Early View

Original article

Transcriptomic analysis of CFTR-impaired endothelial cells reveals a pro-inflammatory phenotype

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Transcriptomic analysis of CFTR-impaired endothelial cells reveals a pro-inflammatory phenotype

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Take-Home message (249/256 characters): CFTR-impaired endothelial cells (ECs) revealed a pro-inflammatory phenotype which can attract and reinforce leukocyte extravasation. Our study suggests that ECs possibly contribute to the excessive inflammatory phenotype observed in cystic fibrosis.

Abstract (250/250 words)

Cystic fibrosis (CF) is a life-threatening disorder characterized by decreased pulmonary mucociliary and pathogen clearance, and an exaggerated inflammatory response leading to progressive lung damage. CF is caused by bi-allelic pathogenic variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes a chloride channel. CFTR is expressed in endothelial cells (ECs) and EC dysfunction has been reported in CF patients, but a role for this ion channel in CF disease progression is poorly described.

We used an unbiased RNA sequencing approach in complementary models of CFTR silencing and blockade (by the CFTR inhibitor CFTRinh-(172)) in human ECs to characterize the changes upon CFTR impairment. Key findings were further validated *in vitro*, *in vivo* in CFTR knock-out mice and *ex vivo* in CF patient-derived ECs.

Both models of CFTR impairment revealed that EC proliferation, migration and autophagy were downregulated. Remarkably though, defective CFTR function led to EC activation and a persisting pro-inflammatory state of the endothelium with increased leukocyte adhesion. Further validation in CFTR knock-out mice revealed enhanced leukocyte extravasation in lung and liver parenchyma associated with increased levels of EC activation markers. In addition, CF patient-derived ECs displayed increased EC activation markers and leukocyte adhesion, which was partially rescued by using CFTR modulators VX770-VX809.

Our integrated analysis thus suggests that ECs are no innocent bystanders in CF pathology, but rather may contribute to the exaggerated inflammatory phenotype, raising the question whether normalization of vascular inflammation might be a novel therapeutic strategy to ameliorate the disease severity of CF.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by impaired function of the CFTR protein, which primarily acts as a chloride channel, mainly in epithelial cells ¹. CF is a multi-systemic disorder characterized by progressive lung disease and pancreatic insufficiency, while also featuring conditions of gastrointestinal involvement, liver disease and diabetes ². Lung disease determines much of the morbidity and mortality in CF patients ³. While the CF lung phenotype is considered to be primarily caused by infections ³, the subsequent inflammation is simultaneously exaggerated and ineffective at eradicating pathogens, initiating a vicious cycle of infections and inflammation ⁴. Persistent high-intensity inflammation inflicts permanent structural damage to the airways and impairs lung function, which ultimately results in respiratory failure and death ^{3,4}.

Endothelial cells (ECs) line the inner wall of blood vessels ⁵. These cells regulate blood clotting, inflammation, angiogenesis and vascular tone ⁶. Although CFTR is expressed and functional in normal ECs ^{7,8}, the vascular role of CFTR is less well studied in comparison to its well established role in epithelial cells. Yet, micro- and macrovascular dysfunction have been documented in CF patients ^{9,10}. Endothelial dysfunction has previously been described to be involved in CF-associated manifestations including diabetes, cardiovascular, lung and liver-associated complications ^{11,12}. Nevertheless, the underlying mechanism of EC dysfunction, and its role in disease progression remain elusive ^{11,12}. Interestingly, as endothelial dysfunction is a predictor of cardiovascular risk, genome wide association studies detected a link between single-nucleotide polymorphisms in *CFTR* and coronary artery disease and flow mediated arterial dilation ^{13,14}. Elucidating the role of CFTR in ECs is therefore relevant for understanding the pathophysiology of CF and cardiovascular diseases ^{13,14}. We thus employed an unbiased transcriptomics approach complemented with functional characterization of EC properties upon CFTR impairment, and studied the possible relevance of our findings for CF patients.

Materials & Methods

Details regarding materials and methods are provided in the supplementary data available online on the ERJ website.

Cell culture

Freshly isolated human umbilical vein endothelial cells (HUVECs) were obtained from different donors and used as single-donor cultures between passage 1 and 4 as previously described^{15,16}. Blood outgrowth endothelial cells (BOECs) were freshly isolated from peripheral blood obtained from different healthy donors and CF patients from the outpatient clinic in their usual state of health without intercurrent infections (clinical information is shown in supplementary table S1) with minor modifications from previously described protocols^{17,18}.

In vivo mouse assays

Leukocyte infiltration into lung and liver was analysed as previously described with minor adaptations²⁴. Briefly, Cfr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J (CFTR^{KO}) mice^{19,20} were perfused with PBS and organs were collected for immunostaining (CD45 and CD105) or digested for flow cytometry analysis (CD45, CD31, ICAM1 and VCAM1).

Bulk RNA sequencing and analysis

RNA extracted with TRIzol was subjected to sequencing library preparation with the Lexogen QuantSeq 3' mRNA-Seq Library prep kit. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies, Leuven, Belgium) with the DNA 1000 kit (Agilent Technologies, Leuven, Belgium). Libraries (2 nM) were sequenced on an Illumina HiSeq4000 platform. Single-end reads of 50 bp length were produced with a minimum of 1M

reads per sample. Quality control of raw reads was performed with FastQC v0.11.7.

²¹.

Immunoblotting

Protein extraction and immunoblot analysis were performed using a modified Laemmli sample buffer (125 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 10% glycerol) in the presence of protease and phosphatase inhibitors (Roche, Anderlecht, Belgium). Mitochondrial isolation was performed using the mitochondria isolation kit for cultured cells according to the manufacturer's instructions (Thermo Fisher Scientific, Geel, Belgium). Lysates were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and analysed by immunoblotting.

In vitro functional assays

Endothelial proliferation, LDH viability assay, scratch wound, trans-endothelial electrical resistance (TEER) and leukocyte adhesion assays were performed as described previously ^{22–25}.

Intracellular and Mitochondrial ROS Analysis

Intracellular and mitochondrial ROS levels were measured using CM-H₂DCFDA (Thermo Fisher Scientific) and MitoSOX (Thermo Fisher Scientific) according to the manufacturer's instructions, and quantified as previously described ²⁶.

Oxygen Consumption Rate (OCR)

Bioenergetics of ECs were determined on a Seahorse XF24 instrument (Agilent, Diegem, Belgium) as previously described ²⁷.

Detection of Glutathione Species and NADPH

Targeted metabolites were measured as previously described ²⁷.

Quantification and statistical analysis

Data represent mean $\pm$ SEM of at least three independent experiments. Statistical significance was calculated by standard two-tailed t-test (with Welch's correction when variances were significantly different between groups), ANOVA (for multiple comparisons within one dataset), one-sample t-test (for comparisons to point normalized data) using Prism v8.2. Bio-informatic analysis was carried out by using the in-house developed BIOMEX software ²⁸. p-value < 0.05 was considered as statistically significant.

Results

CFTR impairment leads to transcriptomic alterations in ECs

We first confirmed earlier findings ^{7,8} of detectable CFTR expression in cultured primary human umbilical vein ECs (HUVECs; hereafter referred as ECs) at the protein level via immunoblotting and immunocytochemistry (figure 1a-b). In many cases, CFTR mutations result in lower levels of functional CFTR protein in the plasma membrane ²⁹. To study CFTR's function, we therefore used two complementary models to mimic this situation: (i) we blocked CFTR activity by using the allosteric CFTR inhibitor CFTRinh-(172) which is commonly used in CF research ³⁰, and (ii) silenced CFTR expression by using lentiviral-mediated expression of two non-overlapping CFTR-specific short hairpin RNAs (shRNAs) to obtain a CFTR knock-down (CFTR^{KD}). Together, we refer to CFTR-blocked or -silenced ECs as "CFTR-impaired" ECs.

CFTR^{KD} lowered CFTR protein levels by more than 55% (figure 1a-c, supplementary figure S1A) without altering protein expression of typical endothelial markers VWF and VE-cadherin (supplementary figure S1b). While CFTR-blockade or silencing only minimally affected EC survival (supplementary figure S1c-d), increasing CFTR^{KD} efficiency (>95% at the protein level) increased cell death (>50%,

supplementary figure S1d-e). We therefore performed further experiments at an intermediate silencing depth.

We then carried out an unbiased transcriptomics analysis to screen for global changes induced upon CFTR impairment. Principal component analysis of all genes and hierarchical clustering analysis of the highly variable genes revealed that control and CFTR-impaired ECs grouped into distinct clusters, suggesting broad transcriptomic changes (figure 1d-e, supplementary figure S2a-b). Gene set enrichment analysis (GSEA) comparing control *versus* CFTR-impaired ECs was used to associate groups of significant differentially expressed genes to biological processes (supplementary table S2)³¹. Upon CFTR impairment, GSEA revealed increased expression of pro-inflammatory, ion transmembrane transport and hypoxia-related pathways, while gene sets involved in proliferation (cell cycle), cell migration and cell adhesion were downregulated (figure 1f, supplementary figure S2c, supplementary table S2).

