39], GSE129605[45], GSE132901[46] and GSE133992[47]) and EMBL-EBI (E-MTAB-7458[14], E-MTAB-8077[16]). The computational code of the analysis and visualizations is deposited on github at https://github.com/yale-p2med/lungendothelialcellatlas.

Immunohistochemistry

Immunohistochemistry was performed as previously described[13]. FFPE (Formalin fixed paraffin embedded) blocks were cut at 5 µm, rehydrated (xylene/ethanol deparaffinization), then boiled at 95°C for 20 min in 1× Tris-based Antigen Unmasking Solution (Vector Laboratories, USA) for heat-induced antigen retrieval. Histology slides were incubated for 10 min in BLOXALL Blocking Solution (Vector Laboratories, USA) to block endogenous peroxidase and alkaline phosphatase activity. Unspecific antibody binding was blocked using 2.5% Normal Horse Serum Blocking Solution (Vector Laboratories, USA) for 20 min. Slides were incubated with the primary antibody (rabbit antibodies: ACKR1 (polyclonal, # PA5-82549, Thermofisher), CA4 (clone #039, # 10472-R039-50, Sino Biological), PRX (polyclonal, # NBP1-89598-25ul, Novus Bio), COL15A1 (polyclonal, # PA553667, Thermofisher), VWA1 (polyclonal, # 14322-1-AP, Proteintech); mouse antibodies: PECAM1 (clone JC/70A, #MA513188, Thermofisher), CLDN5 (clone A-12, # sc-374221, Santa Cruz), GJA5 (clone B-3, # sc-365107, Santa Cruz), PDPN (clone D2-40, # 916601, Biolegend), HPGD (clone H-3, # sc-271418, Santa Cruz); goat antibody:

LYVE1 (polyclonal , # AF2089, Novus Bio), diluted in 2.5% Normal Horse Serum Blocking Solution for 30 min at room temperature. Histology slides were incubated for 30 min with secondary antibodies (anti-mouse/-rabbit/-goat ImmPRESS reagent, Vector Laboratories, USA), conjugated with horseradish peroxidase. Specimen were incubated for 10 min in DAB working solution (Vector Laboratories, USA). Slides were counterstained in Hematoxilin Solution Gill no. 1 (Sigma-Aldrich, USA) for 3 min, and then washed with tap water. Slides were dehydrated in ethanol/xylene and mounted with VectaMount permanent mounting solution (Vector Laboratories, USA). Stained slides were digitalized on an Aperio Scanner (Leica) and then analyzed using the softwares QuPath and ImageJ. Raw Aperio Scanner files have been deposited at Zenodo under DOI: 10.5281/zenodo.4503718 [8].

Immunofluorescence microscopy

Deparaffinization and blocking was performed as described above for conventional immunohistochemistry. Slides were incubated with the primary antibodies (same clones as described above) diluted in 2.5% Normal Horse Serum Blocking Solution over night at 4°C. Histology slides were incubated for 1h with secondary antibodies (Horse DyLight 594 Anti-Rabbit IgG, Horse DyLight 488 Anti-Mouse IgG, Vector Laboratories, USA) at room temperature. Autofluorescence was quenched using the Vector TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories, USA) according to the manufacturer's instructions with an incubation time of 3min. Slides were mounted using the Vectashield Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories, USA). Representative regions of stained slices were digitalized on a Leica SP5 Confocal Microscope and analyzed using the software ImageJ.

RNA in situ hybridization

RNAscope technology (Advanced Cell Diagnostics (ACD), Newark, CA) was used for RNA in situ hybridization (RNA-ISH). FFPE blocks were cut at 5 µm, mounted on slides, baked for 1 h at 60°C, then deparaffinized in xylene and 100% ethanol. Hydrogen peroxide (ACD 322381) was applied for 10 min at room temperature, followed by a mild boil at 98-102°C for 15 min in 1x target retrieval reagent buffer (ACD 322001). Sections were treated with Protease Plus (ACD 322381) at 40°C for 30 min in HybEZ Oven (ACD). Hybridization with target probes (FCN3 - ACD 818741, SOSTDC1 - ACD 469921), preamplifier, amplifier, label and wash buffer (ACD 320058) were performed following the ACD manual. Parallel sections were incubated with ACD positive (HsPPIB; ACD 313901, MnPpib; ACD 313911) and negative (DapB; ACD 310043) control probes. Stained slides were digitalized on an Aperio Scanner (Leica), and representative regions were digitalized on a Leica SP5 Confocal Microscope, as the used dye Vector Red is also fluorescent.

Supplemental Results

Lung endothelial cells in Mendelian genetic diseases of the lung

To showcase the utility of a scRNAseq-based atlas of ECs within the human lung, we analyzed the EC-specific gene expression patterns in Mendelian lung disorders involving lung ECs. For our analysis of Mendelian genetic diseases of the lungs, we used the "Online Mendelian Inheritance in Man" (omim.org) website to extract genes associated with the following monogenetic lung diseases: primary pulmonary hypertension (PH), pulmonary venooccclusive disease (PVOD), alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) and primary pulmonary hyperplasia. All disease-associated genes showed the highest expression within the endothelial cells of our integrated dataset. However, expression of most of these genes was not confined to a singular EC subpopulation (Supplemental Figure V). Gene associated with PH and expressed in several EC populations include BMPR2, EIF2AK4, SMAD9, CAV1, ENG, ACVRL1 and AQP1, with BMPR2 and EIF2AK4 being additionally associated with PVOD. While most of these genes are expressed mainly in larger vessels, only CAV1, AQP1 and ACVRL1 show the highest expression in aerocytes. Additionally, SOSTDC1, antagonist to BMPR2 ligands BMP2/4/6/7, is specifically expressed in aerocytes. PH-associated genes specifically expressed in arterial ECs are SOX17 and ATP13A3 while genes specifically expressed in venous ECs are GDF2 and ABCC8. FOXF1, associated with ACDMPV, is mainly expressed in aerocytes, whereas ZFPM2, associated with primary pulmonary hypoplasia, is almost exclusively expressed in lymphatic ECs.

