

# Endothelial dysfunction in pulmonary arterial hypertension: loss of cilia length regulation upon cytokine stimulation

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## Abstract

Pulmonary arterial hypertension (PAH) is a syndrome characterized by progressive lung vascular remodelling, endothelial cell (EC) dysfunction, and excessive inflammation. The primary cilium is a sensory antenna that integrates signalling and fine tunes EC responses to various stimuli. Yet, cilia function in the context of deregulated immunity in PAH remains obscure. We hypothesized that cilia function is impaired in ECs from patients with PAH due to their inflammatory status and tested whether cilia length changes in response to cytokines. Primary human pulmonary and mouse embryonic EC were exposed to pro- (TNF $\alpha$ , IL1 $\beta$ , and IFN $\gamma$ ) and/or anti-inflammatory (IL-10) cytokines and cilia length was quantified. Chronic treatment with all tested inflammatory cytokines led to a significant elongation of cilia in both control human and mouse EC (by  $\sim 1 \mu\text{m}$ ,  $P < 0.001$ ). This structural response was PKA/PKC dependent. Intriguingly, withdrawal of the inflammatory stimulus did not reduce cilia length. IL-10, on the other hand, blocked and reversed the pro-inflammatory cytokine-induced cilia elongation in healthy ECs, but did not influence basal length. Conversely, primary cilia of ECs from PAH patients were significantly longer under basal conditions compared to controls ( $1.86 \pm 0.02$  vs.  $2.43 \pm 0.08 \mu\text{m}$ ,  $P = 0.002$ ). These cilia did not elongate further upon pro-inflammatory stimulation and anti-inflammatory treatment did not impact cilia length. The missing length modulation was specific to cytokine stimulation, as application of fluid shear stress led to increased cilia length in the PAH endothelium. We identified loss of cilia length regulation upon cytokine stimulation as part of the endothelial dysfunction in PAH.

## Keywords

primary cilia, pulmonary endothelium, inflammation, chemokines and cytokines

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## Introduction

Pulmonary arterial hypertension (PAH) represents a group of lung diseases characterized by high pulmonary artery pressure (PAP) ( $> 25 \text{ mmHg}$ ) eventually leading to right heart failure.<sup>1,2</sup> Blood vessels in the lungs of PAH patients are highly remodeled due to genetic changes, altered cellular signaling, metabolic changes, aberrant pressures, and chronic inflammation.<sup>2–4</sup>

PAH patients have a high inflammatory status both in the systemic circulation and in the lung vasculature.<sup>4</sup> Vascular inflammation involves various cytokines, including

the pro-inflammatory TNF $\alpha$ , IL1 $\beta$ , and IFN $\gamma$ , as well as the anti-inflammatory cytokine IL10. Under the influence of pro-inflammatory cytokines, endothelial cells (ECs) express proteins, such as cell adhesion molecules, for the recruitment of blood borne inflammatory cells into the vessel wall.<sup>5,6</sup>

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Chronic or dysregulated inflammation leads to EC dysfunction involving a number of factors, such as loss of barrier integrity.<sup>5,7</sup>

As with most mammalian cell types, ECs carry primary cilia. The main structural part of the cilium is the rigid axoneme consisting of 9+0 microtubule doublets, which protrudes from the cellular membrane into the lumen or extracellular space.<sup>8</sup> The base of the cilium, the transition zone, functions as an active barrier for both the cytoplasmic and membrane content preventing free exchange and contributing to a unique subcellular environment.<sup>9</sup> The endothelial primary cilium is a highly regulated and specified antenna that senses and orchestrates responses to chemical and mechanical cues from the flowing blood.<sup>10,11</sup> Emerging data further identify the primary cilium as a specialized organelle involved in intracellular signaling processes from hedgehog proteins, growth factors, calcium, and others.<sup>12–19</sup> Furthermore, EC primary cilia are important for vascular integrity and homeostasis, since absence of primary cilia has been shown to promote endothelial-to-mesenchymal transition (EndoMT).<sup>20</sup> In accordance, primary cilia were found in areas of disturbed flow and are therefore suggested to protect against shear-induced EndoMT.<sup>21,22</sup>

Cilia length is instrumental for cilia function and is controlled by intraflagellar transport (IFT). Cilia length itself can regulate cargo loading of the IFT particles (e.g. with receptors), suggesting that signaling directly links to cilia length.<sup>23</sup> In addition, many signaling pathways have been shown to influence cilia length.<sup>24–26</sup> As such, cilia length increases in response to pro-inflammatory cytokines and was thereby proposed to mediate inflammatory responses.<sup>27</sup>

In diseases with dysregulated vascular inflammation, such as PAH, ECs are chronically exposed to vast amounts of pro-inflammatory cytokines. Hence, we hypothesized that the loss of cytokine-induced cilia length control is part of the EC dysfunction in PAH and tested whether cytokines can affect cilia length of human pulmonary microvascular EC from patients.