We then set out to obtain congruent markers, conserved across CFTR blockade and silencing, hypothesizing that congruent pathways are related to the chloride channel function of CFTR whose malfunction is shared between all CF patients. Interestingly, congruent upregulated gene sets suggested a role in EC activation (TNF α , STAT3, HIF1 α , IL-2 & IL-6-related pathways), antigen binding and processing (HLA class I, transporters associated with antigen processing (TAPs) and cathepsins), matrisome remodelling, ion transmembrane transport, while congruent downregulated gene sets implied a possible role in the cell cycle, migration and adhesion (supplementary figure S2d, supplementary table S3). We confirmed these findings by performing a meta-analysis of control *versus* CFTR-impaired ECs to rank differentially regulated genes and gene sets (figure 1g, supplementary table S3). Among the top 20 ranking genes were *CD9*, *CYR61*, *CXCL1* and *CCL2*, which have been linked to leukocyte recruitment to the endothelial membrane and trans-endothelial leukocyte migration^{32–35} (supplementary table S3). Furthermore, gene sets involved in EC activation, STAT3 signalling and H⁺ transmembrane transport

ranked in the top three upregulated gene sets, while gene sets involved in the cell cycle and cell migration ranked as the top three downregulated gene sets (figure 1g, supplementary table S3). Taken together, CFTR impairment induced a prominent pro-inflammatory and EC activation gene signature, accompanied by a downregulated gene signature of EC migration and proliferation.

Several key EC functions are malfunctioning upon CFTR impairment

To investigate in more detail the compromised EC proliferation transcriptome signature upon CFTR silencing, we compared the expression of genes involved in various phases of the cell cycle. Genes coding for proliferation markers, transcriptional regulators, cyclins and cyclin-dependent kinases were downregulated upon CFTR impairment (figure 2a). These transcriptomics findings were confirmed by a decrease in EC proliferation as measured by tritium-labelled thymidine incorporation into DNA upon CFTR impairment (figure 2b).

The transcriptomics analysis further revealed that CFTR silencing downregulated the expression of genes involved in integrin and VEGF signalling, and actin remodelling, suggesting a novel role of CFTR in EC migration (figure 2c). In agreement, the scratch wound assay confirmed reduced migration of CFTR-impaired ECs (figure 2d). Interestingly, overexpression of an exogenous HA-tagged CFTR in ECs (to facilitate visualization) followed by immunostaining for the HA-tag revealed that CFTR was primarily expressed and enriched in membrane ruffling structures (e.g. lamellipodia) of migrating ECs (supplementary figure S3a), further supporting a role in EC migration. Confluent monolayers of CFTR^{KD} ECs displayed compromised junctional integrity as measured by higher proportion of discontinuous and reticulated VE-cadherin and CD31 junctions ^{18,36,37} (figure 2e-f, supplementary figure S3b-c). Functionally, this corresponded to a decrease in the TEER of CFTR-impaired ECs (figure 2g), which is in line with previously published findings on CF in ECs ^{38,39}. Our data thus indicate an underappreciated role for CFTR in EC proliferation and migration, and to maintain endothelial barrier integrity.

CFTR impairment induces oxidative stress and mitochondrial dysfunction

Increased oxidative stress is a well-known and noxious hallmark of CF pathophysiology⁴⁰. CFTR silencing led to the induction of several gene classes related to an anti-oxidative stress response, including transcription factors capable of binding antioxidant response elements (ARE), glutathione-consuming enzymes, superoxide dismutase, peroxiredoxin, thioredoxin, DNA repair and chaperone proteins (figure 3a). We confirmed these transcriptomic changes by flow cytometry analysis of total cellular oxidative stress, which indeed revealed increased oxidative stress upon CFTR impairment (figure 3b). Furthermore, when key cellular antioxidant scavengers were measured by LC-MS, both glutathione (GSH) as well as its reducing co-factor NADPH were affected as indicated by the increased ratios of oxidized/total GSH and NADP⁺/NADPH (figure 3c-d). Interestingly, the mRNA transcript levels of glutamate-cysteine ligase (GCLC), the rate controlling enzyme in GSH biosynthesis, were unchanged in CFTR-silenced ECs (supplementary figure S4a), suggesting increased GSH consumption rather than reduced GSH synthesis contributed to the elevated oxidative stress.

Measurements of mitochondrial reactive oxygen species (ROS) using the MitoSOX dye by flow cytometry revealed that upon CFTR-impairment, mitochondrial O²⁻ levels were significantly elevated (figure 3e). This was associated with decreased mitochondrial respiration as reflected by a reduced basal oxygen consumption rate (OCR_{BASAL}), ATP production (OCR_{ATP}) and maximal oxygen consumption rate (OCR_{MAX}) (figure 3f-g), suggesting mitochondriopathy. The clearance of damaged mitochondria through autophagy, a process called mitophagy, is vital for cellular functioning and survival⁴¹. Immunoblotting revealed increased mitochondrial levels of DRP1 and Parkin (mediating mitochondrial fission), but reduced levels of OPA1 (mediating mitochondrial fusion), indicative of increased mitochondrial fission but reduced fusion (figure 3h-k). Together, CFTR deficiency induces an oxidative stress response, more damaged mitochondria with defective mitochondrial function.

Autophagy deficiency in CFTR-impaired ECs

Differential gene expression analysis of our transcriptomic data revealed that autophagy markers p62 (*SQSTM1*) and LC3B (*MAP1LC3B*) were elevated upon CFTR silencing (figure 4a). Real time qPCR analysis of these marker genes further confirmed these findings (figure 4b). Corroboratively, the protein levels of the autophagic substrate p62, and autophagosome markers LC3BI & LC3BII were also increased (figure 4c-f), suggesting an accumulation of autophagosomes and a defect in autophagy flux rather than increased functional autophagy. To validate our findings, we performed transmission electron microscopy (TEM) which highlighted a two-fold increase in the autophagosomes abundance upon CFTR impairment, confirming defective autophagy (figure 4g, supplementary figure S4b). CFTR^{KD} ECs displayed up-regulated mTORC1 activity (a known inhibitor of autophagosome formation and lysosome biogenesis ⁴²) as measured by the increased phosphorylation of its downstream canonical target P70S6K and its substrate S6 (figure 4h). Our results suggest that CFTR impairment may hinder endothelial autophagy through mTORC1 activation and autophagosome accumulation.

CFTR impairment induces EC activation

Differential expression analysis in control *versus* CFTR^{KD} ECs revealed a pro-inflammatory phenotype with *CXCL1*, *CCL2* and *ICAM1* ranking as the top three up-regulated genes in CFTR^{KD} cells (supplementary table S4). Notably, CFTR-silenced ECs upregulated transcripts of various pro-inflammatory genes, adhesion molecules and TNF-related signalling (figure 5a). In baseline conditions and upon pro-inflammatory stimulus (LPS challenge), CFTR^{KD} ECs had a more pronounced pro-inflammatory phenotype than control cells, as *VCAM1*, *ICAM1*, *SELE* (E-selectin) and *IL8* expression levels were elevated. Notably, while LPS stimulation effectively increased activation markers' expression in control ECs, it had no further effect on their already increased expression in CFTR^{KD} ECs (figure 5b). Emerging evidence

highlights that a loss in autophagy could contribute to EC activation ⁴³. However, rescuing the pro-inflammatory phenotype with rapamycin (a mTORC1 inhibitor inducing autophagy) did not alter the expression levels of both conditions (figure S4c). We hypothesized that increased expression of adhesion molecules and chemokines by ECs could result in more attraction and binding of leukocytes onto the EC surface. Therefore, we performed an *in vitro* leukocyte adhesion assay and confirmed that, compared to control ECs, more leukocytes adhered to the surface of CFTR-impaired ECs at both baseline and after LPS stimulation (figure 5c-d).

We then investigated the role of CFTR in a bi-transgenic mouse model of CFTR harbouring a targeted knock-out (CFTR^{KO}) mutation of CFTR and expressing the human CFTR transgene under the expression of the *FABP1* promoter (rescuing the lethal intestinal occlusion phenotype occurring at birth ²⁰). Immunostaining of CD45, a marker of leukocytes, showed increased leukocyte infiltration into the lung and liver parenchyma of adult CFTR^{KO} mice compared to wildtype littermates (figure 6a-b). Flow cytometry measurements of single cell lung suspensions further confirmed a heightened CD45⁺ cell fraction, and an increased expression of VCAM1 and ICAM1 in viable CD31⁺CD45⁻ cells isolated from CFTR^{KO} mice (figure 6c-e, supplementary figure S4d). Our data thus suggests that across different model organisms, CFTR impairment leads to a pro-inflammatory phenotype in ECs, which can attract leukocytes and reinforce their extravasation across the endothelium.

CFTR mutations cause a pro-inflammatory phenotype in CF patient-derived ECs

In the clinical context that patients with CF can suffer sustained periods of inflammation with an exaggerated immune response ⁴⁴, we validated our findings in an *ex vivo* model using blood outgrowth endothelial cells (BOECs), cells derived from circulating EC progenitors that express typical EC markers ⁴⁵. BOECs were cultured from peripheral blood taken from CF patients with severe mutations (Class I-III, patients' clinical information is shown in supplementary table S1), and retained endothelial

features when cultured *in vitro* (figure 7a, supplementary figure S4e). Confirming our previous results in HUVECs, wound closure and barrier integrity were also impaired in CF patient-derived BOECs (figure 7b-c). Similar to CFTR^{KD} ECs, CF patient-derived BOECs displayed increased discontinuous and reticulated VE-cadherin junctions as compared to BOECs from healthy donors (figure 7d-f). Such reticulated junctions were suggested to be important in controlling leukocyte transmigration^{37,46,47}, corroborating the role of CFTR in barrier integrity and inflammation. Furthermore, mRNA levels of the pro-inflammatory *ICAM1*, *SELE* and *IL-8* were increased in BOECs from CF patients as compared to healthy donors which was mirrored in the increased adherence of leukocytes to CF patient-derived ECs (figure 7g-h). These results correlate with our earlier findings, where CFTR loss of function in our models resulted in a pro-inflammatory phenotype in ECs. More importantly, we found that rescuing CFTR function with the combination of CFTR-modulators VX-770 and VX-809 (Orkambi®) was also able to partially rescue the pro-inflammatory gene expression signature, and significantly diminished the leukocyte adhesion onto CF patient-derived ECs (figure 7h-i).