Single cell RNA sequencing of commercially available, primary lung endothelial cells reveals a loss of a native lung phenotype

17,476 cells were from three samples – cultured human primary pulmonary arterial, venous, and microvascular endothelial cells from commercial sources - were profiled by scRNAseq. All profiled cells were clearly ECs as highlighted by the expression of PECAM1, CDH5, ERG and TIE1 (Supplemental Figure VII). As one can appreciate in the heatmap of marker genes was designed exactly the same as in Fig 3 in the main manuscript (with addition of few general EC marker genes; Supplemental Figure VII), commercially obtained cell populations do not resembled the native lung endothelial phenotype; most of the marker genes are either not expressed in the huge majority of cells or the marker genes do not show an expected specific expression in cells from a respective sample. Instead, we observed transcriptomic features which are mainly driven by the culture in flasks itself: throughout all cells, we observed a clear endothelial to mesenchymal transition like phenotype as shown by the expression of CDH2, VIM, FN1, LTBP1, ZEB1, ZEB2, TWIST2 and SNAI1 (Supplemental Figure VIII). Furthermore, actively proliferating endothelial subpopulations could be identified based on expression of MKI67, TOP2A, CENPF and ASPM), tip-like subpopulations based

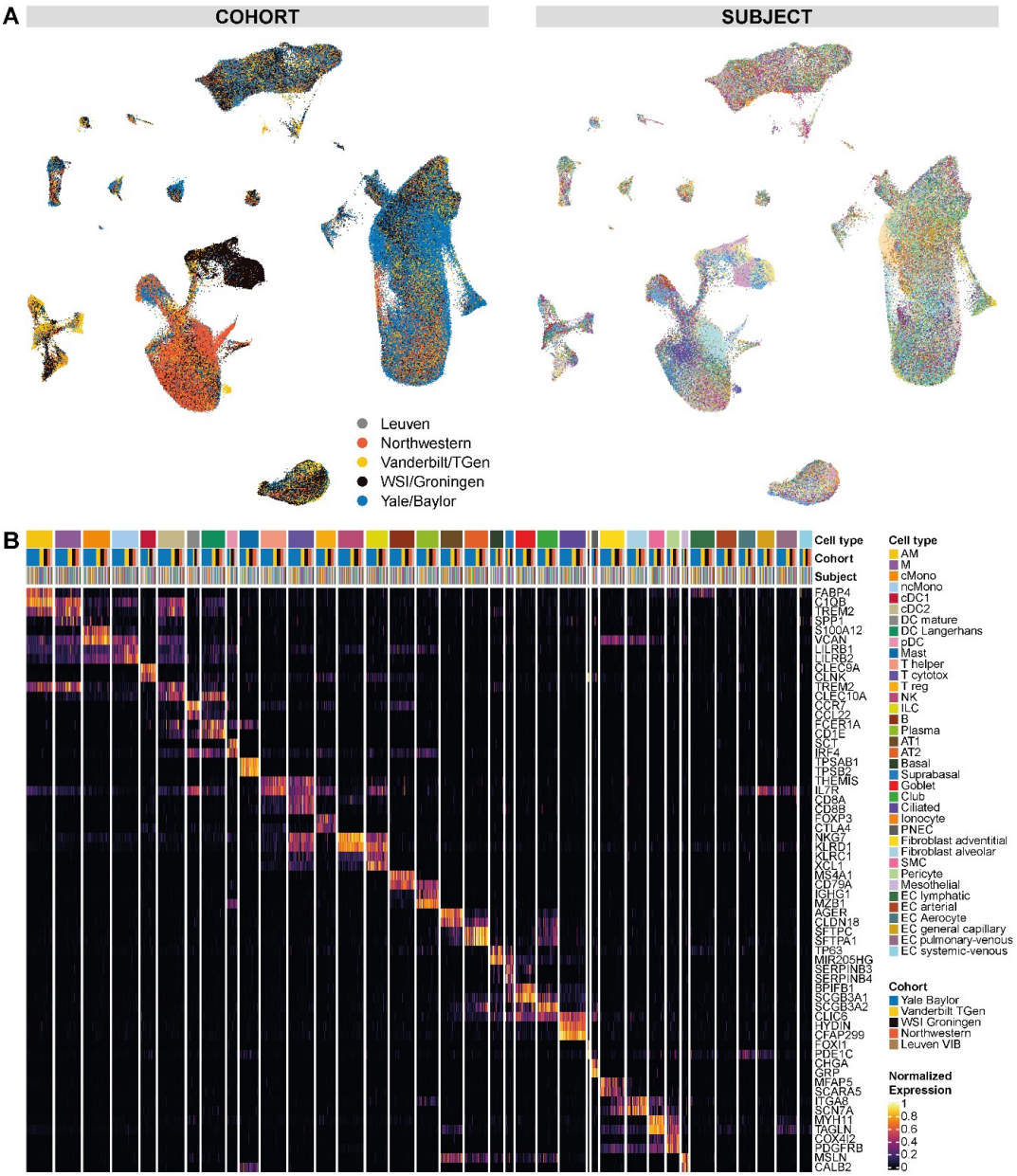
on expression of CXCR4, DLL4, UNC5B and CD34, and a lymphatic-like subpopulation based on expression of PDPN, RELN, PROX1 and TBX1 (Supplemental Figure VIII). Taken together, commercially available, primary lung endothelial cells loose at their respective native lung phenotype and but exhibit a bland, de-differentiated phenotype instead. Many of the observed transcriptional characteristics can be associated with the culture of these cells. For in vitro experiments which depend on a native lung endothelial phenotype, isolation of lung endothelial cells from fresh lung tissue seems to be superior compared to commercially available primary cells: Comhair at al. convincingly showed that it is indeed possible to retain distinct characteristics of lung arterial and microvascular endothelial cells in vitro[63].

Proof-of-concept: Vascular endothelial subpopulation can be identified in a scRNAseq data set of pulmonary arterial hypertension

2,569 vascular ECs from a scRNAseq data set of 6 controls and 3 patients with pulmonary hypertension[44] were re-analyzed that had not been characterized with respect to endothelial subpopulations in the original publication. All five EC subpopulation could be identified, and marker genes of them exhibited expected expression patterns (see Supplemental Figure IX and X). The small sample size of patients with pulmonary hypertension (n=3) precluded a meaningful comparison of the disease status within endothelial subpopulations. Nonetheless, this proof-of-concept analysis highlights, that endothelial subpopulations can be characterized in a disease setting as well and that endothelial diversity in pulmonary hypertension could be studied by scRNAseq, ideally in a much larger cohort.