## Methods

### Cell culture

Cells were grown on 0.1% gelatin coated eight-chamber slides (BD Biosciences) until confluency. Ciliated mouse embryonic endothelial cells (MEC)<sup>28</sup> were cultured as previously described.<sup>20</sup> Primary human pulmonary microvascular endothelial cells (MVEC) were obtained from end-stage PAH patients and healthy tissues of lobectomy donors, as described before.<sup>29</sup> The tissue harvest and MVEC isolations were approved by the IRB of the VU University Medical Center (VUmc, Amsterdam, The Netherlands) and consent was given. MVEC were cultured in complete ECM medium supplemented with 1% pen/strep, 1% endothelial cell growth supplement, and 5% FCS (ScienceCell). Shear stress was applied, as previously described,<sup>29</sup> by culturing

cells on  $\mu$ -slides I Luer (ibidi) and applying medium flow at 15 dyn/cm<sup>2</sup> over the adherent cells with the ibidi pump system for five days.

### Treatments

Treatments were performed in starvation medium with 1% FCS and pen/strep. Stimuli were provided in fresh medium for 24 h. The following concentrations were used: TNF $\alpha$  10 ng/mL, IL1 $\beta$  10 ng/mL, IFN $\gamma$  100 U/mL, and IL10 10 ng/mL. Forskolin (FK) was used in a concentration of 100  $\mu$ M (Sigma-Aldrich). H89 and Gö6983 (Sigma-Aldrich) were applied at 10  $\mu$ M and 2  $\mu$ M, respectively. The NF $\kappa$ B inhibitor BAY 11-7085 (Cayman Chemicals) was applied at 1  $\mu$ M final concentration.

### Cilia immunostaining

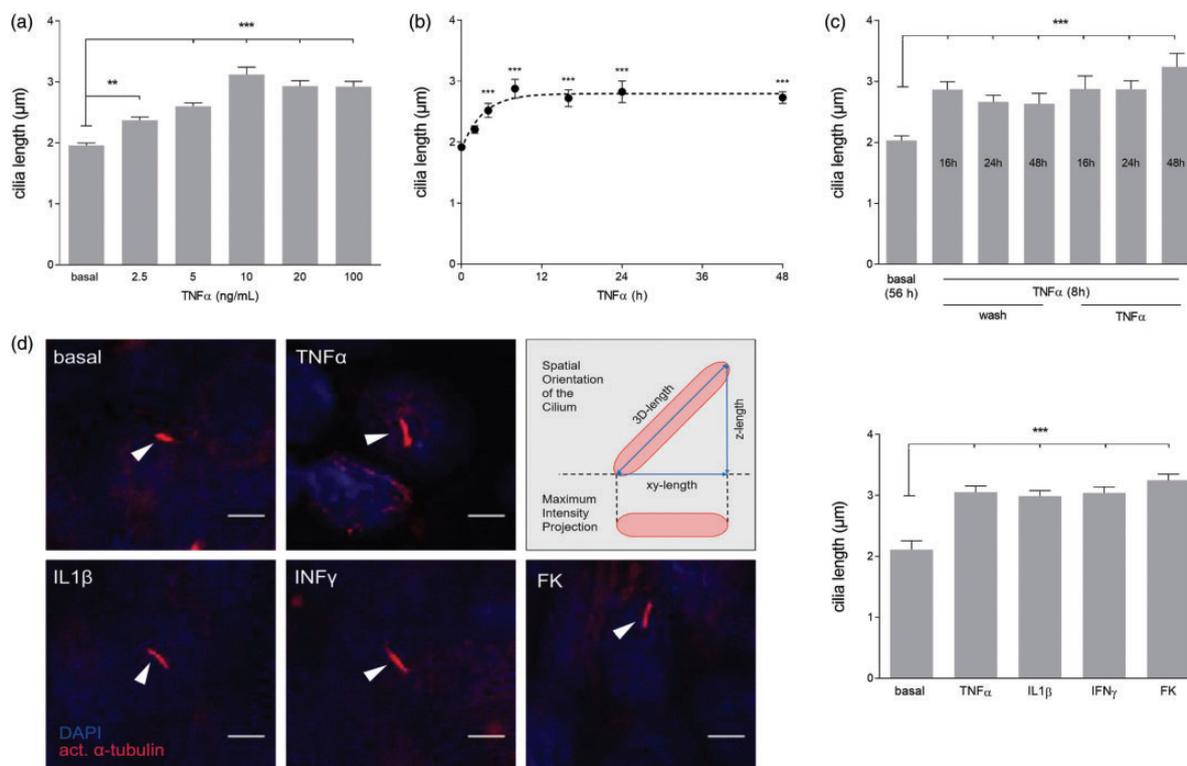
Cells were fixed in 4% paraformaldehyde (Merck) in PBS for 10 min at room temperature (RT). Fixed cells were permeabilized with 0.05% Tween 20 (Merck) in PBS. Incubation with the primary antibody against acetylated- $\alpha$ -tubulin (6-11B-1, 1:2000, Sigma-Aldrich) was performed overnight at 4°C. This was followed by 1 h incubation with secondary Cy3-labeled goat-anti-mouse antibody (1:500, Vector Laboratories) and DAPI nuclear counterstaining (1:1000, Molecular Probes) for 5 min at RT. Samples were mounted in Prolong Gold (Molecular Probes).