Discussion

Inflammation plays a major role in CF pathophysiology affecting mainly the lungs but also other organs such as the liver, intestines and pancreas². The origin of the inflammation in CF-affected organs is a topic of debate as it is yet unclear whether it is of primary or secondary origin^{4,48}. A pro-inflammatory phenotype has already been observed in different cell types independent of pro-inflammatory challenges^{49–51}. For instance, this has been demonstrated in HUVECs treated with CFTR inhibitor which produced more IL-8^{39,52}. Our bulk transcriptomic data revealed an upregulated expression of multiple pro-inflammatory genes in CFTR-impaired ECs. *In vitro* upon CFTR impairment and *ex vivo* in CF patient-derived ECs, the expression of several EC pro-inflammatory genes (*ICAM1*, *IL-8* and *SELE*), which are essential molecules

for leukocyte adhesion and diapedesis, was upregulated. This was mirrored by increased leukocyte adhesion to CFTR-impaired ECs. These findings confirm that CFTR dysfunction by itself leads to a pro-inflammatory phenotype even in the absence of a concomitant infection. CFTR-modulator treatment was already successfully used at rescuing CFTR function in other *in vitro* systems, even showing clinical correlation to patient response^{53,54}. Accordingly, the combination treatment of VX-770 + VX-809 (Orkambi®) was able to partially rescue the pro-inflammatory phenotype of CF patient-derived ECs as measured by inflammatory marker gene expression and leukocyte adhesion. Also, our results are in accordance with earlier findings in CF patients where increased soluble EC activation molecules were detected in the plasma and serum, and correlating in some cases, with impaired lung function^{55–60}. Additionally, in the gut-corrected CFTR^{KO} mouse model, microscopic quantifications revealed more leukocyte infiltration in lung and liver, together with increased expression of leukocyte adhesion molecules in lung ECs. Despite the fact that these CFTR^{KO} mice do not display the inflammatory lung phenotype^{19,61}, they do show signs of subsymptomatic inflammation, for which the vasculature could thus at least be partly responsible by promoting leukocyte adhesion and infiltration. Hence, CFTR impairment leads to an inflammatory EC phenotype with increased activation and leukocyte extravasation, highlighting the potential, but overlooked, role of the endothelium as a mediator in the inflammatory process in CF. In support of a role of ECs in immune regulation, we recently observed that freshly isolated tumour ECs from human lung cancers expressed a transcriptome signature associated with diverse immune functions, suggesting a more important function of disease ECs in immune surveillance than previously anticipated⁶².

We propose three potential interlinked underlying mechanisms to give rise to this increase in inflammation: (i) defective autophagy, (ii) increased oxidative stress and (iii) mitochondrial dysfunction (figure 8). Our study provides several lines of evidence that autophagy might be defective in ECs with impaired CFTR function. We

observed increased protein levels of autophagy marker LC3BI, thus confirming autophagy induction. However, analysis of autophagic flux using LC3BII and TEM images revealed autophagosome accumulation, indicating decreased autophagic flux due to defective autophagosome maturation and/or autolysosome function. Although an autophagy impairment has been reported in CF-deficient epithelial cells and macrophages, in which accumulation of p62 is documented together with a decrease in LC3BII and autophagosomes ^{63,64}, our mechanism seems to be distinct as it is characterized by an accumulation of autophagosomes. Autophagy is a highly dynamic process with several components and mediators involved including mTORC1, a key regulator of autophagosome formation and lysosome biogenesis ⁶⁵. We observed increased mTORC1 activity in CFTR^{KD} ECs, suggesting inhibition of autophagy. Indeed, a prominent function of mTORC1 is to restrict autophagy ⁴², however reactivation of mTORC1 is required for the termination of autophagy ⁶⁶. Defective autophagy has already been associated with a pro-inflammatory phenotype in different cell types including ECs ^{67,68} and could contribute to the pro-inflammatory phenotype in CF. In addition, it is already well established that impaired cellular proteostasis and autophagy are involved in the pathogenesis of CF ⁶⁹. In fact, correcting the underlying proteostasis and autophagy defect has emerged as a novel intervention strategy in CF ⁶⁹.

Increased oxidative stress is another hallmark of CF pathophysiology ⁴⁰. We observed increased oxidative stress in CFTR-impaired ECs, together with a striking transcriptomic signature where multiple antioxidant-related genes were upregulated. Although our data revealed that the rate controlling enzyme for glutathione synthesis (a key intracellular antioxidant) was unchanged upon CFTR silencing, GSSG and NADP⁺ levels were increased suggesting increased oxidative stress. Our findings confirm earlier studies in CF patients that documented heightened vascular oxidative stress, probably scavenging the vasodilator nitric oxide (NO), leading to reduced NO bioavailability, EC activation and dysfunction ^{70–72}. The role of excessive ROS levels

in the respiratory system has mostly been characterized in connection with chronic pulmonary infections and persistent inflammation ⁴⁰. Additionally, low GSH levels were found in plasma and blood neutrophils from CF patients ^{73,74} and systemic GSH dyshomeostasis has already been suggested in CF ⁴⁰. Because of the heightened oxidative stress in CF patients, CF research also explored the potential of antioxidant treatment ^{40,75}. Recently, an antioxidant cocktail treatment in CF patients improved both vascular and lung function ⁷¹. Although these results are promising, the beneficial effect of antioxidant therapy remains difficult to evaluate in CF patients with chronic infections on intensive antibiotic treatments and warrants a large scale, long-term study ⁷⁵.

Mitochondria can be ROS-producing organelles and CFTR function is necessary for optimal mitochondrial function ^{76,77}. Consistently, we found increased mitochondrial ROS in CFTR-impaired ECs. A previous study in human bronchial epithelial cells reported that oxygen consumption and both mitochondrial complex I and IV activities were impaired in CF epithelial cells, concomitantly associated with an increased mitochondrial ROS production and membrane lipid peroxidation ⁷⁸. We confirmed these findings in ECs as both basal and maximal respiration as well as mitochondrial ATP production were decreased in CFTR-impaired ECs. Currently, the molecular mechanisms explaining how CFTR impairment affects so many parameters of mitochondrial function remain unknown ⁷⁸. In the absence of efficient removal of damaged mitochondria (mitophagy), it would be expected that ROS levels rise. Our observations of increased mitochondrial fission and impaired mitophagy suggest that increased mitochondrial damage is at least partly responsible for the observed mitochondrial phenotype. However, we cannot conclude which mechanism is initiating the mitochondriopathy: (mitochondrial) ROS inducing autophagy/mitophagy or vice versa (figure 8). The process of autophagy/mitophagy in ECs might be particularly important in CF since impairment of this pathway contributes to inflammasome activation ^{67,79}.

From our transcriptomics data, we observed downregulation of essential marker genes for proliferation, as well as cyclins and cyclin-dependent kinases ⁸⁰, consistent with our functional validation *in vitro*. The mechanisms of how chloride fluxes are affecting interactions between cyclins and cyclin-dependent kinases, thereby regulating cell cycle, are poorly characterized, especially in ECs. Although interesting, how and if an EC proliferation defect might contribute to CF disease progression is currently unknown.

EC migration is a regulated multistep process involved in tissue formation, wound healing and regeneration ⁸¹. Upon CFTR impairment, ECs exhibited decreased migration which is in accordance with their transcriptomic signature. We also obtained evidence that CFTR is expressed at the lamellipodia of migrating ECs and therefore is possibly involved in EC polarization and migration. Our findings are consistent with reports that CFTR regulates lamellipodia protrusion and cell migration in non-EC types ^{82,83}. It is becoming increasingly obvious that CFTR regulates cell migration across a diverse range of cell types, however the underlying mechanisms remain to be further uncovered, since delayed wound repair may augment inflammation in CF ⁸².

The endothelium also functions as a semi-permeable barrier where EC junctions dynamically open to allow the passage of ions, nutrients and inflammatory cells for tissue homeostasis and immune surveillance. In accordance with published literature ^{38,39}, we also observed increased membrane permeability in CFTR^{KD} cells and CF-patient derived ECs. This was probably caused by the larger amount of discontinuous junctions present when CFTR was impaired. More interestingly, CFTR^{KD} cells and CF-patient derived ECs displayed higher fractions of reticulated adherens junctions. Such reticulated junctions were previously correlated to be important in controlling leukocyte transmigration ^{37,46,47}. This reinforces our hypothesis that CFTR impairment is associated with a pro-inflammatory and leaky barrier which could pos-

sibly contribute to the excessive leukocyte extravasation observed in the lung of CF patients⁸⁴.