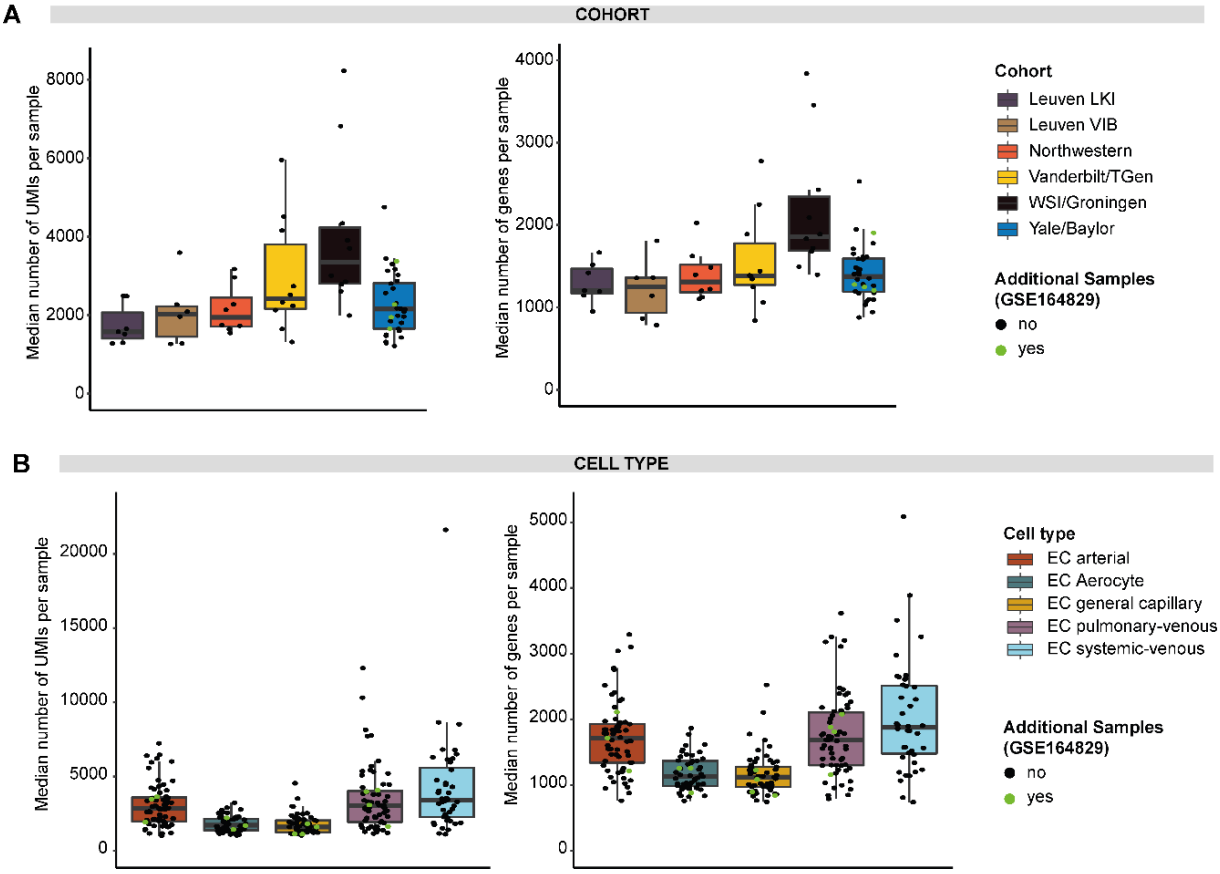
Supplemental Figures

Supp. Fig. I



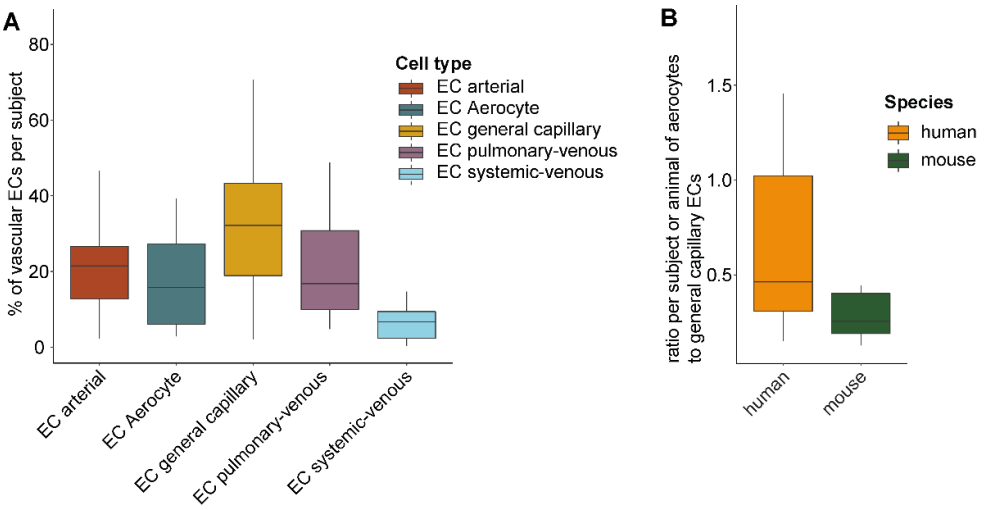
Supp. Fig. I: **(A)** Additional annotations of the UMAP in Fig. 1B in the main manuscript. UMAP of the full dataset colored by cohort and subjects. Each dot represents a single cell. In the UMAP colored by subjects, each color represents a distinct subject. **(B)** Enlarged heat map of Fig. 3A zooming in on marker gene expression of all non-EC populations. Each column represents the average expression value for one subject, grouped by cell type and cohort. All gene expression values are unity normalized from 0 to 1 across rows. AM: alveolar macrophage; AT1/2: alveolar cell type 1/2; B: B cell; cDC1/2: classical dendritic cell type 1/2; cMono: classical monocyte; DC: dendritic cell; EC: endothelial cell; M: macrophage; NK: natural killer; ILC: innate lymphoid cell; ncMono: non-classical monocyte; NK: natural killer cell; pDC: plasmacytoid dendritic cell; PNEC: pulmonary neuroendocrine cell; SMC: smooth muscle cell; T: T cell.

Supp. Fig. II



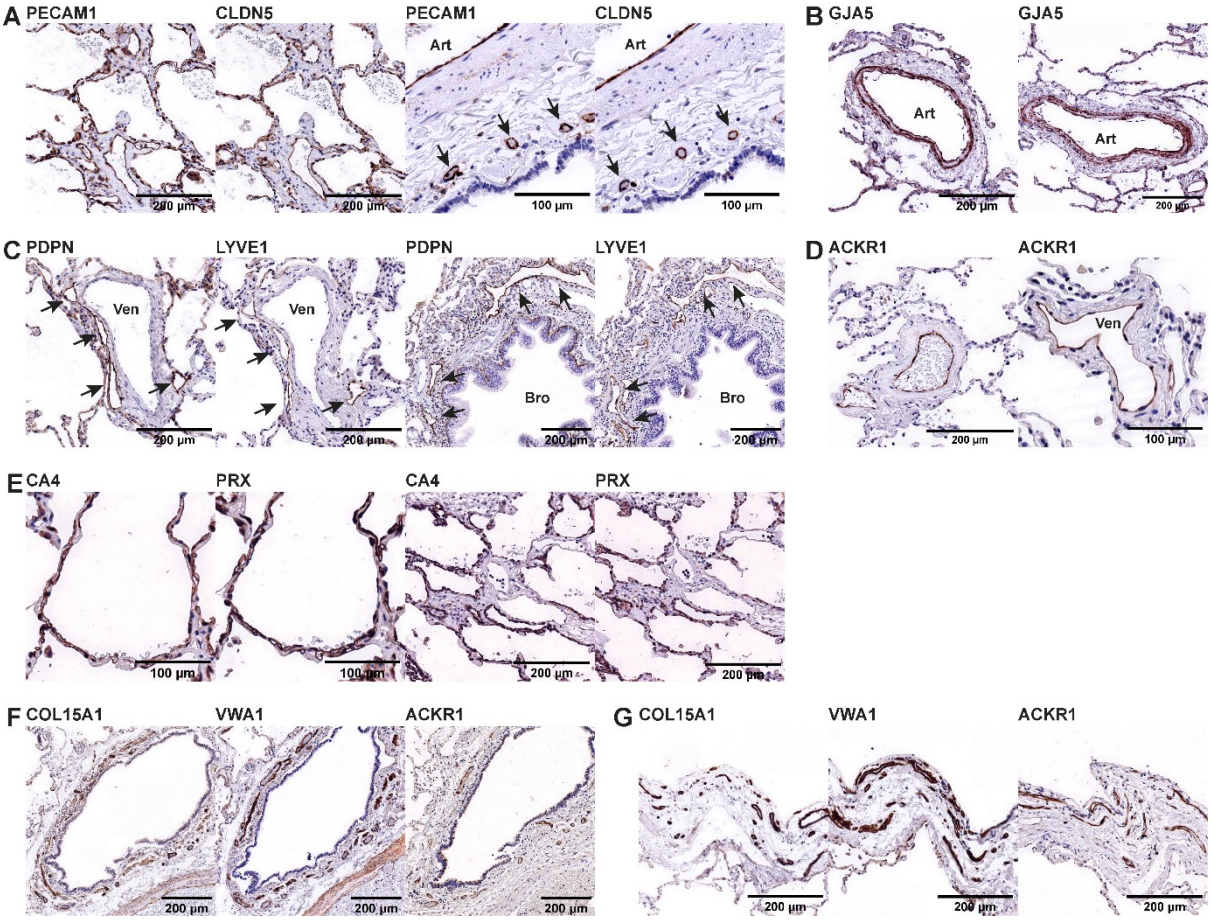
Supp. Fig. II: **(A)** Distribution of median number of unique mRNAs (UMIs) and unique genes per subject, split by cohort, in vascular ECs. **(B)** Distribution of median number of unique mRNAs (UMIs) and unique genes per subject and cell type in vascular ECs. Additional samples are highlighted in green and do not represent outsiders.