### Cilia length measurements

Confocal z-stacks were taken with a fixed step distance of 0.25  $\mu$ m using a SP5 confocal microscope (Leica). Image acquisition and cilia length measurements were performed as described previously.<sup>30</sup> In short, a random population of at least ten cilia per condition were measured using the Pythagoras (PyT) method. Herefore, cilium length was determined in the xy- as well as in z-direction with ImageJ (NIH) and the three-dimensional length was calculated based on the Pythagorean theorem  $a^2+b^2=c^2$ , with  $a$  being the xy-length based on a maximum intensity projection (MIP) and  $b$  the z-length (Fig. 1, schematic). With the PyT method, the spatial orientation of the cilium is accounted for, wherefore selection bias and standard deviation are minimized.

### Statistics

Experiments in MEC were performed in duplicate and repeated three times, experiments with MVEC were performed in duplicate in at least three donors. Statistics were calculated based on the averaged cilia length per donor with the total number of donors used as independent n. Data visualization and statistics were generated with GraphPad Prism 7. Data are presented as mean  $\pm$  SEM. Samples were tested for Gaussian distribution by D'Agostino-Pearson



**Fig. 1.** Various pro-inflammatory cytokines elongate primary cilia permanently. (a) TNF $\alpha$  was applied to MEC in different concentrations for 24 h ( $n \geq 100$  per concentration). (b) Time course of cilia elongation was quantified in MEC after addition of 10 ng/mL TNF $\alpha$  ( $n \geq 43$  cilia per time point). (c) Cilia length was determined after an initial trigger of 8h TNF $\alpha$  (10 ng/mL) followed by additional stimulation or an alternative wash step after 16 h, 24 h, or 48 h. All conditions were fixed and quantified after 56 h ( $n \geq 34$  cilia per condition). (d) Maximum intensity projections (MIP) of representative (flat) cilia (arrow heads) upon 24 h treatment with TNF $\alpha$  (10 ng/mL), IL1 $\beta$  (10 ng/mL), IFN $\gamma$  (100 U/mL), or the positive control FK (100  $\mu$ M) (scale bar = 5  $\mu$ m). Schematic depicts differences between MIP used to determine xy-length and three-dimensional (3D) length calculation applied for cilia length analysis and statistics. Quantification of average 3D cilia length is shown to the right ( $n \geq 53$  per condition).

omnibus normality test. If not otherwise indicated, significance was determined by one-way ANOVA with Kruskal–Wallis test and Dunn’s post hoc test. \* $P = 0.033$ , \*\* $P = 0.002$ , \*\*\* $P < 0.001$

## Results

### TNF $\alpha$ induces sustained cilia elongation

We sought to determine whether TNF $\alpha$  influences cilia length dose dependently. Therefore, different concentrations of TNF $\alpha$  were tested on MEC (Fig. 1a). A low concentration of 2.5 ng/mL TNF $\alpha$  induced significant cilia elongation compared to unstimulated controls ( $1.96 \pm 0.04$  to  $2.37 \pm 0.05$   $\mu$ m,  $P = 0.002$ ). Cilia length plateaued at an average length of approximately 3  $\mu$ m with a concentration of 10 ng/mL or higher ( $P < 0.001$ ). An additional increase in TNF $\alpha$  concentration did not further increase cilia length.

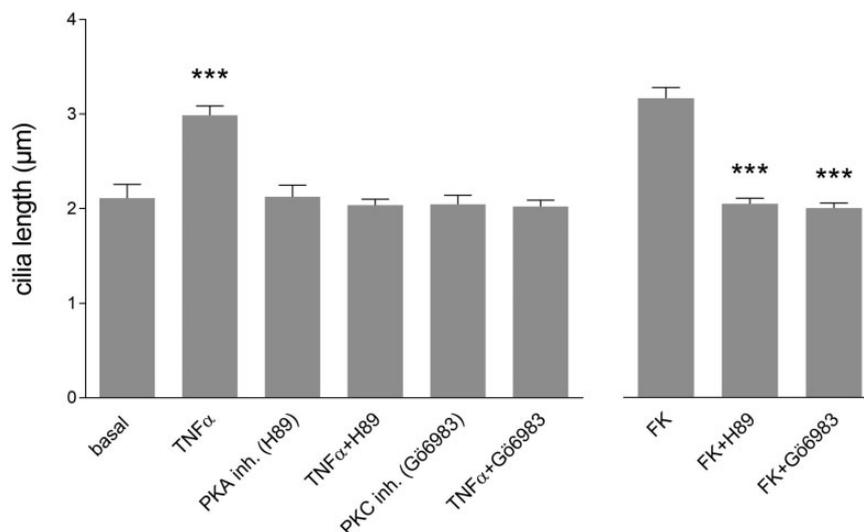
To examine the time course of cilia elongation upon TNF $\alpha$  stimulation, cilia length was measured at various time points (Fig. 1b). Indeed, cilia elongation upon TNF $\alpha$  stimulation was time-dependent. At 2 h after treatment, cilia elongation was visible, although not significant. Length was

significantly increased 4 h after treatment ( $P < 0.001$ ) and reaches a plateau after 8 h. The stimulation with TNF $\alpha$  caused sustained elongation and no significant differences in cilia length were found between 8 h and 48 h after treatment.