Our study also has limitations and outstanding questions. For instance, how does CFTR, a protein in the plasma membrane that regulates ion conductivity, regulates diverse processes such as endothelial proliferation, migration, autophagy and inflammation. CFTR is a well-known promiscuous protein which also functions as a protein hub for scaffolding proteins at the plasma membrane and in the Golgi apparatus^{85–87}. Protein-protein interactome analysis revealed that CFTR is involved in a wide range of processes (including proteostasis, cytoskeleton remodelling, immune response, ROS signalling, metabolism and proliferation), highlighting the complex underlying regulatory network⁸⁸. Limitations to our study are the low endogenous CFTR levels in ECs, making its detection challenging, and the fact that we had to use a limited CFTR^{KD} efficiency in order to avoid cellular toxicity at higher CFTR^{KD} efficiency. At the same time, this incomplete CFTR^{KD} sufficed to induce the observed vascular inflammation and other phenotypes, indicating its physiological relevance. CF carriers (50% CFTR expression) were thought to be sufficient for maintaining health^{89,90}, however, it was recently observed that CF carriers also have an increased risk for a wide range of CF-related conditions⁹¹. Thus, studying and comparing carrier- *versus* CF patient-derived ECs would give insightful information. It is well established that the gut corrected CFTR^{KO} mice are not an ideal model organism to study CF since it does not accurately reflect the human phenotype^{19,61}. Since CFTR is absent in almost all cell types, the influence of other cell types could also have an effect on the increased leukocyte extravasation found in murine CF lungs. However, endothelial leukocyte adhesion markers were increased in murine CFTR^{KO} lung ECs suggesting that the endothelium could partially mediate the excessive extravasation. Obtaining primary ECs from CF patients is challenging. We therefore used BOECs as an alternative, though it remains to be studied in how much these cells reflect *in situ* organ-derived ECs from CF patients. Nevertheless, we observed

a similar pro-inflammatory signature in BOECs from CF patients as in ECs with impaired CFTR function.

In conclusion, our findings provide new evidence that EC function is altered upon CFTR impairment with defective cell proliferation, migration and autophagy. Remarkably, altered CFTR expression led to EC activation and a pro-inflammatory state increasing leukocyte adhesion and extravasation *in vitro*, *in vivo* and *ex vivo*. Our data raise the question whether ECs should no longer be considered passive bystanders in CF pathology, but in fact could actively co-determine the exaggerated inflammatory response. Hence, our study may also provide an incentive to consider EC normalization as a new therapeutic approach to reduce the pro-inflammatory status of ECs and thereby improve the quality of life of CF patients.

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Author Contributions

MDeclercq, LT, PC, DC and PW designed research. MDeclercq, LT, PDZ, MGC, KB, SV and BG performed research. PDZ, NVC, VG, ME, MC, AR, MB, MP, FV, LD, BG, GE and MDewerchin contributed vital new reagents or analytic tools. MDeclercq, LT, PC and PW analysed data. MDeclercq, LT, PC and PW wrote the paper. All authors discussed the results and commented on the manuscript.

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Disclosure of Conflicts of Interest

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1: Transcriptomic signature of CFTR-silenced endothelial cells

a) Representative immunoblot for CFTR expression in ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA (CFTR^{KD}). Bands B and C indicate immature (native) and mature (glycosylated) CFTR respectively. GAPDH was used as loading control. Densitometric quantification of the ratio of CFTR to GAPDH is shown on the right. Mean + SEM; n=10; ***p<0.001 by one-sample *t*-test. **b)** Representative confocal images of ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA

(CFTR^{KD}) stained for CFTR (red), actin (green) and nuclei (Hoechst; blue). Scale bar: 100 μ m. **c)** Fluorescence quantification of CFTR of ECs transduced with scrambled shRNA (Ctrl) *versus* silenced CFTR (CFTR^{KD}). Mean + SEM; n=3; *p<0.05 by one-sample *t*-test. AU, arbitrary units. **d)** Principal component analysis (PCA) on all genes of Ctrl and CFTR^{KD} ECs obtained after bulk RNA sequencing. **e)** Correlation heatmap of the highly variable genes in Ctrl and CFTR^{KD} ECs. Colour scale: red, high correlation; blue, low correlation. **f)** Barplots representing the top deregulated gene sets (with a significant adjusted p-value) ranked based on their normalized enrichment score. Upregulated gene sets induced upon CFTR^{KD} are shown in red; downregulated genes sets are in blue. For official gene set names see supplementary table 2. Numbers between parentheses indicate alternative gene sets pertaining to the same biological function or signalling pathway. **g)** Violin plots representing top (dots) and low (diamonds) ranking gene sets obtained after meta-analysis of Ctrl *versus* CFTR-impaired ECs (CFTR^{KD} and CFTR(inh)-172-treated, CFTR_{inh}). Symbols indicate where gene sets are located in the distribution.

Figure 2: Endothelial proliferation and migration are reduced upon CFTR impairment

a) Heatmap showing the expression levels of differentially expressed genes between ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA (CFTR^{KD}). Selected genes are involved in cell proliferation and have adjusted p-values<0.05. **b)** [³H]-thymidine incorporation into DNA (proliferation assay) in ECs transduced with scrambled shRNA (Ctrl) *versus* silenced CFTR (CFTR^{KD}, red, top panel, n=10), or treated with the CFTR(inh)-172 (CFTR_{inh}, yellow, lower panel, n=6) *versus* the corresponding DMSO-treated control (Ctrl). Mean + SEM; ***p<0.001; by two-tailed paired *t*-test. **c)** Heatmap showing differentially expressed genes important in cell migration between Ctrl and CFTR^{KD} ECs. Selected genes have adjusted p-values<0.05. **d)** Scratch wound migration assay with Ctrl and CFTR-impaired ECs (CFTR^{KD}, n=8 or CFTR_{inh}, n=7). Mean + SEM; *p<0.05; **p<0.01 by two-tailed paired *t*-test. **e-f)** Quan-

tification of the fraction of discontinuous and continuous VE-cadherin-stained junctions (n=4) (e), and the fraction of reticulated VE-cadherin junctions in Ctrl and CFTR^{KD} ECs (n=5) (f). Mean + SEM; *p<0.05; **p<0.01 by two-tailed unpaired *t*-test. **g)** Trans-endothelial electrical resistance (TEER) assay with Ctrl and CFTR-impaired ECs (CFTR^{KD}, n=3 or CFTR_{inh}, n=3). Mean ± SEM; **p<0.01; ***p<0.001 by two way ANOVA.

Figure 3: CFTR defect in endothelial cells induces oxidative stress and mitochondrial dysfunction

a) Heatmap showing the expression levels of differentially expressed genes between ECs transduced with scrambled shRNA (Ctrl) or CFTR^{KD}. ARE: transcription factors binding antioxidant response elements; glutathione: glutathione consuming enzymes. Selected genes are involved in cell response to oxidative stress and have adjusted p-values<0.05. **b-g)** ECs were transduced with Ctrl shRNA and compared to silenced CFTR cells (CFTR^{KD}, red, top panel), or treated with the CFTR(inh)-172 (CFTR_{inh}, yellow, lower panel) *versus* DMSO-treated control (Ctrl). Intracellular ROS levels (CM-H₂DCFDA, measured by flow cytometry and expressed in arbitrary units (AU)) (b), GSSG/(GSH+GSSG) ratios (c) and NADP⁺/NADPH ratios (d) (measured by LC-MS and normalized to protein content). Mean + SEM; CFTR^{KD} n=4; CFTR_{inh} n=4; *p<0.05; **p<0.01 by one-sample *t*-test. Mitochondrial ROS levels, measured by flow cytometry after incubation with the MitoSOX dye (e). Mean + SEM; CFTR^{KD} n=3; CFTR_{inh} n=6; **p<0.01 by two-tailed paired *t*-test. Basal, ATP-producing and maximal oxygen consumption rates (OCR) are measured by Seahorse after Oligomycin (Oligo), FCCP and Antimycin A (AA) treatments (f-g). Mean + SEM; CFTR^{KD} n=4; CFTR_{inh} n=4; *p<0.05; **p<0.01 by one-sample *t*-test. **h)** Representative immunoblots for DRP1 (mitochondrial fission), OPA1 (mitochondrial fusion) and PARKIN (mitophagy) in Ctrl or CFTR^{KD} ECs and subjected to mitochondrial (mito.) and cytoplasmic (cyto.) fractionation. VDAC1 was used as loading control for mitochondrial fraction, while GAPDH was used for the cytoplasmic fraction. Densitometric

quantifications are shown in **i-k**). Mean + SEM; n=5; *p<0.05 by one-sample *t*-test.