Supp. Fig. III



Supp. Fig. III: **(A)** Boxplots representing the percentage of each vascular EC types among all vascular ECs per subject for all subjects in which all five vascular ECs had been profiled (n=29 subjects). The length of each whisker represents 1.5×IQR. **(B)** Boxplots representing the ratio of aerocyte to general capillary EC counts per human subject in which both cell types had been profiled (n=46 subjects) in orange and per mouse in green. The length of each whisker represents 1.5×IQR.

Supp. Fig. IV



Supp. Fig. IV:

(A) Representative IHC staining of pan-endothelial markers CLDN5 and PECAM1, with positive brown staining in capillaries and veins in the first figure pair and arterial ("Art") and peribronchial vessels (arrows) in the second figure pair.

(B) Representative IHC staining of arterial marker GJA5 with positive staining of an arteriole ("Art") in brown.

(C) Representative IHC staining of lymphatic markers PDPN and LYVE1, with positive brown staining in lymphatic vessels (arrows) surrounding a larger vein ("Ven") in the first figure pairs and a bronchus ("Bro") in the second figure pair. As predicted by the transcriptomic scRNAseq data, PDPN also stains basal cells and AT1.

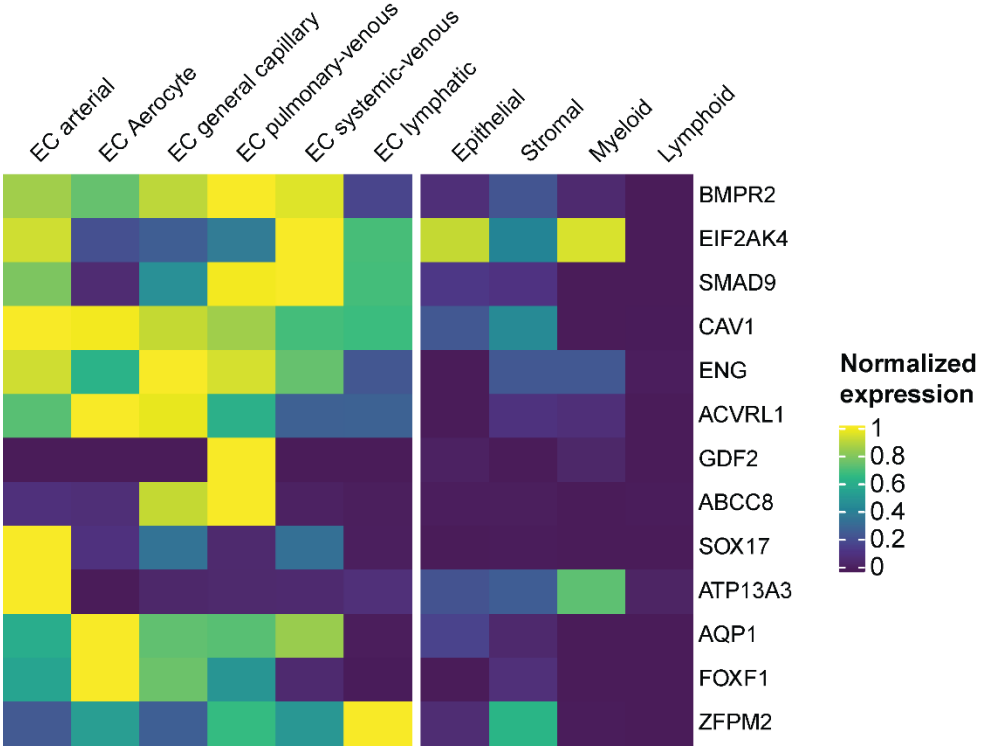
(D) Representative IHC staining of venous marker ACKR1 with positive staining of venules ("Ven") in brown.

(E) Representative IHC staining of capillary markers PRX and CA4 with positive brown staining in capillaries, in higher magnification in the first two figure pairs focusing on an alveolus and a lower magnification in the second two figure pairs in which the negative staining of a larger central vessel can be appreciated

(F) Representative IHC staining of systemic-venous EC markers COL15A1, VWA1 and ACKR1 with positive brown staining in small vessels surrounding a larger bronchus.