Additionally, we tested whether the removal of TNF $\alpha$  would reverse cilia length (Fig. 1c). To our surprise, washing steps after 16 h, 24 h, or 48 h did not alter cilia length. Moreover, additive treatment after 16 h, 24 h, or 48 h with TNF $\alpha$  did not show an extra effect on cilia length. Taken together, 8 h of TNF $\alpha$  treatment with a concentration of 10 ng/mL is sufficient to reach TNF $\alpha$ -induced maximal average cilia length and retain cilia elongation for at least 48 h.

### Various pro-inflammatory cytokines stimulate cilia elongation

To investigate whether various pro-inflammatory cytokines stimulate cilia elongation, MEC were exposed to either TNF $\alpha$ , IL1 $\beta$ , or IFN $\gamma$  (Fig. 1d). In general, cilia length significantly increased from approximately 2  $\mu$ m to approximately 3  $\mu$ m after stimulation ( $P < 0.001$ ). FK was used as



**Fig. 2.** Cilia elongation upon inflammatory cytokines is PKA/PKC-dependent. MEC were stimulated for 24 h with different combinations of TNF $\alpha$  (10 ng/mL) and/or the PKA inhibitor H89 (10  $\mu$ M) or PKC inhibitor Gö6983 (2  $\mu$ M) ( $n \geq 43$  cilia per condition). The PKA activator FK was used as positive control.

a positive control and did show the same average cilia elongation as the inflammatory cytokines.

Since cells responding to stimulation have considerable cilia length variations, their frequency distribution was analyzed (Suppl. Fig. 1). The cytokine and FK stimulated samples showed a shift in frequency distribution towards longer cilia compared to controls. Cilia of 2  $\mu$ m were still present, indicative for cells that did not respond to stimulation, but the majority elongated to approximately 3  $\mu$ m and some individual cilia even up of 7  $\mu$ m.

### Cytokine-induced cilia elongation is dependent on PKC-dependent/PKC signaling

It has been shown that primary cilia elongate upon direct stimulation of cyclic AMP with concomitant activation of PKC-dependent.<sup>31</sup> Involvement of PKC-dependent/PKC signaling in ciliary extension upon TNF $\alpha$  was tested by chemical inhibition with H89 or Gö6983 (Fig. 2). H89 and Gö6983 alone did not alter cilia length in MEC compared to basal condition. In agreement with the previous experiments, cilia length significantly increased to an average of approximately 3  $\mu$ m upon TNF $\alpha$  stimulation ( $P < 0.001$ ). Importantly, inhibition of PKA and PKC prevented TNF $\alpha$ -induced elongation. Similar results were obtained with IL1 $\beta$ , IFN $\gamma$ , or the direct PKA activator FK (Suppl. Fig. 2A). In the presence of the inhibitors, none of the tested stimuli altered cilia length suggesting that cilia elongation by inflammatory cytokines is transduced through PKA/PKC dependent signaling.

To confirm the importance of PKC signaling more specifically, PKC knockdown was performed by shRNA. The knockdown was sufficient to prevent TNF $\alpha$ -induced elongation (Suppl. Fig. 2B). The use of the lentiviral construct

resulted in viable cells with a 60% decrease in PKC mRNA levels (Suppl. Fig. 2C).