Figure 4: Autophagy defect in CFTR-impaired ECs

a) Boxplots showing normalized expression levels of *SQSTM* and *MAP1LC3B* (encoding p62 and LC3B, respectively) in ECs transduced with scrambled shRNA (Ctrl) and CFTR shRNA (CFTR^{KD}). n=6; ***adjusted p<0.001 by two-tailed paired *t*-test. **b)** qRT-PCR analysis of *SQSTM* and *MAP1LC3B* expression levels in ECs transduced with scrambled shRNA (Ctrl), CFTR shRNA (CFTR^{KD}, red), and treated with CFTR(inh)-172 (CFTR_{inh}, yellow) *versus* the corresponding DMSO control (Ctrl). Individual data points with median; CFTR^{KD} n=4; CFTR_{inh} n=4; *p<0.05; **p<0.01 by two-tailed paired *t*-test. **c-f)** Representative immunoblots (c) and corresponding densitometric quantifications (d,f) for p62 and LC3BI/II in Ctrl, CFTR^{KD} or CFTR_{inh} ECs. GAPDH was used as loading control. Mean + SEM; CFTR^{KD} n=4; CFTR_{inh} n=8; *p<0.05; **p<0.01; ***p<0.001 by one-sample *t*-test. **g)** Representative transmission electron microscopy of cultured Ctrl *versus* CFTR^{KD} ECs indicate an accumulation of autophagosomes (white arrowheads). Scale bars: 500 nm. Quantification of autophagosome area is presented in the right panels for EC upon CFTR^{KD} or CFTR_{inh}. Mean + SEM; CFTR^{KD} n=3; CFTR_{inh} n=4; *p<0.05; **p<0.01 by two-tailed paired *t*-test. **h)** Representative immunoblots and corresponding densitometric quantifications for p-P70S6K, P70S6K, p-S6 and S6 in Ctrl and CFTR^{KD} ECs. GAPDH was used as loading control. Mean + SEM; n=7; *p<0.05; **p<0.01; ***p<0.001 by one-sample *t*-test.

Figure 5: CFTR impairment induces EC activation

a) Heatmap showing the expression levels of differentially expressed genes between ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA (CFTR^{KD}). Selected genes are involved in inflammation and endothelial activation processes, and have adjusted p-values<0.05. **b)** qRT-PCR analysis of *SELE*, *IL8*, *ICAM1* and *VCAM1* expression levels in Ctrl and CFTR^{KD} ECs treated or not with LPS. Expression levels

are normalized to *HPRT1*. Individual data points with median; $n \geq 6$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA. **c-d)** Representative microscopy pictures of leukocyte adhesion assay to a monolayer of Ctrl and CFTR^{KD} (c) or Ctrl and CFTR_{inh} (d) ECs treated (+) or not (-) with LPS. Leukocytes are labelled with calcein (blue). Scale bars: 100 μ m. Quantifications of leukocytes adhering to EC monolayer are shown in the insets. Mean + SEM; CFTR^{KD} $n=4$; CFTR_{inh} $n=4$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA.

Figure 6: CFTR loss causes a pro-inflammatory phenotype *in vivo*

a-b) Representative confocal images of perfused lung (a) and liver (b) sections isolated from WT and CFTR^{KO} littermates, and stained for CD105 (vessels; green) and CD45 (leukocytes; red). Scale bars: 100 μ m. Quantifications of CD45 area are provided on the right. Mean + SEM; $n \geq 4$; ** $p < 0.01$ by two-tailed unpaired *t*-test. **c-e)** Flow cytometry analysis of WT and CFTR^{KO} mouse lung cell suspension for viable CD45⁺CD31⁻ (c), ICAM1⁺CD31⁺CD45⁻ (d) and VCAM1⁺CD31⁺CD45⁻ (e) cells. Mean + SEM; $n \geq 4$; * $p < 0.05$ by two-tailed unpaired *t*-test.

Figure 7: In CF patients, CFTR loss promotes EC dysfunction and activation

a) Representative confocal images of blood outgrowth endothelial cells (BOECs) from healthy donors (Ctrl) and CF patients stained for VE-Cadherin (green), CD31 (cyan) and vWF (red). Scale bar: 50 μ m. **b)** Scratch wound migration assay with Ctrl and CF BOECs ($n=3$). Mean + SEM; ** $p < 0.01$ by two-tailed unpaired *t*-test. **c)** Trans-endothelial electrical resistance (TEER) assay with Ctrl and CF BOECs ($n=6$). Mean $\pm$ SEM; *** $p < 0.001$ by two way ANOVA. **d-e)** Quantification of the fraction of discontinuous and continuous VE-cadherin-stained junctions (d), and the fraction of reticulated VE-cadherin junctions (e) in Ctrl and CF BOECs ($n=4$). Mean + SEM; *** $p < 0.001$ by two-tailed unpaired *t*-test. **f)** Representative confocal images of reticulated adherens junctions in Ctrl and CF patient-derived BOECs stained for VE-Cadherin. Yellow arrowheads indicate discontinuous junctions. Scale bar: 25 μ m. **g)**

qRT-PCR analysis of *ICAM1*, *SELE* and *IL8* expression levels in Ctrl and CF BOECs. Expression levels are normalized to *HPRT1*. Individual data points with median; $n \geq 6$; * $p < 0.05$ by two-tailed unpaired *t*-test. **h)** Quantifications of leukocytes adhering to Ctrl and CF BOEC monolayers, treated or not with the Orkambi CFTR modulators combination (VX-770 & VX-809) ($n=3$). Mean + SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA. **i)** qRT-PCR analysis of *ICAM1*, *SELE* and *IL8* expression levels in BOECs from CF patients treated or not with Orkambi. Expression levels are normalized to *HPRT1*. Individual data points with median; $n=4$; * $p < 0.05$; *** $p < 0.001$ by two-tailed paired *t*-test.

Figure 8: Visual abstract summarizing key events occurring in ECs upon CFTR-impairment

Upon endothelial CFTR-impairment three potential interlinked underlying mechanisms could give rise to the increase in endothelial activation and subsequent heightened inflammation: (i) increased oxidative stress and decreased antioxidant properties, (ii) defective autophagy characterized by autophagosome accumulation, and (iii) mitochondrial dysfunction. Other observed EC phenotypes included decreased viability (when CFTR expression level/activity is almost completely abolished), proliferation and wound closure, and increased membrane permeability.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Supplementary Data

Supplementary Methods

Cell Lines and Culture

Human Primary Endothelial Cells

Human umbilical vein endothelial cells (HUVECs, UZ Leuven, Leuven, Belgium) were freshly isolated from umbilical cords obtained from different donors under the ethical approval protocol S57123 (Ethics Committee Research UZ/KU Leuven) and with informed consent from all subjects, as previously described ¹. Unless specified, ECs were routinely cultured in endothelial cell basal medium (EGM2; containing 2% fetal bovine serum (FBS); PromoCell, Heidelberg, Germany) supplemented with endothelial cell growth medium supplement pack (ECGM-2; PromoCell, Heidelberg, Germany) and 100 IU/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific, Geel, Belgium). Alternatively, M199 medium (1 mg/mL D-glucose, Thermo Fisher Scientific, Geel, Belgium) supplemented with 10% FBS (Thermo Fisher Scientific, Geel, Belgium), 2 mM L-glutamine (Thermo Fisher Scientific, Geel, Belgium), Endothelial Cell Growth Supplement (ECGS)/ Heparin (PromoCell, Heidelberg, Germany), 100 IU/mL penicillin and 100 mg/mL streptomycin was used. In all experiments, HUVECs were used as single-donor cultures and were used between passage 1 and 4. All experiments were performed with ECs from different donors. Unless otherwise indicated, experiments were done on sub-confluent ECs cultures and CFTRinh-(172) (Merck, Darmstadt, Germany) was used at 10 μ M and added 24 h before cell lysis.

Blood Outgrowth Endothelial Cells

Blood outgrowth endothelial cells (BOECs) were freshly isolated from peripheral blood obtained from different healthy donors and CF patients (clinical information is shown in supplemental Table 4) under the ethical approval protocol S57123 (Ethics Committee Research UZ/KU Leuven) and with informed consent from all subjects. Our protocol is based on a published method with minor adaptations ². Briefly, six

EDTA-coated tubes (Vacutest Kima, Arzergrande, Italy) or ~54 mL of blood were collected by venipuncture per individual. Under sterile conditions, equal parts of the blood were transferred into three 50 mL conical tubes and diluted with Dulbecco's Phosphate-Buffered Saline (DPBS) (Thermo Fisher Scientific, Geel, Belgium) to a final volume of 50 mL per tube. Six new 50 mL conical tubes containing 12.5 mL of Ficoll-PaqueTM PLUS density gradient centrifugation medium (GE Healthcare, Chicago, USA) were prepared (1 tube for every 25 mL of diluted blood, 6 tubes per donor), tilted to an angle of 20° with the work surface, and 25 mL of diluted blood was slowly added on top of the medium. Afterwards, the samples were centrifuged at 1000 g for 20 min in a cooled centrifuge (4°C) with the brake off. The buffy coat layer was collected using a pipette and transferred into two new 50 mL conical tubes and washed with DPBS in a final volume of 50 mL. The tubes were centrifuged at 540 g for 7 min at 4°C with the brake on. The supernatant was discarded and the cellular pellet was resuspended in 10 mL of EGM2 supplemented with ECGM-2 containing 20% heat-inactivated embryonic stem-cell FBS (Thermo Fisher Scientific, Geel, Belgium) hereafter called BOEC medium. Cells were centrifuged for 5 min at 300g at room temperature and the cell pellet resuspended in BOEC medium. A 6-well plate was coated with a 50 µg/mL type I collagen (Collagen Type I, rat tail; Merck, Darmstadt, Germany) solution in 0.02 M acetic acid in PBS for 30 minutes, and washed twice with DPBS before adding 2 mL of the cell suspension per well. The culture plate was put in the incubator at 37°C in a 95% air/ 5% CO₂ atmosphere saturated with H₂O. The next day the BOEC medium was refreshed and hereafter every 2-3 days until colonies started to form. The checking for colonies with a cobblestone-like morphology started on day 7 onward and was performed on a daily basis. The colonies appeared between days 8 and 25 and were further grown in BOEC medium until the colony reached a 50% confluency. The cells were then trypsinized with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Geel, Belgium) and reseeded in a 0.1% gelatin-coated 25 cm² flask with full EGM2. The cells were grown until they reached 70-90% confluency and were then transferred to a 75 cm²

flask. Hereafter, BOECs were cultured in EGM2 + 15% FBS. In all experiments, BOECs were used as single-donor cultures and were used between passage 4 and 10. All experiments were performed with ECs from different donors. Unless otherwise indicated, experiments were done on sub-confluent ECs cultures. CFTR modulators VX-770 (3 μ M) and VX-809 (3 μ M) (Selleckchem, Houston, USA) were used as previously reported³⁻⁵.