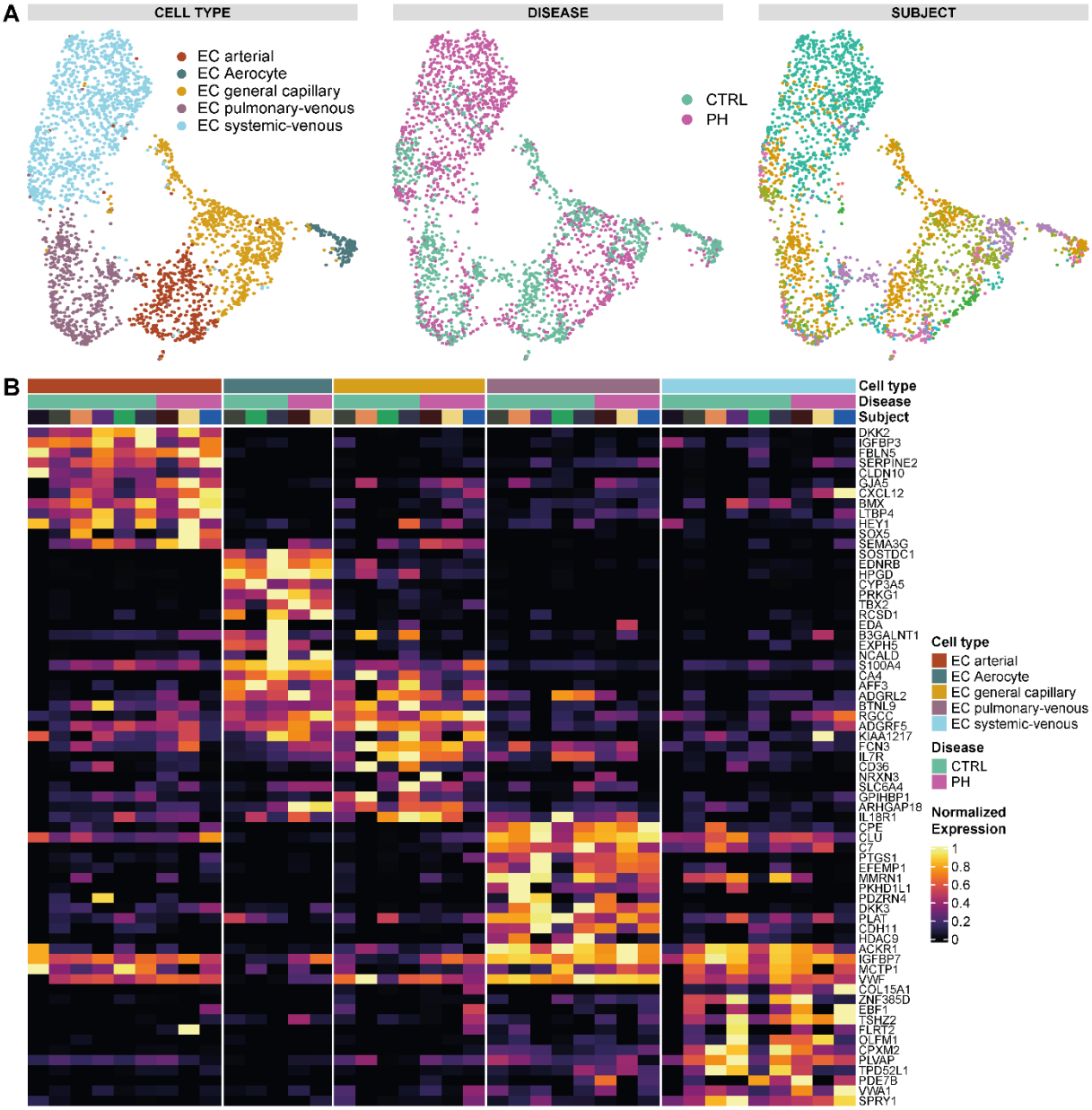
(G) Representative IHC staining of systemic-venous EC markers COL15A1, VWA1 and ACKR1 in small vessels of the visceral pleura.

Supp. Fig. V



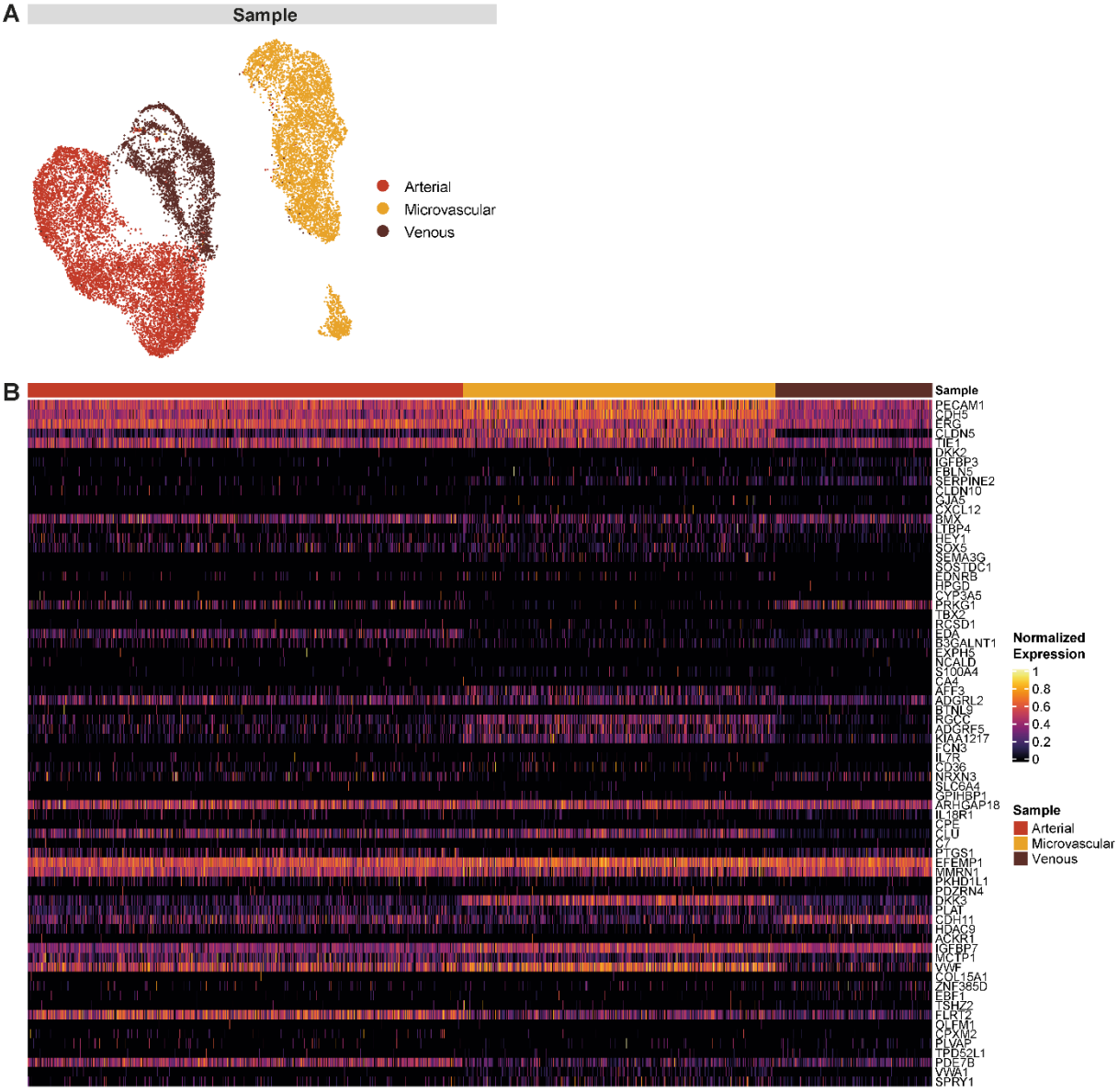
Supp. Fig. V: Heat map of genes associated with Mendelian genetic diseases of the lung with involvement of ECs. Each column represents the average expression value per cell type for all ECs and per lineage for all non-ECs of. All gene expression values are unity normalized from 0 to 1 across rows.