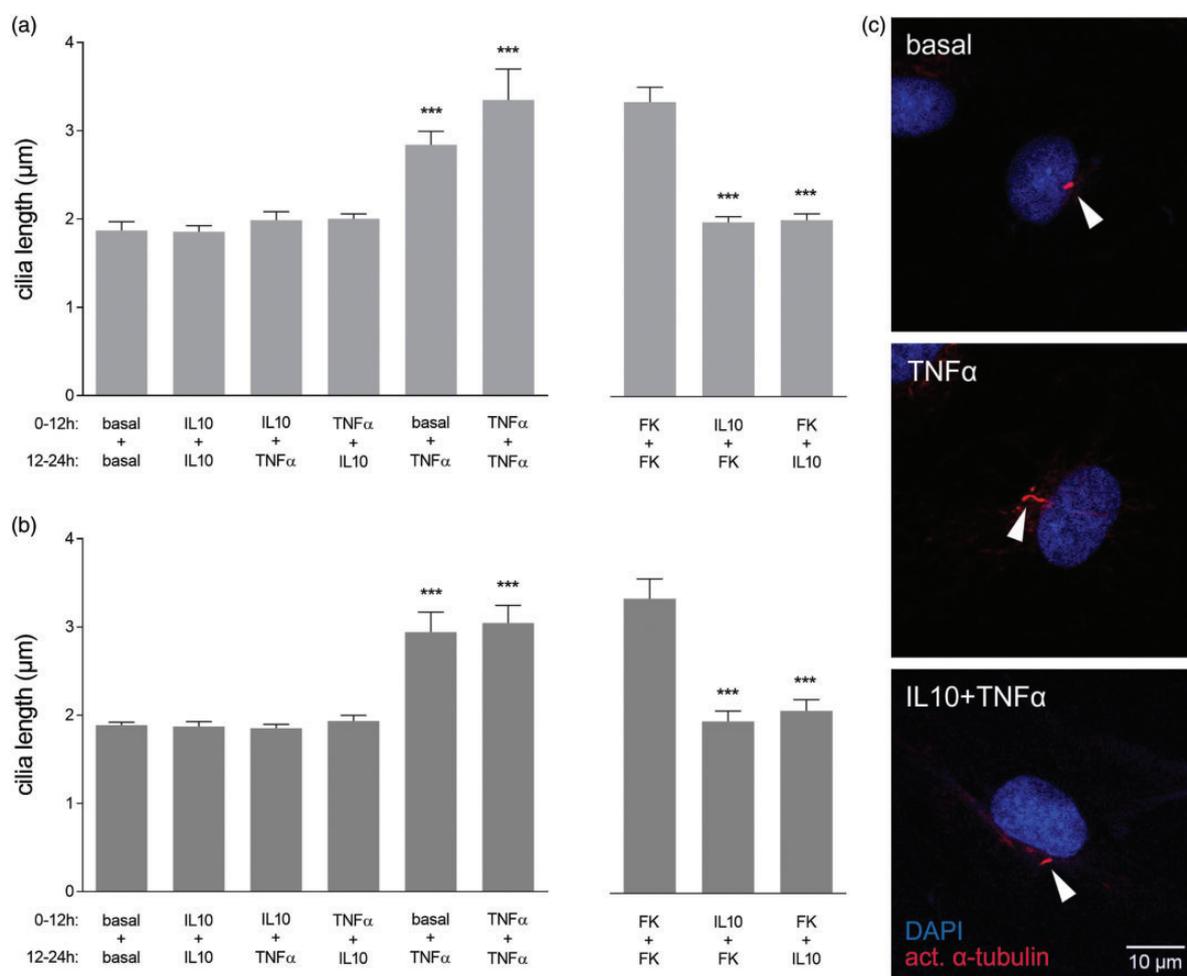
### IL10 blocks and reverses cilia elongation upon cytokine stimulation

We reasoned that when cells react to inflammatory cytokines by elongating their primary cilia, anti-inflammatory stimuli might counteract this response. Therefore, we tested whether IL10 prevents and reverses pro-inflammatory cytokine-induced cilia elongation.

MEC were treated with basal medium, TNF $\alpha$ , or IL10 alone for 12 h. Afterwards either IL10 or TNF $\alpha$  were added on top of the previous stimulus for additional 12 h (Fig. 3a). In agreement with our previous experiments, 12 h or 24 h of TNF $\alpha$  stimulation alone showed significant cilia elongation to  $\sim 3$   $\mu$ m ( $P < 0.001$ ). IL10 alone did not influence cilia length compared to basal condition. Importantly, application of IL10 to TNF $\alpha$  blocked cilia elongation and IL10 added after TNF $\alpha$  reversed cilia length back to basal levels. The effect of IL10 on cilia length was not exclusive to TNF $\alpha$ , but identical upon IL1 $\beta$ , IFN $\gamma$ , and IL10 blocked the effect of direct PKA activation by FK stimulation (Suppl. Fig. 3A). Interestingly, its inhibitory function was dependent on the actual presence of IL10. Pre-treatment with IL10 followed by FK without IL10 in the medium was not sufficient to prevent elongation (Suppl. Fig. 3B). In conclusion, IL10 blocked and reversed cilia elongation upon stimulation with various inflammatory cytokines.