CFTR Mice

Animal housing and all experimental procedures were approved by the Institutional Animal Ethics Committee of the KU Leuven (Leuven, Belgium) under the project number P036/2019. *Cftr*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice were used which re-express human CFTR in the gut under control of the FABP1 promoter, which prevents morbidity and mortality in CFTR knock-out mice due to acute intestinal obstructions^{6,7}.

Knockdown and Overexpression Strategy

cDNA for human CFTR was obtained from Origene (Rockville, USA). Lentiviral expression constructs were obtained by cloning the respective cDNAs into pRRLsinPPT.CMV.MCS MM WPRE-vector. To generate short hairpin RNA (shRNA) vectors against CFTR (two different shRNAs, labeled KD1 and KD2, were used), oligonucleotides were cloned into the pLKO-shRNA2 vector (No. PT4052-5; Takara Bio, Saint-Germain-en-Laye, France). CFTR KD1 and KD2 shRNA were from Origene (TRCN0000082964: CCGGCCTGGAATTGTCAGACATATACTCGAGTATATGTCTGACAATTCCAGGTT TTTG and TRCN0000082967: CCGGGCAGTACGATTCCATCCAGAACTCGAGTTCTGGATGGAATCGTACTGCTT TTTG; Rockville, USA). A nonsense scrambled shRNA sequence was used as a negative control. Lentiviral particles were produced in 293T cells as previously described⁸. For lentiviral transduction, a MOI of 10 was used throughout the study

and results from the main figures display KD1 and KD2 constructs merged. MOI of 30 was only used for the initial screening (supplemental Figure 1). Transductions were performed on day 0 and after 24 h the cells were refed with fresh medium. All experiments were performed from day 3 or 4 onwards. Knockdown efficiency was monitored for each experiment at the protein level. For 3HA-CFTR localization studies, lentiviral vectors were used encoding 3HA-WT-CFTR under the control of the human CMV promoter (with the 3HA-tag inserted into the fourth extracellular loop of CFTR (described in Sharma *et al.* ⁹) (CFTR cDNA sequence NCBI GenBank CCDS 5773.1)).

Bulk RNA Sequencing

RNA of control vs CFTR^{KD} and vehicle vs CFTR(inh)-172 (Merck, Darmstadt, Germany) treated ECs was extracted using the TRIzol kit (Thermo Fisher Scientific, Geel, Belgium) following the manufacturer's instructions; quality and quantity were measured on a Nanodrop (Thermo Fisher Scientific, Geel, Belgium). Sequence libraries were prepared with the Lexogen QuantSeq 3' mRNA-Seq Library prep kit according to the manufacturer's protocol. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies, Leuven, Belgium) with the DNA 1000 kit (Agilent Technologies, Leuven, Belgium) according to the manufacturer's recommendations. Libraries were diluted to a final concentration of 2 nM and subsequently sequenced on an Illumina HiSeq4000 platform according to the manufacturers recommendations. Single-end reads of 50 bp length were produced with a minimum of 1M reads per sample. Quality control of raw reads was performed with FastQC v0.11.7. (available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) ¹⁰. Adapters were filtered with ea-utils fastq-mcf v1.05. (available online at: <https://github.com/ExpressionAnalysis/ea-utils>) ¹¹. Splice-aware alignment was performed with STAR v2.6.1b against the human reference genome hg38 using the

default parameters¹². Reads mapping to multiple loci in the reference genome were discarded. Resulting BAM alignment files were handled with Samtools v1.5.¹³ Quantification of reads per gene was performed with HT-seq Count v2.7.14. Raw sequencing data are available in ArrayExpress under accession number E-MTAB-8599.

Gene Set Enrichment Analysis

Bio-informatic analysis was carried out by using the in-house developed BIOMEX software¹⁴. We used gene set enrichment analysis (GSEA) as implemented in the clusterProfiler package (version 3.6.0) to compare gene expression signatures between controls and CFTR knockdown or CFTR inhibitor-treated HUVEC samples. Gene set analysis was performed using a set of vascular related gene sets selected from the Molecular Signatures Database (MSigDB version 7.0 downloaded from <http://bioinf.wehi.edu.au/software/MSigDB/>), as previously described¹⁵. GSEA scores were calculated for sets with a minimum of 10 detected genes, all other parameters were default. Individual genes shown in heatmaps were selected out of relevant MSigDB gene lists whereafter they were filtered based on significant adjusted p-value and endothelial function.

RNA Isolation and Gene Expression Analysis

Total RNA was extracted with the PureLink RNA mini kit (Thermo Fisher Scientific, Geel, Belgium) according to the manufacturer's instructions; RNA quality and concentration were measured on a Nanodrop (Thermo Fisher Scientific, Geel, Belgium). cDNA synthesis was performed with the iScript cDNA synthesis kit (BioRad, Temse, Belgium) with a starting RNA material of 500 µg. RNA expression analysis was performed by Taqman quantitative RT-PCR using premade primer sets (Applied Biosystems, Carlsbad, CA and IDT, Belgium): HPRT Hs.PT.58.2145446 (NM_000194); VCAM1 Hs.PT.56a.24988808 (NM_001199834); ICAM1 Hs.PT.56a.4746364 (NM_000201); SELE Hs.PT.58.1165629 (NM_000450); IL8

Hs.PT.58.39926886.g (NM_000584); SQSTM1 Hs.PT.58.39829257 (NM_003900); MAP1LC3B Hs.PT.58.27295455.g (NM_022818); GCLC Hs.PT.58.246294 (NM_001498).

Immunoblotting

Protein extraction and immunoblot analysis were performed using a modified Laemmli sample buffer (125 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 10% glycerol) in the presence of protease and phosphatase inhibitors (Roche, Anderlecht, Belgium). Mitochondrial isolation was performed using the mitochondria isolation kit for cultured cells according to the manufacturer's instructions (Thermo Fisher Scientific, Geel, Belgium). Lysates were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and analyzed by immunoblotting. Primary antibodies used were: CFTR (MAB25031; R&D Systems, Abingdon, United Kingdom), p62 (P0067; Merck, Darmstadt, Germany), LC3B (ab51520; Abcam, Cambridge, United Kingdom), p-P70S6K (#9205; Cell Signaling, Frankfurt am Main, Germany), P70S6K (#9202; Cell Signaling, Frankfurt am Main, Germany), p-S6 (#4858; Cell Signaling, Frankfurt am Main, Germany), S6 (#2217; Cell Signaling, Frankfurt am Main, Germany), OPA1 (612606; R&D Systems, Abingdon, United Kingdom), DRP1 (NB110-55237; R&D Systems, Abingdon, United Kingdom) and Parkin1 (ab15954; Abcam, Cambridge, United Kingdom). Equal loading was verified by GAPDH immunoblotting (#2118; Cell Signaling, Frankfurt am Main, Germany) for total and cytosolic fractions and VDAC1 (sc390991; Santa Cruz, Heidelberg, Germany) for mitochondrial fractions. All antibodies were used in 5% bovine serum albumin (BSA) diluted in TBST buffer (Tris-buffered saline, 0.1% Tween 20). Appropriate secondary antibodies were from Cell Signaling Technology (Anti-Rabbit IgG HRP linked #7074; Anti-Mouse IgG HRP linked, #7076; Frankfurt am Main, Germany) in a 1:2000 dilution in TBST with 5% BSA. Signal was detected using the ECL and/or Femto systems (GE Healthcare, Chicago, USA) according to

the manufacturer's instructions. Densitometric quantifications of bands were done with Fiji software (<https://fiji.sc>)¹⁶.

Immunocytochemistry

All methods for histology and immunostainings have been previously described^{1,17–20}. Cells grown for 24 h on coverslips were fixed with 4% PFA for 15 min at room temperature and processed for immunocytochemistry. In general, PBS-Triton 0.5%-2%BSA was used to permeabilize the cells or PBS-2%BSA where non-permeabilized cells were required. Primary antibodies were incubated with the cells overnight, and secondary antibodies were incubated for 2 h. On tissues, immunostainings were performed using the following primary antibodies: anti-CD31 (550274; BD Biosciences, Erembodegem, Belgium), anti-VE-cadherin (555289; BD Biosciences, Erembodegem, Belgium), anti-CFTR (MAB25031; R&D Systems, Abingdon, United Kingdom), anti-HA (11583816001; Roche, Anderlecht, Belgium), anti-vWF (ab6994; Abcam, Cambridge, United Kingdom). Sections were then incubated with the appropriate fluorescently conjugated secondary antibodies (Alexa 488, 546, 568, 633 or 647; Thermo Fisher Scientific, Geel, Belgium), or followed by amplification with the proper tyramide signal amplification systems when needed (Perkin Elmer, Zaventem, Belgium). Actin was counterstained with Alexa-568 Fluor conjugated phalloidin (Thermo Fisher Scientific, Geel, Belgium). Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Geel, Belgium).

