Supplementary materials and methods

Primary airway epithelial cell sampling

The patients and healthy samples were obtained between 2012 and 2015. Information on the PCD and respiratory control patients and healthy volunteers is summarised in Supplementary Table S1. The clinical history of PCD patients is summarised in Table 1. We performed 2 nasal brushings per PCD patient or healthy volunteer to obtain primary human airway epithelial cells, using 2 mm diameter cytology brushes (ConMed, Linvatec, UK). Brushes were placed into 1.5 ml Medium 199 (Gibco, Life Technologies, UK) and cells dislodged by vigorous pipetting. Cells were pelleted by centrifugation at 1,400 rpm (using a Jouan CR312 centrifuge) for 5 minutes.

Air-liquid interface culture

Costar™ culture-ware (Fisher Scientific, ThermoFisher Scientific, UK) was pre-coated with 300 mg/ml PureCol (Nutacon, NL) before use. Cells were re-suspended in 1 ml bronchial epithelial growth medium (BEGM) supplemented with Clonetics™ SingleQuots (Lonza, UK) and additional 50 Units/ml penicillin and 50 μg/ml streptomycin (Gibco, Life Technologies, UK), and 20 Units/ml nystatin (Sigma-Aldrich, UK) and seeded into wells of 12-well plates. Cultures were incubated at 37^{o}C (5% CO2, 100% relative humidity) and fed three times per week. At approximately 80% confluence the cells were passaged in 0.25% trypsin-EDTA solution (5 minutes at 37^{o}C), pelleted by centrifugation and resuspended in 5 ml BEGM into a T-25 flask. The cells were passaged again, at approximately 80% confluence, before the pellet was re-suspended in 1 ml air-liquid interface medium (ALI medium) containing a 1:1 ratio of BEBM:Dulbecco's modified eagle medium (Gibco, Life Technologies, UK) supplemented with growth Clonetics™ SingleQuots (using only 1/5th volume of the hEGF solution), antibiotics and anti-fungal additives. Cells (~500,000 per 0.5 ml) were seeded submerged into 12-well Costar Transwell inserts and when confluent taken to an air-liquid interface (ALI). At ALI the cells were fed baso-laterally with ALI medium, supplemented with additional 100 nM retinol (Sigma-Aldrich, UK) to promote basal epithelial cell differentiation

to a ciliated columnar cell phenotype (approximately 3 weeks). ALI-culture ciliation was confirmed by light microscopy and ALI-cultures used when cells were ciliated maximally (variable coverage). Polarized ciliated epithelial cells from PCD patients or non-PCD volunteers were cultured for two days at ALI in ALI-medium without antibiotic or anti-fungal treatment prior to NTHi co-culture.

Characterisation of NTHi on ciliated airway cells

Fluorescence in situ hybridization (*16S rRNA FISH*) was performed as previously described using the *H. influenzae* 16S ribosomal probe sequence: Hinf Cy3 labeled (Integrated DNA Technologies Inc, Lueven, Belgium) 5'- CCGCACTTTCATCTCCG-3' (16S [185-202]). ALI-cultured specimens were fixed with fresh 4% paraformaldehyde in HBSS followed by washes with HBSS and HBSS-ethanol (1:1), with subsequent 3-minute incubations in 80% and 100% ethanol. Cilia on epithelial cells were specifically immuno-labelled using an anti-β-tubulin antibody so that the localisation of FISH-labelled NTHi could be judged in proximity to cilia. Samples were rinsed with sterile PBS and blocked with (20% Newborn calf serum in DMEM, with 10% BSA and 0.05% Triton X) for 30 min, rinsed, and incubated with the T5293 anti-β-tubulin monoclonal antibody (Sigma-Aldrich, UK) (diluted 1:2,000 in blocking buffer) to specifically label cilia. Samples were washed 3 times before incubation for 90 minutes with the secondary Alexa Fluor® 594 chicken anti-mouse IgG antibody (A21201, Molecular Probes®, Life Technologies, UK). Epithelial cell nuclei were counterstained for 30 minutes with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Life Technologies, UK) diluted 1:360 in 1x PBS before being washed in 1xPBS and mounted on glass slides. Samples were analysed by confocal microscopy (SP5, Leica Microsystems, UK) at x400 magnification and every consecutive field of view was assessed across the width of the membrane. The volume of adherent NTHi bacterial micro-colonies on the epithelial cell layers were calculated by Volocity 3D image analysis software (version 6.0.1, PerkinElmer, UK).