### Cilia cytokine responses are similar in mouse and human endothelial cells

Primary cilia are highly conserved among species, wherefore their mechanisms might be as well. To examine, whether the



**Fig. 3.** IL10 blocks and reverses cilia elongation upon inflammatory cytokine stimulation in mouse and human endothelial cells. (a) MEC were either incubated with IL10 (10 ng/mL), TNF $\alpha$  (10 ng/mL), or left untreated for 12 h. Subsequently, different combinations of TNF or IL10 were added directly into the previous conditions and incubated for another 12 h ( $n \geq 26$  cilia per condition). (b) The combination treatments were repeated in human pulmonary MVEC of healthy individuals (donor = 3,  $n \geq 28$  cilia per condition). (c) MIP of representative cilia (arrowheads) on MVEC under the different conditions.

primary cilia of human lung EC react in a similar manner to the pro-inflammatory TNF $\alpha$  and anti-inflammatory IL10 as mouse EC, we repeated the combination treatments in MVEC from healthy control lungs (Fig. 3b and c). Indeed, 12 h and 24 h of TNF $\alpha$  showed a significant cilia elongation to approximately 3  $\mu$ m ( $P < 0.001$ ) compared to basal length of  $\sim 2$   $\mu$ m. Again, IL10 alone had no effect on basal cilia length. When combined with TNF $\alpha$ , IL10 blocked cilia elongation, while IL10 applied 12 h after TNF $\alpha$  reversed cilia length back to basal levels.

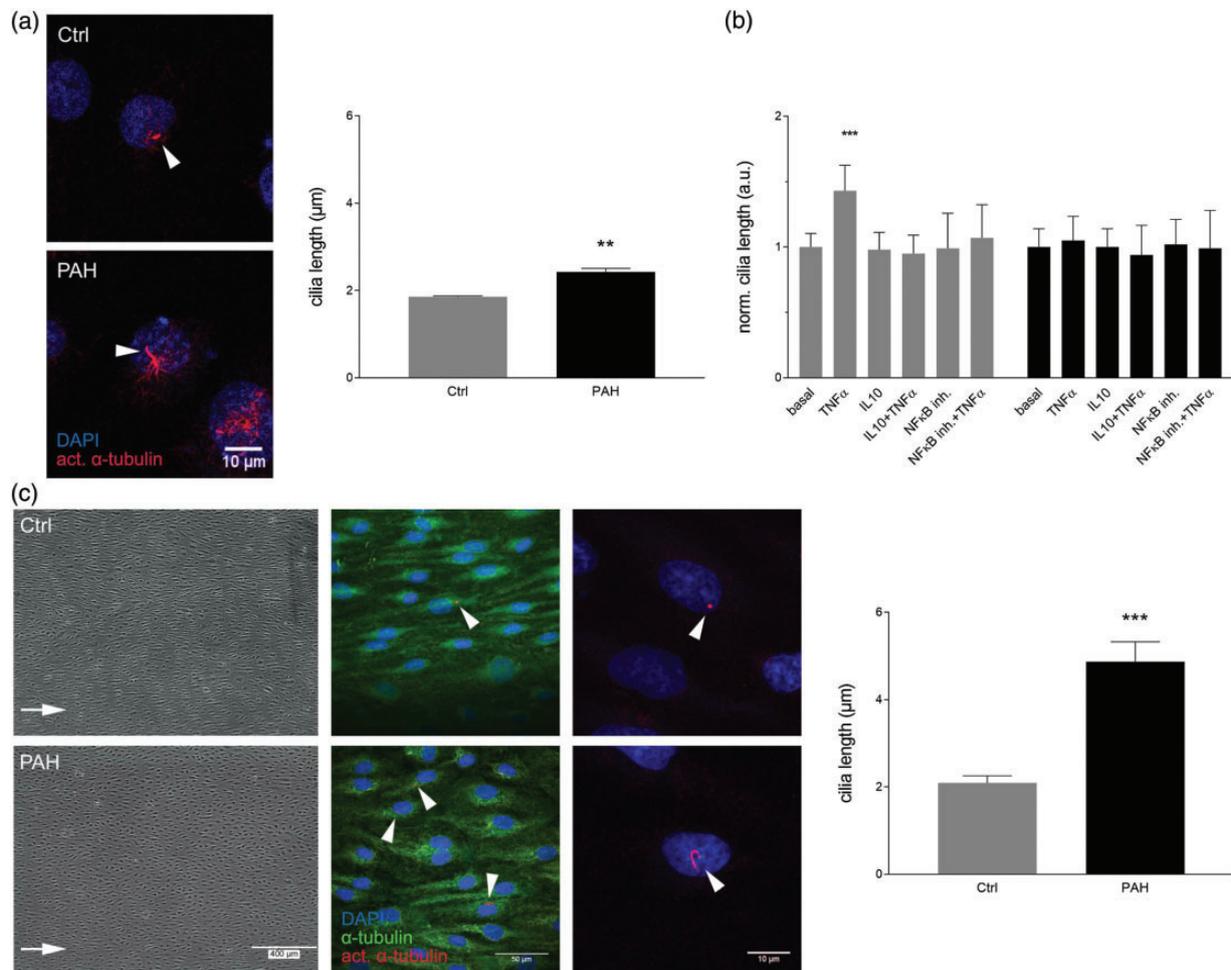
#### Endothelial cells of PAH patients display elongated cilia

To test, whether cilia length differs between patient and control MVEC, cilia were quantified in samples of three control and three donors with PAH (Fig. 4a). Under basal conditions the average length of cilia on EC from PAH patients was significantly increased to

$2.43 \pm 0.08$   $\mu$ m compared to  $1.86 \pm 0.02$   $\mu$ m of the controls ( $P = 0.002$ ).