In Vitro Functional Assays

Proliferation

Endothelial proliferation was quantified by incubating the cells with 1 mCi/mL [³H]-thymidine in full EGM2 as previously described²⁰. Thereafter, cells were fixed with 100% ethanol for 15 min at 4°C, precipitated with 10% trichloroacetic acid for 10 min at 4°C, and lysed with 0.1 N NaOH. The amount of [³H]-thymidine incorporated into DNA was measured by scintillation counting. CFTR(inh)-172 in 10 µM or vehicle

(DMSO) were added to the medium 24 h before cell lysis. [³H]-thymidine was added together with the supplement 6 h before cell lysis. The data were normalized to protein content, determined by BCA assay (Roche, Anderlecht, Belgium) immediately after the assay.

LDH Viability Assay

Cell survival was assessed by lactate dehydrogenase (LDH) release into the media using the Cytotoxicity Detection kit (Roche, Anderlecht, Belgium) as indicated in manufacturer's specifications, whereby low LDH release signifies low cell death and high survival. CFTR(inh)-172 at 10 μ M or vehicle (DMSO) were added to the medium 24 h before cell lysis.

Scratch Wound Migration Assay

A scratch wound was applied on confluent EC monolayers (pretreated for 8 h with 1 μ g/mL Mitomycin C to block proliferation) using a 20 μ l tip, 24 h after seeding. After scratch wounding and photography (T0), the cultures were further incubated in fully supplemented EGM2 medium with CFTR(inh)-172 at 10 μ M or vehicle (DMSO) for 18 h (until near closure was reached in the control condition) and photographed again (T18) using a Leica DMI6000 microscope (Leica Microsystems, Wetzlar, Germany). Migration was measured with the Fiji software (<https://fiji.sc>)¹⁶ and is expressed as percentage of wound closure (gap area at T0 minus gap area at T18 in % of gap area at T0).

Trans-endothelial Electrical Resistance

25,000 ECs were seeded in growth medium on 6.5 mm 0.1% gelatin-coated polyester transwells, 0.4 μ m pore size (Costar ref. 3470; Merck, Darmstadt, Germany). The trans-endothelial electrical resistance (TEER) was measured using the Endohm-6 electrode (World Precisions Instruments, Friedberg, Germany) connected to an EVOM2 Volt/Ohm meter (World Precisions Instruments, Friedberg, Germany). Gelatin-coated wells without cells were used to measure the intrinsic electrical resistance of the inserts and these values were then subtracted from the values measured in the presence of cells. Measurements were performed every day,

taking at least 2 measurements per treatment. The medium was changed every day with fully supplemented EGM2 medium with CFTR(inh)-172 at 10 μ M or vehicle (DMSO).

Staining and quantification of VE-cadherin junctions

VE-cadherin staining and quantification of junctional length was performed as previously described^{21,22}. First, the total junctional length (100%) was determined by summing up all segments, then the sum of all continuous segments was calculated as the percentage of total junctional length. The percentage difference between total and continuous represents the discontinuous length. Reticulated fraction was determined with the formula reticulated length/total junctional length using the VE-cadherin staining²³. Junctional lengths, reticulated and total junctional length were defined manually with the Fiji software (<https://fiji.sc>)¹⁶. For each condition, a minimum of 2 fields were quantified (20-40 cells per field on average) per condition. The intensity plot profile was created with Fiji to show overlapping signal between VE-cadherin and CD31, justifying the validity of using VE-cadherin to measure reticulated junctions.

Intracellular and Mitochondrial ROS Analysis

Intracellular and mitochondrial ROS levels were measured using CM-H₂DCFDA (Thermo Fisher Scientific, Geel, Belgium) and MitoSOX (Thermo Fisher Scientific, Geel, Belgium) according to the manufacturer's instructions. The ROS levels were determined by pre-incubation of the ECs for 30 min with the dye in serum free M199. The fluorescent intensity was measured by flow cytometry. CFTR(inh)-172 in 10 μ M or vehicle (DMSO) were added to the medium 24 h before the addition of the fluorescent dye.

Leukocyte Adhesion Assay

Peripheral blood mononuclear cells (PBMC) were collected from healthy human volunteers, isolated by gradient centrifugation as described above and labelled with

calcein according to the manufacturer's instructions (C1430, Thermo Fisher Scientific, Geel, Belgium). Confluent monolayers of HUVECs were grown in 35 mm high glass bottom ibidi dishes (Ibidi, Beloeil, Belgium) and incubated either with vehicle (DMSO), CFTR(inh)-172 (10 μ M) or LPS (1 mg/mL) overnight (37°C, 5% CO₂). After this period, medium was removed and ECs were washed with PBS. The mononuclear cells were added (1.0 x 10⁶/ibidi dish) and incubated for 60 min (37°C, 5% CO₂). Non-adherent cells were removed by gently washing 5 times with PBS and cells were fixed using 4% PFA. Five fields of view per well (magnification 20X, Leica DMI6000B microscope, Wetzlar, Germany), randomly chosen, were analyzed, and the number of adherent leukocytes per field was determined.

Metabolism Assays

Oxygen Consumption Rate (OCR)

Cells were seeded at 50,000 cells per well in Seahorse XF24 tissue culture plates (Seahorse Bioscience Europe, Copenhagen, Denmark) the day before the assay. The measurement of oxygen consumption was performed at 10 min intervals (2 min mixing, 2 min recovery, 6 min measuring) for 3 h using the Seahorse XF24 analyzer. Inhibitors and substrates were used at the following concentrations: oligomycin (1.2 μ M), antimycin (1 μ M), FCCP (5 μ M). At any condition, at least 4 consecutive measurements of OCR are done. The data were normalized to protein content, determined by BCA assay upon lysis of the cells (using RIPA buffer) immediately after the assay.

Detection of Glutathione Species and NADPH

Metabolites from ECs grown on a 6-well plate were extracted in 300 μ L of a 80% methanol, containing 2 μ M d27myristic acid extraction buffer. Extraction samples were then spun down in a cooled centrifuge for 15 min at 20.000 g and the supernatant was transferred to LC-MS vials. Targeted measurements of GSSG, GSH, NADP⁺, NADPH were performed using a Dionex UltiMate 3000 LC System (Thermo Fisher Scientific, Geel, Belgium) coupled to a Q Exactive Orbitrap mass

spectrometer (Thermo Fisher Scientific, Geel, Belgium) operated in negative mode. Practically, 35 μ L of sample was injected on a SeQuant ZIC/ pHILIC Polymeric column (Merck, Darmstadt, Germany). The gradient started with 20% of solvent B (10 mM NH_4 -acetate in MQH₂O, pH 9.3) and 80% solvent A (LC-MS grade acetonitrile) and remained at 20% B until 2 min post injection. Next, a linear gradient to 80% B was carried out until 29 min. At 38 min the gradient returned to 40% B followed by a decrease to 20% B at 42 min. The chromatography was stopped at 58 min. The flow was kept constant at 100 μ L per min at the column was placed at 25°C throughout the analysis. The MS was operated both in targeted MS2 mode using a spray voltage of 3.5 kV, capillary temperature of 320°C, sheath gas at 10.0, auxiliary gas at 5.0. For the targeted MS2 mode, AGC was set at 2e^5 , maximum IT at 100 ms, a resolution of 17.500 and an isolation window of 1.2 m/z. Data collection was performed using Xcalibur software (Thermo Fisher Scientific, Geel, Belgium).

Transmission Electron Microscopy

Transmission electron microscopy was performed on a JEOL JEM1400 (JEOL Europe BV, Zaventem, Belgium) (VIB Bio Imaging Core, Leuven Platform). For TEM observations samples were fixed for 24 h with 2.5% glutaraldehyde at pH 7.3, buffered with 0.05 M sodium cacodylate. Prior to embedding in Agar 100 Resin (Agar Scientific, Stansted, UK) the material was post fixed in 2% OsO₄ (buffered with 0.05 M sodium cacodylate, pH 7.3) and dehydrated in a graded acetate series. Semi-thin ($\pm 1 \mu\text{m}$) sections were cut with a Reichert Jung Ultracut E microtome and stained with 0.1% thionin - 0.1% methylene blue. The ultra-thin ($\pm 70 \text{ nm}$) sections, on copper grids, were stained with uranyl acetate and lead citrate. 7-20 pictures were taken from each condition.

Mouse Experiments

Leukocyte Infiltration Into the Lungs and Liver

Liver and lungs of 11-14 weeks old mice were perfused with PBS, thereafter they were dissected and fixed in 4% PFA overnight at 4°C, dehydrated, embedded in paraffin and sectioned into 7 µm-thin sections. Immunostaining of leukocytes was done with rat anti-Mouse CD45 antibody (30-F11; BD Biosciences, Erembodegem, Belgium) and staining of the endothelium was done with a goat anti-mouse CD105 antibody (AF1320; R&D Systems, Abingdon, United Kingdom). Sections were then incubated with an appropriate secondary antibody (Alexa-647; Jackson Immuno Research, Uden, The Netherlands) and TSA Cy3 amplification (Perkin Elmer, Zaventem, Belgium). Imaging was performed using a Zeiss LSM 780 confocal microscope (Zeiss, Jena, Germany). Morphometric analysis of the area of CD45⁺ dots in % of CD105 lung area was done using Fiji analysis software.