Supp. Fig. VI



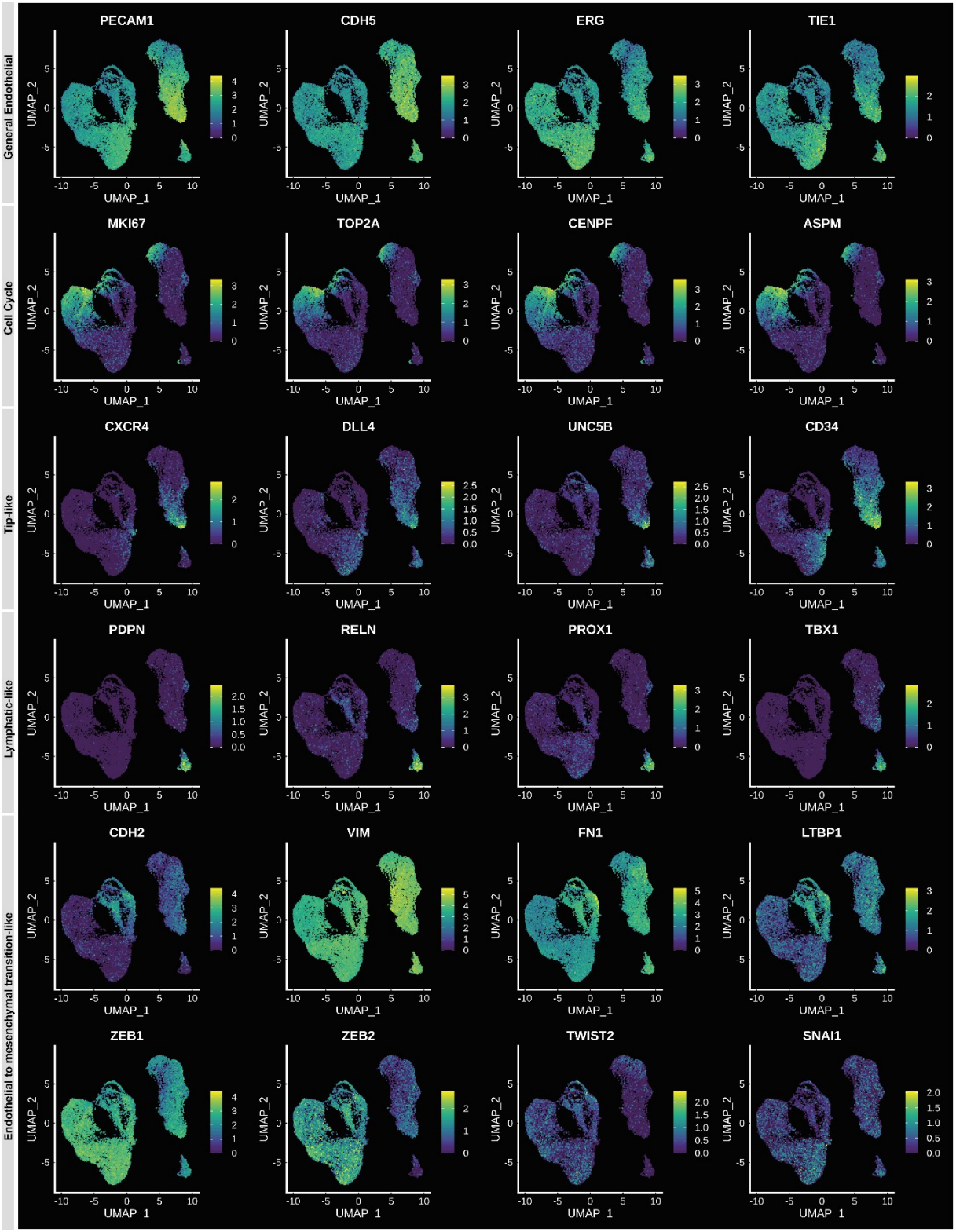
Supp. Fig. VI: Reanalysis of 2,569 vascular ECs from a scRNAseq data set of 6 controls and 3 patients with pulmonary hypertension[44]. (A) UMAP representations of said 2,569 vascular ECs in which each dot represents a single cell, and cells are labelled by cell type, disease status, and subject. In the UMAP colored by subject, each color represents a distinct subject. (B) Heat map of marker genes all five EC populations. The heat map is designed exactly the same as the heat map in Fig. 3 of the main manuscript. Each column represents the average expression value for one subject, grouped by cell type and disease status. All gene expression values are unity normalized from 0 to 1 across rows. CTRL: control subject; EC: endothelial cell; PH: patient with pulmonary hypertension.

Supp. Fig. VII



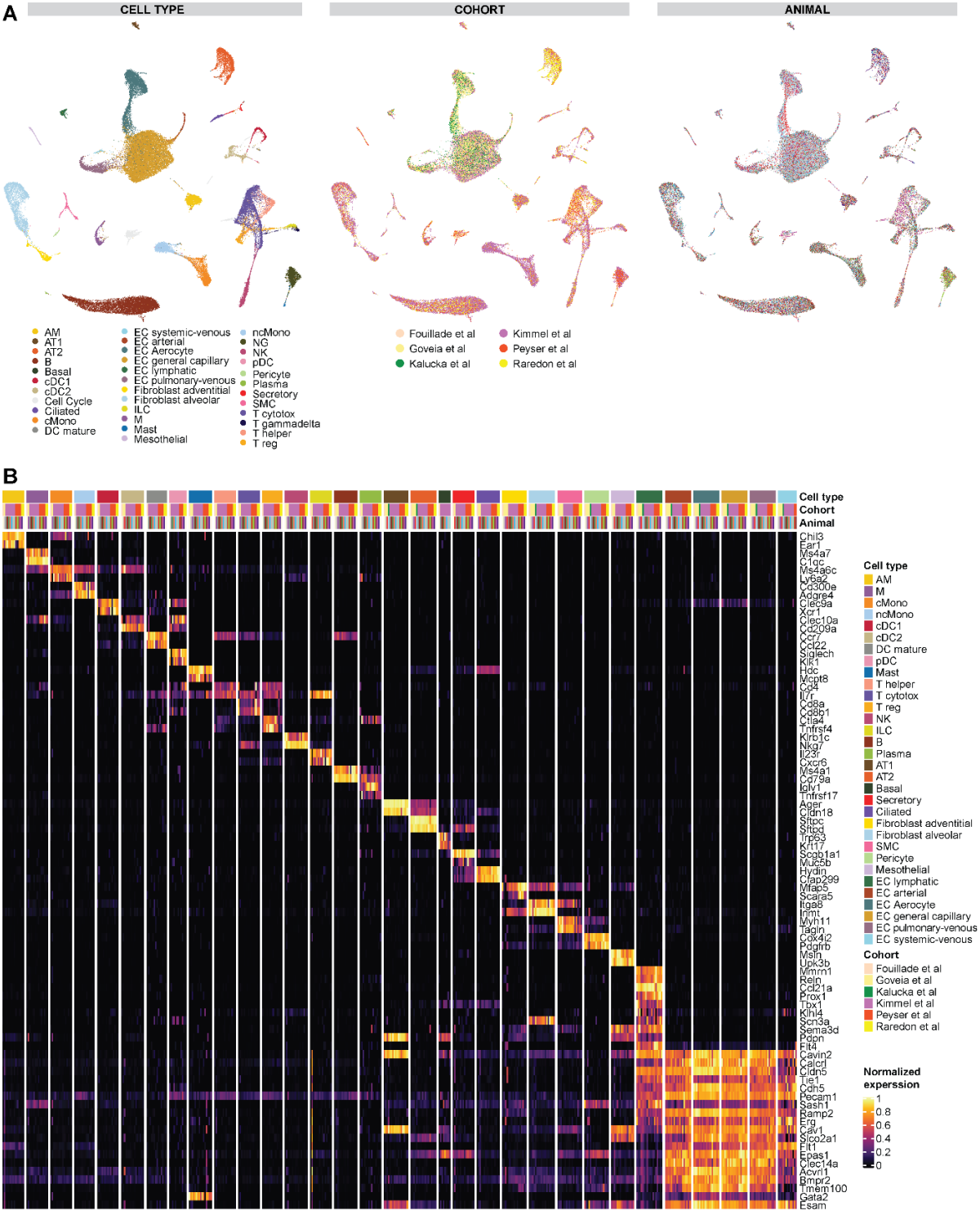
Supp. Fig. VII: (A) UMAP representation of scRNAseq data of 17,476 cultured, commercially obtained, primary arterial, microvascular and venous lung endothelial cells. Each dot represents a single cell, and cells are labelled by sample source. (B) Heat map of marker genes all five EC subpopulations of the cultured primary lung endothelial cells. Each column represents the gene expression of a cell, grouped by sample source. The heat map is designed exactly the same as the heat map in Fig. 3 of the main manuscript with addition of five general endothelial marker genes (PECAM1, CDH5, ERG, CLDN5, TIE1) at the top of the heat map. All gene expression values are unity normalized from 0 to 1 across rows.