#### TNF $\alpha$ and IL10 do not affect cilia length in PAH cells

Healthy MEC and MVEC showed cilia elongation upon stimulation with inflammatory cytokines to  $\sim 3$   $\mu$ m. IL10 could shorten cilia and block the effects of TNF $\alpha$ . PAH patient-derived MVEC exerted elongated cilia under basal conditions already (Fig. 4a), wherefore we tested cilia length responses in these patient cells (Fig. 4b). To our surprise, treatment with TNF $\alpha$  had no additional effect on cilia length and IL10 treatment of PAH MVEC did neither reduce basal cilia length nor affected the response to TNF $\alpha$ . To answer whether specifically the response to IL10 was lost or if cilia of PAH cells are generally unresponsive to anti-inflammatory treatment, the NF $\kappa$ B inhibitor BAY 11-7085 was tested. BAY 11-7085



**Fig. 4.** PAH patient-derived endothelial cells exert elongated cilia that are non-responsive to pro- and anti-inflammatory treatment but respond to fluid shear stress. (a) MVEC from controls and PAH lungs were grown to confluency and did not receive additional treatment. Cilia length was quantified. Samples were normally distributed and significance was calculated using an unpaired student's t-test on their average lengths (donor = 3,  $n \geq 10$  per donor). (b) Healthy controls (gray) and PAH-derived MVEC (black) were stimulated with  $\text{TNF}\alpha$ , IL10 (both 10 ng/mL),  $\text{NF}\kappa\text{B}$  inhibitor (1  $\mu\text{M}$ ) alone, or a combination of  $\text{TNF}\alpha$  and a inhibitor. Cilia length was normalized to intra-experimental controls (basal) (donor  $\geq 3$ ,  $n \geq 10$  per donor and condition). (c) Control and PAH MVEC were subjected to high fluid shear stress (15  $\text{dyn}/\text{cm}^2$ ) for five days and cilia length was quantified. Representative phase-contrast and fluorescence staining are shown. Arrows indicate direction of flow. Cilia length was quantified and differences were calculated with an unpaired student's t-test.

showed similar responses like IL10 blocking the effects of  $\text{TNF}\alpha$  in controls but leaving elongated basal PAH cilia unchanged.

#### The sheared PAH endothelium shows increased cilia length

To further elucidate, if PAH MVEC have completely lost their cilia length regulation ability, fluid shear stress was tested as trigger (Fig. 4c). Primary cilia of PAH MVEC were found significantly elongated to  $4.87 \pm 0.46 \mu\text{m}$  compared to  $2.09 \pm 0.17 \mu\text{m}$  of controls ( $P < 0.001$ ) after five days of high shear stress. Additionally, while cilia of the sheared controls were localized towards the leading edge of the cells in the direction of flow and migration, cilia of PAH MVEC were found randomly positioned around the nucleus.

## Discussion

Microvascular EC from lungs of patients with PAH display elongated cilia. These cells are incapable to adapt their cilia length in response to pro- and anti-inflammatory cytokine stimulation.

Primary cilia play a pivotal role in vascular integrity and homeostasis. Cilia dysfunction is implicated in several pathologies, such as atherosclerosis and developmental diseases. Among the variety of ciliopathies, cilia can be elongated, truncated, less present, or completely absent.<sup>26</sup> In the Joubert syndrome, patients show less and shortened cilia.<sup>32</sup> In contrast, the phenotype of Meckel-Gruber syndrome is elongated cilia. Most patients with Bardet-Biedl syndrome show truncated cilia,<sup>26</sup> although patients with one specific subtype have elongated renal epithelial cilia.<sup>33</sup> In addition to

variations in cilia form across different ciliopathies, there is considerable heterogeneity in cilia function dependent on host cell and vascular bed. Renal cilia play a part in repair processes; they elongate upon renal injury and decrease in length during renal repair.<sup>34</sup> In chondrocytes, cilia shorten upon mechanical loading to minimize cell sensitivity to prolonged activation.<sup>35</sup> In mesenchymal stem cells, cilia elongation has been shown important for differentiation.<sup>36</sup> Taken together, cells change their cilia length in response to environmental cues. When this process is dysfunctional, initial adaptation is disturbed and homeostasis and repair impaired.

PAH is a fatal group of diseases with a high inflammatory status.<sup>4</sup> Cilia length increases in response to pro-inflammatory cytokines and was thereby proposed to mediate inflammatory responses.<sup>27</sup> Hence, we tested the pro-inflammatory cytokines TNF $\alpha$ , IL1 $\beta$ , and IFN $\gamma$  on healthy EC and all three cytokines provoked a similar increase in average cilia length to  $\sim 3\mu\text{m}$ . Thereby, not only the increase in length but also the length distribution was comparable between the various cytokines. This indicates a generic mechanism for cilia elongation upon inflammatory stimuli, since the cytokines themselves act through unique receptors and signaling pathways.<sup>5</sup> Moreover, similar effects were observed between mouse and human ECs, which point towards a conserved mechanism among species.