Cytokine and LL-37 responses to NTHi infection

Baso-lateral medium from ALI-cultures was frozen before inoculation and daily following co-culture with NTHi. We measured basic fibroblast growth factor (FGF-b), granulocyte- and granulocyte-macrophage- colony stimulating factors (G-CSF, GM-CSF), Interleukins- (IL) -6

and -8, IL-1receptor antagonist (IL-1ra), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor (TNF-α) and vascular endothelial growth factor (VEGF) by Fluorokine® Human MultiAnalyte Profiling (MAP) assay. Cytokine specific antibodies were pre-coated on colour-coded polystyrene beads and incubated with thawed supernatants, blank controls and standards (50 µl per 96-well in duplicate) for 3 hours. After washing away unbound sample, a cocktail of biotinylated antibodies specific to each cytokine was added for 1 hour, washed, and a streptavidin-phycoerythrin (streptavidin-PE) conjugate was added for 30 minutes to bind to the biotinylated detection antibodies. All incubations were done at room temperature and in the dark on a horizontal orbital shaker (550 rpm). Unbound streptavidin-PE was finally washed away before analysis with a Bio-Plex® 200 Analyser (R&D systems®, UK). Lasers detected bead type and PE signal, directly proportional to the amount of cytokine bound [33]. Interleukin-8 was measured separately by human IL-8 DuoSet ELISA kit (DY208, R&D systems®, UK). Basolateral supernatants were diluted 1:5 with 1% bovine serum albumin in 1x phosphate buffered saline (1% BSA/PBS) and 50 µl incubated at room temperature for 2 hours in duplicate wells of a 96-well plate (pre-coated overnight at room temperature with 4 µg/ml monoclonal anti-human IL-8 antibody, washed and blocked in 1% BSA/PBS for 1 hour at room temperature prior to use) with blank controls and standards. After washing samples, controls and standards were incubated for 2 hours at room temperature with 20 ng/ml biotinylated goat anti-human IL-8, washed and further incubated for 45 minutes with 50 µl streptavidin-horseradish peroxidase (HRP). An HRP substrate (50 µl 1:1 solution of H_2O_2 :tetramethybenzidine) was added for 20 minutes and the reaction was stopped by addition of 50 µl 2N H_2SO_4. Light emissions (at a wavelength of 450 nm) were detected in wells by a ThermoMax Microplate Reader (Molecular Devices, US) and mean IL-8 concentration was determined from a standard curve.

Nitric oxide production by airway epithelial cells

Transwell membranes of live ALI-cultured epithelial cells were treated with 10 µM 4-amino-5-methylamino-2',7'-difluororescein diacetate (DAF-FM) [34], which was added directly into the basolateral medium (at 37^{o}C) of Transwells inserts and imaged by live confocal microscopy (SP5 TCS Confocal Microscope, Leica Microsystems UK) to assess the presence of NO. DAF-FM diacetate is weakly fluorescent until the diacetate is cleaved in the presence

of NO to produce highly fluorescence DAF-FM. Images were collected within an hour of exposure to DAF-FM diacetate.

Immunofluorescence labelling was used to localise NO synthase (NOS) isoenzymes, (neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) in fixed epithelial cell cultures [32] using confocal microscopy. Transwell membranes sections containing cells were fixed in ice cold methanol for 10 minutes, blocked for 30 minutes in 1% BSA/PBS containing additional 0.05% triton X-100 (blocking buffer). Calbiochem rabbit anti-human polyclonal NOS antibodies (Merck, UK) to nNOS (1414–1434), iNOS (1131–1144) and eNOS (599–913) were diluted 1:200 (from 1 mg/ml stock) in blocking buffer and incubated with samples for 90 minutes at room temperature. Excess unbound antibody was washed away before incubating samples in the dark for a further 90 minutes at room temperature with a secondary Alexa488 conjugated goat anti-rabbit antibody (Invitrogen, UK) diluted 1:500 (from 2 mg/ml stock). We have previously demonstrated that nNOS co-localised with β-tubulin labelling in cilia [32]. Therefore for nNOS labelled samples, cilia were co-localised with a T5293 monoclonal anti-human β-tubulin antibody (Sigma-Aldrich, UK) diluted 1:2000, followed by an Alexa594 conjugated chicken anti-mouse antibody (Molecular Probes, Life Technologies, UK) diluted 1:500 (from 2 mg/ml stock) [32]. Cell nuclei were stained for 30 minutes with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) diluted 1:360 in 1x PBS (Molecular Probes, Life Technologies, UK). Slides were mounted under coverslips with Mowiol. Fluorescence labelling was imaged by confocal microscopy.