Supp. Fig. VIII



Supp. Fig. VIII: UMAPs of cultured primary lung endothelial cells colored by the expression of selected genes, thematically sorted by row, from top to bottom: general endothelial marker genes; genes associated with cell cycle; marker genes of tip ECs; marker genes of lymphatic ECs; and genes associated with endothelial-to-mesenchymal transition (last two rows).

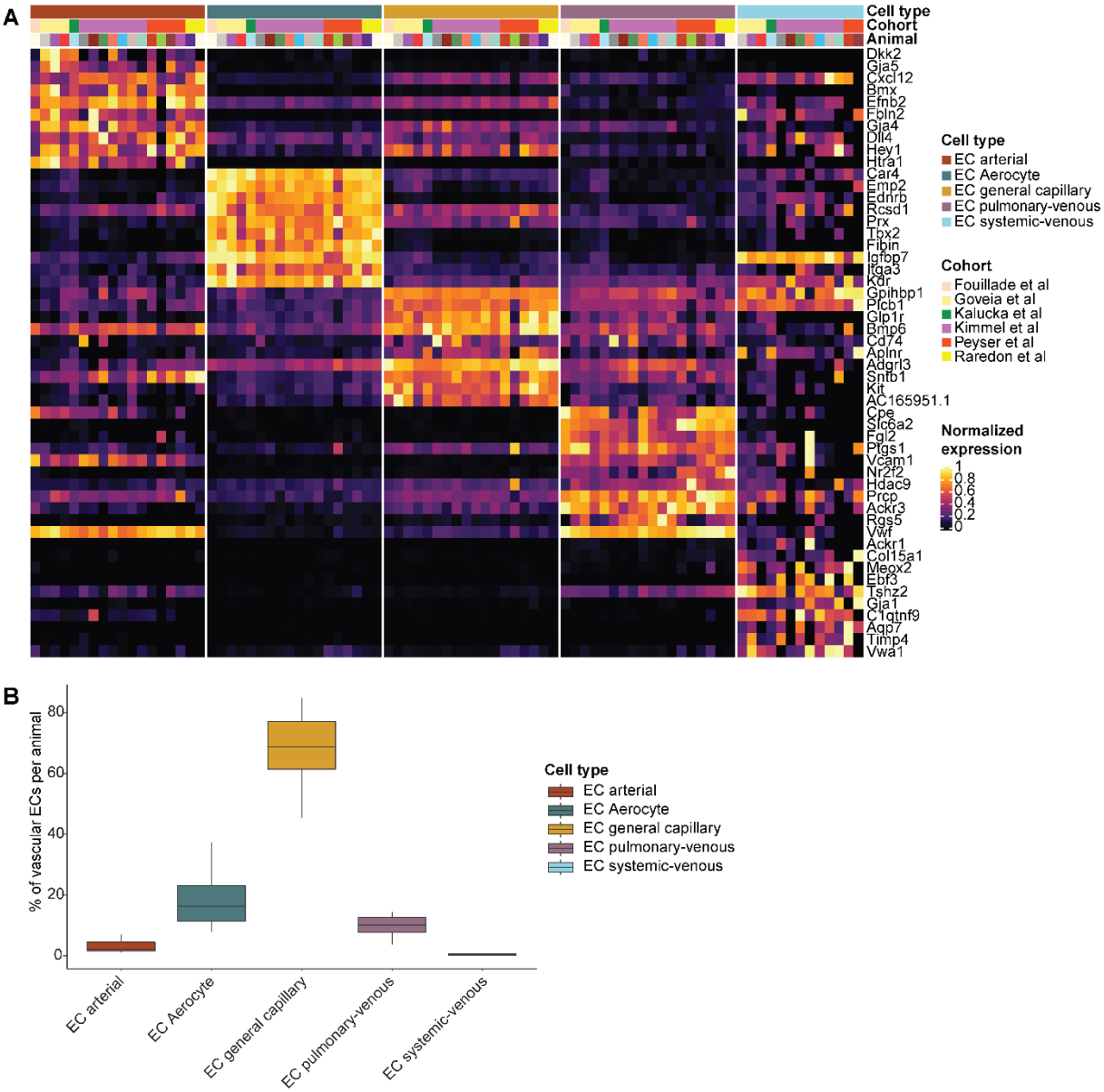
Supp. Fig. IX



Supp. Fig. IX: **(A)** Additional annotations of the UMAP in Fig. 5A in the main manuscript. UMAPs of the full mouse dataset are colored – from left to right - by cell type, cohort