Chondrocytes have been shown to elongate cilia upon IL1 $\beta$  stimulation in a PKA/PKC-dependent manner.<sup>27</sup> In line with this finding, we show that PKA and PKC inhibition prevented cilia elongation after stimulation with the inflammatory cytokines. Interestingly, PKA/PKC signaling was not necessary to express cilia and basal length was not affected by PKA or PKC inhibition. Therefore, PKA/PKC signaling is predominantly needed for cilia elongation and might present a common integrator for various stimuli.

To consider, the individual stimuli might cause subtle differences in absolute cilia length. Using the PyT method to determine average cilia length keeps selection bias minimal. However, subtle length differences  $< 0.2\mu\text{m}$  might be underestimated.<sup>30</sup> What controls maximal cilia length and what absolute minimal change of length is functionally important remains to be resolved.

To reverse cilia length, removing the inflammatory stimulus was not sufficient, but application of the anti-inflammatory cytokine IL10 was needed to shorten the extended cilia back to basal levels demonstrating the need for active cues to switch EC from a pro- to anti-inflammatory state. In addition, IL10 (or NF $\kappa\text{B}$  inhibition) could block cilia elongation upon inflammatory cytokines and FK. Here, the effect was direct and reversible and left basal cilia length unaffected. Interestingly, the effect of IL10 was general for all tested cytokines and conserved in mouse and human EC. The precise mechanism of IL10 regulating cilia length remains to be determined. A direct effect of IL10 on PKA/PKC was not yet shown, while PKA itself is involved in IL10 production.<sup>37</sup> Recent literature showed that the effects of TNF $\alpha$ , INF $\gamma$ ,

and IL10 might, at least in part, be regulated independent of PKA via the SOCS (suppressor of cytokine signaling) pathway.<sup>37</sup> The authors found a synergistic activation of SOCS-3 when combining IL10 and cyclic AMP treatment that was independent from PKA activation.

When repeating the stimulations in MVEC from patients with PAH, we found that cilia were already elongated under basal conditions compared to controls. Therefore, we assumed that treatment with IL10 would reduce cilia length. To our surprise, PAH ECs did neither respond to pro- nor anti-inflammatory cytokines with a variation of cilia length. The basal elongation and loss of TNF $\alpha$  response might be an adaptation to the excessive amounts of pro-inflammatory cytokines produced by the diseased cells and the chronic state of inflammation in patient lungs.<sup>4</sup> However, the failure of IL10 to reduce cilia length might alternatively indicate that the basal elongation is independent from inflammatory signaling but rather due to metabolic changes.<sup>38</sup> Of interest, longer cilia are known to be associated with decreased proliferation<sup>39</sup> and thereby might be the cells attempt to counteract the hyper-proliferative phenotype characteristic to the PAH endothelium.<sup>40</sup> In accordance with this line of reasoning, PAH patients with high levels of IL10 have a worse prognosis.<sup>41</sup> On the contrary, higher IL10 levels are also suggested as compensatory mechanism in more advanced stages of the disease. In addition, administration of IL10 prevented development of PH in the monocrotaline rat model a model characterized by severe inflammation.<sup>42</sup>

Application of fluid shear stress (instead of cytokines) significantly altered cilia length of PAH cells, wherefore the loss of cilia length control seems specific to cytokine stimulation. Nevertheless, cilia of sheared PAH cells were longer than of controls and randomly localized around the nucleus, although they should be oriented towards the leading edge of the shear adapted cells, such as seen in the controls. This indicates defective mechano-responses and cell polarity in the sheared PAH cells, which might be a consequence of the defective shear sensing and delayed morphological adaptation that we reported earlier.<sup>29</sup> However, mono-motile cilia are linked to cell polarity, directed migration, and wound repair<sup>43</sup> pointing towards a generally dysfunctional PAH endothelial responsiveness to micro-environmental cues that might manifest through or, at least partly, be caused by the dysfunctional cilium.

To summarize, longer cilia are an inherent feature of PAH MVEC. The sustained elongation of primary cilia and a loss of length regulation function upon cytokine stimulation might represent an adaptive response to the chronic inflammation and act as a rescue mechanism to prevent further recruitment of blood-borne inflammatory cells into the vessel wall of patient lungs and decrease EC proliferative rates. However, the functional consequences of cilia non-responsiveness on intra-endothelial signaling and the surrounding tissue remain subject to further studies. Here, investigating defective cilia elongation in response to

inflammatory cytokines might reveal downstream cellular changes contributing to disease progression. Additionally, restoring cilia responses to anti-inflammatory treatment in ECs from PAH patients might decelerate disease progression by maintaining the EC phenotype.

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### Conflict of interest

The author(s) declare that there is no conflict of interest.

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