Flow Cytometry Analysis

Mice were first anesthetized using Ketamine/Xylazine (500 mL/mouse of Ketamine 0.65%; Xylazine 0.05% diluted in saline solution) and perfused with 10 mL PBS followed by perfusion with 5 mL of full DMEM-based digestion buffer containing 0.1% collagenase II (Thermo Fisher Scientific, Geel, Belgium), 0.1% collagenase I (Thermo Fisher Scientific, Geel, Belgium) and DNase I 0.075 mg/ml (Merck, Darmstadt, Germany). Lungs were dissected and placed into a gentleMACS dissociation tubes (Miltenyi, Leiden, The Netherlands) containing 5 mL of digestion buffer, and processed as per the manufacturer's instructions. The organs were incubated in a water bath at 37°C, 30 min with shaking every 10 min. Next, tissue was homogeneously dissociated and the reaction was stopped by adding 10 mL PBS/0.1% BSA. Subsequently, the cell suspension was spun down for 5 min at 300g before staining and flow cytometry analysis. The staining was performed using a mix containing CD34 (48-0341-80; Thermo Fisher Scientific, Geel, Belgium); CD31 (551262 & 561410; BD Biosciences, Erembodegem, Belgium), CD45 (45-0454-82; Thermo Fisher Scientific, Geel, Belgium), ICAM1 (550287; BD Biosciences, Erembodegem, Belgium) and VCAM1 (553330; BD Biosciences, Erembodegem, Belgium) antibodies. The vibrant dye (V35003; Thermo Fisher Scientific, Geel,

Belgium) was used to gate for viable cells. After removing doublet cells, endothelial cells were identified based on viable cells with CD45⁻ CD31⁺ expression. Data were recorded by flow cytometry on the FACS ArialIII (BD Biosciences, Erembodegem, Belgium), and analyzed with the FlowJo 8.8.6 software (<https://www.flowjo.com>).

Quantification and Statistical Analysis

Data represent mean $\pm$ SEM of at least three independent experiments. Statistical significance was calculated by standard two-tailed t-test (with Welch's correction when variances were significantly different between groups), ANOVA (for multiple comparisons within one dataset), one-sample t-test (for comparisons to point normalized data) using Prism v8.2. Bio-informatic analysis was carried out by using the in-house developed BIOMEX software ¹⁴. p-value < 0.05 was considered as statistically significant.

Supplementary Tables

Supplementary table S1: Patient genotypes used for BOEC generation

The table shows CFTR genotype, gender, age and forced expiratory volume in one second (FEV1) from each CF patient enrolled for the generation of blood outgrowth endothelial cells (BOECs).

Supplementary table S2: GSEA and meta-analysis of CFTR-impaired ECs

Bulk RNA sequencing was performed in primary HUVECs after inducing CFTR impairment with: (i) the highly specific CFTR inhibitor CFTRinh-(172) (CFTR_{inh}), or (ii) CFTR-specific short hairpin RNAs leading to CFTR knockdown (CFTR^{KD}). The table presents the results of gene set enrichment analysis (GSEA) for (i) Ctrl (DMSO) *versus* CFTRinh-(172) and (ii) Ctrl (PLKO) *versus* CFTR^{KD}. The table also contains the results of congruent significantly up/down-regulated gene sets between CFTR^{KD} and CFTR_{inh}. A curated list of human vascular gene sets was used. Only

gene sets with a minimal set size of 10 and with a significant adjusted p-value were considered for GSEA. For each top up- or down-regulated gene sets, enrichment score, normalized enrichment score (NES, used to generate figure 1f and supplemental figure 2c), p-values and adjusted p-values are indicated. Each gene set is presented with its official identifier and the corresponding name was used. This annotation was made according to the known gene functions within each gene set.

Supplementary table S3: Meta-analysis upon CFTR impairment

The table presents the results of meta-analysis of differential analysis between Ctrl *versus* CFTR^{KD}, and Ctrl *versus* CFTRinh-(172) at the gene and gene set levels. Low rank numbers mean genes/gene sets upregulated upon CFTR impairment, while high rank numbers translate into genes/gene sets that are downregulated upon CFTR impairment. The product rank method was applied to generate meta-analysis scores.

Supplementary table S4: Differential analysis of CFTR-impaired ECs

The table presents the results of differential analysis expressed as Log fold change for: (i) Ctrl (DMSO) *versus* CFTRinh-(172) and (ii) Ctrl (PLKO) *versus* CFTR^{KD}. For each detected gene, the average expression, p-values and adjusted p-values are indicated.

Supplementary Figure Legends

Supplementary figure S1: CFTR silencing in HUVEC

a) Representative immunoblot for CFTR expression in ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNAs (CFTR^{KD1} and CFTR^{KD2}). Bands B and C indicate immature (native) and mature (glycosylated) CFTR respectively. GAPDH was used as loading control. Densitometric quantification of the ratio of CFTR to GAPDH is shown on the right. Mean + SEM; CFTR^{KD1}, n=5, CFTR^{KD2}, n=5; **p<0.01

by one-sample *t*-test. **b)** Representative image of endothelial markers VWF (red) and VE-cadherin (green) in Ctrl and CFTR^{KD} HUVECs. **c-d)** Cell death is assessed by LDH release in ECs treated with CFTR(inh)-172 (CFTR_{inh}, yellow, n=5) *versus* the corresponding DMSO-treated control condition (Ctrl) (c), and in ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA (CFTR^{KD}, red) at multiplicity of infection (MOI) 10 (n=5) and 30 (n=6) (d). Mean + SEM; **p*<0.05; ***p*<0.01 by two-tailed paired *t*-test. **e)** Representative immunoblot for CFTR expression in ECs transduced with scrambled shRNA (Ctrl) or CFTR shRNAs (CFTR^{KD}) at MOI 30. Bands B and C indicate immature and mature CFTR respectively. GAPDH was used as loading control. Densitometric quantification of the ratio of CFTR to GAPDH is shown on the right. Mean + SEM; n=3; ****p*<0.001 by one-sample *t*-test.

Supplementary figure S2: Transcriptomic analysis upon CFTR impairment

a) Principal component analysis (PCA) on the highly variable genes of Ctrl (DMSO) and CFTR_{inh} ECs obtained after bulk RNA sequencing. **b)** Correlation heatmap of highly variable genes in Ctrl and CFTR_{inh} ECs. **c)** Barplots representing the top deregulated gene sets (with a significant adjusted *p*-value) ranked based on their normalized enrichment score. Upregulated gene sets induced upon CFTR_{inh} are shown in red while downregulated genes sets are in blue. Numbers between parentheses indicate alternative gene sets pertaining to the same biological function or signaling pathway. For official gene set names see supplemental Table 1. **d)** Venn diagrams indicating congruent vascular gene sets (with a significant *p*-value) between CFTR^{KD} *versus* Ctrl (scrambled shRNA) and CFTR_{inh} *versus* Ctrl (DMSO) differential analyses.

Supplementary figure S3: Endothelial junction alterations upon CFTR impairment

a) Representative image of cultured HUVECs transduced with a CFTR overexpression vector (3HA-CFTR; anti-HA staining in green) allowing to determine selective plasma membrane localization under non-permeabilized conditions; actin

(red) and nuclei (Hoechst; blue). Yellow signal in the merged image signifies co-localization of CFTR and actin. White arrowhead depicts ruffling structures enriched in CFTR. Scale bar: 100 μ m. **b)** Representative confocal images of HUVECs transduced with scrambled shRNA (Ctrl) or CFTR shRNA (CFTR^{KD}) stained for VE-Cadherin. Red arrows indicate discontinuous junctions, yellow arrowheads indicate reticulated junctions. The magnification shows a detailed view of the reticular network. Scale bar: 25 μ m. Scale bar magnification: 25 μ m. **c)** Representative confocal images of CFTR^{KD} ECs stained for VE-Cadherin (green) or CD31 (red). Scale bar: 25 μ m. Yellow straight segment [AB] was used to generate the intensity plot profile created with Fiji to show overlapping signal between VE-cadherin (green) and CD31 (black).

Supplementary figure S4: Effect of CFTR impairment and FACS gating strategy

a) qRT-PCR analysis of GCLC expression levels in Ctrl and CFTR^{KD} ECs. Expression levels are normalized to *HPRT1*. Individual data points with median. n=6. **b)** Representative transmission electron microscopy of Ctrl versus CFTR_{inh} ECs indicate an accumulation of autophagosomes (white arrowheads). Scale bars: 500 nm. **c)** qRT-PCR analysis of inflammation markers upon LPS and rapamycin combination in Ctrl and CFTR^{KD} HUVECs. n=3; ^{NS}p>0.05; two-tailed paired *t*-test. **d)** Gating strategy for flow cytometry analysis of wildtype and CFTR^{KO} mouse lung cell suspension. **e)** Flow cytometry analysis of Ctrl BOECs stained for CD34 (hematopoietic progenitor marker) and CD31 (endothelial marker).

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Supplementary figure S1

Supplementary figure S2

Supplementary figure S3

Supplementary figure S4