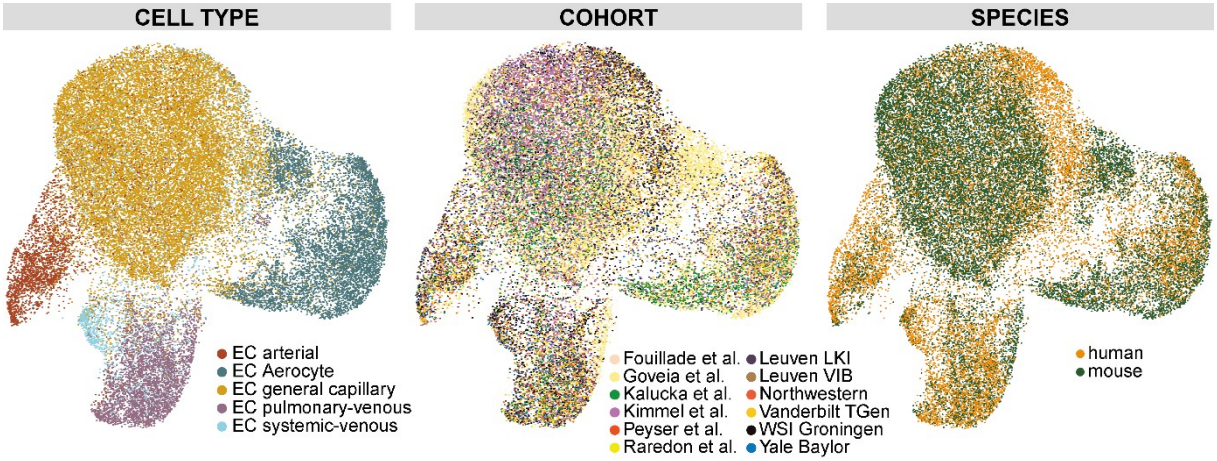
and animals. In the UMAP colored by animals, each color represents a distinct animal. **(B)** Heat map of murine marker genes for all non-EC cell types and of lymphatic ECss, as well as pan-endothelial (specifically expressed in all EC sub-populations) and pan-vascular (specifically expressed in all EC populations but lymphatic ECs) marker genes. Each column represents the average expression value for one animal, grouped by cell type and cohort. All gene expression values are unity normalized from 0 to 1 across rows. AM: alveolar macrophage; AT1/2: alveolar cell type 1/2; B: B cell; cDC1/2: classical dendritic cell type 1/2; cMono: classical monocyte; DC: dendritic cell; EC: endothelial cell; M: macrophage; NK: natural killer; ILC: innate lymphoid cell; ncMono: non-classical monocyte; NK: natural killer cell; pDC: plasmacytoid dendritic cell; PNEC: pulmonary neuroendocrine cell; SMC: smooth muscle cell; T: T cell.

Supp. Fig. X



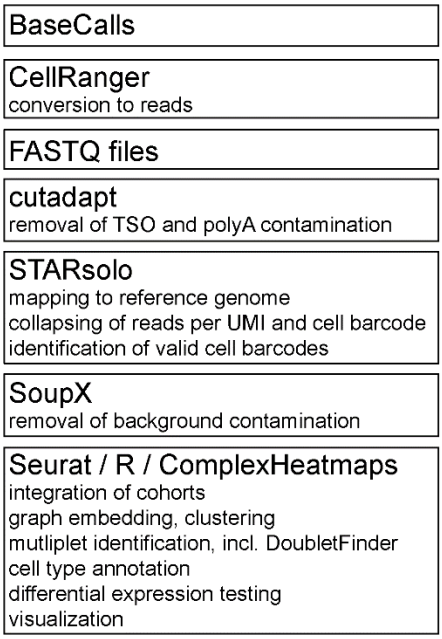
Supp. Fig. X: **(A)** Heat map of marker genes all five EC populations of the mouse dataset. Each column represents the average expression value for one animal, grouped by cell type and cohort. All gene expression values are unity normalized from 0 to 1 across rows. **(B)** Boxplots representing the percentage of each murine vascular EC type among all murine vascular ECs per animal. The length of each whisker represents 1.5×IQR.

Supp. Fig. XI



Supp. Fig. XI: UMAPs of integrated human and murine vascular EC datasets. Each dot represents a single cell, and cells are labelled – from left to right - by cell type, cohort and species.

Supp. Fig. XII



Supp. Fig. XII: Schematic of the computational workflow

Supplemental Tables

Supplemental tables are available as additional excel files.

Supp. Table I: Table of libraries of all human data sets
Table on basic characteristics of all human libraries included in this study.

Supp. Table II: Summary table of basic characteristics by cohort for human and mice
Summary table on basic characteristics by cohort for human subjects and mice. If age was given as range in the original publication, the range was averaged for this purpose here. Age is given with interquartile range in square brackets. If the sums of certain variables do not add up to the total number of subjects per cohort, the corresponding information was not available for respective subjects. NA: not available; n. a.: not applicable.

Supp. Table III: Human cell type marker table and table of pan-endothelial and pan-vascular marker genes
Results of Wilcoxon rank-sum test testing each cell type against all others. Each gene is annotated whether it was identified as pan-endothelial or pan-vascular marker gene. “pct.1” represents the fraction of cells within a specific cell type expressing a specific gene; “pct.2” represents the fraction of cells outside a specific cell type expressing a specific gene.

Supp. Table IV: Technical summaries of library read processing of the human samples
Technical summaries of computational read processing pipeline for each library.

Supp. Table V: Human vascular endothelial subset marker table
Results of Wilcoxon rank-sum test of each vascular endothelial cell type against the other vascular endothelial varieties. “pct.1” represents the fraction of cells within a specific cell type expressing a specific gene; “pct.2” represents the fraction of cells outside a specific cell type expressing a specific gene.

Supp. Table VI: Connectomic edgelist
Connectomic edgelist generated on the basis of the full human dataset, filtered such that 20% cells within a given cell type express a specific ligand or receptor.

Supp. Table VII: Summary table of libraries of all murine data sets
Summary table on basic characteristics of animals and basic information of data sets for all murine libraries included in this study.

Supp. Table VII: Mouse cell type marker table and table of pan-endothelial and pan-vascular marker genes

Results of Wilcoxon rank-sum test of the mouse full dataset testing each EC type against all others. Each gene is annotated whether it was identified as pan-endothelial or pan-vascular marker gene. “pct.1” represents the fraction of cells within a specific cell type expressing a specific gene; “pct.2” represents the fraction of cells outside a specific cell type expressing a specific gene.

Supp. Table IX: Mouse vascular endothelial subset marker table

Results of Wilcoxon rank-sum test of each mouse vascular endothelial cell type against the other mouse vascular endothelial varieties. “pct.1” represents the fraction of cells within a specific cell type expressing a specific gene; “pct.2” represents the fraction of cells outside a specific cell type expressing a specific gene.

Supp. Table X: Technical summaries of library read processing of the mouse samples

Technical summaries of computational read processing pipeline for each mouse library.

Supp. Table XI: Conserved marker table

Table concatenating the results of the balanced differential testing per cell type including annotation whether a gene was identified as conserved marker in human and mice. The column “species” signifies whether results are derived from a Wilcoxon rank-sum test performed in the human or mouse dataset, the column “dataset” signifies whether the test was performed on the full lung dataset or on the subset of bona fide pulmonary ECs. Each gene is annotated whether it was identified as pan-endothelial, pan-vascular or lymphatic conserved marker gene or a conserved marker gene of the bona fide pulmonary EC subvarieties. If the test was performed on the human dataset, the column “Gene.name.mouse” represents the translated homologue if a one-to-one homologue in the respective other species was available. If there is none one-to-one homologue, it was flagged as “NA” in the “Gene.name.mouse” column. Vice versa, a gene name translation was performed to human genes, when the test was performed on the mouse dataset. “pct.1” represents the fraction of cells within a specific cell type expressing a specific gene; “pct.2” represents the fraction of cells outside a specific cell type expressing a specific gene.