ONLINE DATA SUPPLEMENT

Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence

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SUPPLEMENTAL METHODS

Collection of cells

Primary bronchial epithelial cells (PBEC) were obtained from tumour-free resected lung tissue at the Leiden University Medical Center, Leiden, the Netherlands. For this, bronchial epithelial cells were isolated from a bronchial ring by enzymatic digestion for 2 h at 37 °C with 0.18% (w/v) proteinase type XIV (Sigma-Aldrich, St. Louis, MO, USA) in $Ca^{2+/}Mg^{2+}$-free Hank's Balanced Salt Solution (Life Technologies Europe B.V., Bleiswijk, The Netherlands). Next, the obtained cell fraction was expanded in serum-free keratinocyte medium (KSFM, Life Technologies Europe B.V.) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 μg/ml bovine pituitary extract (Life Technologies Europe B.V.), 1 μM isoproterenol (Sigma-Aldrich), 100 U/mL Penicillin (Lonza, Verviers, Belgium), 100 μg/ml Streptomycin (Lonza) and 5 μg/ml Ciproxin. Upon reaching confluence, cells were trypsinized in 0.03% (w/v) trypsin (Difco, Detroit, USA), 0.01% (w/v) EDTA (BDH, Poole, England), 0.1% glucose (BDH) in PBS and stored in liquid nitrogen until further use. For our cultures, cells were thawed in KSFM medium supplemented with the above mentioned supplements until near confluence, seeded on semipermeable transwell inserts with 0.4 μm pore size (Corning Costar, Cambridge, USA) that were coated with a mixture of bovine serum albumin, collagen and fibronectin and cultured as described [1].

RNA isolation, cDNA synthesis and qPCR

Cells were lysed using lysis buffer from Promega, Leiden, the Netherlands. Next, RNA was extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, DE). cDNA synthesis was performed using oligo dT primers (Qiagen, Venlo, the Netherlands) and M-MLV Polymerase (Promega) in the presence of RNAsin (Promega). For qPCR analysis,

diluted cDNA was mixed with primers (sTable 1) and iQ™ SYBR® Green Supermix (Bio-Rad, Veenendaal, the Netherlands). Reactions were performed in triplicate and results were corrected for the geometric mean of expression of 2-3 reference genes selected using the Genorm method. Expression values were determined by the relative gene expression of a standard curve as determined by CFX manager software (Bio-Rad).

Confocal microscopy

Following fixation with 1% PFA, cell culture inserts and/or cytospins containing luminal epithelial cells were treated with methanol for 10 min at 4 °C, washed with PBS and cells were permeabilized with 1% w/v BSA, 0.3% v/v Triton-X100 in PBS (PBT) for 30 min at 4 °C. After washing with PBS, cells were pre-treated with SFX-signal enhancer (Life Technologies Europe B.V.) followed by incubation with primary antibodies in PBT for 1 h at RT (sTable 2). Next inserts were washed in PBS and incubated with an Alexa Fluor 488 or 568-labeled secondary antibody (Alexa Fluor 488 donkey-anti--mouse IgG; Alexa Fluor 568 donkey--anti-rabbit IgG, Life Technologies Europe B.V.) together with DAPI in PBT for 30 min at RT. Images were acquired using a TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems B.V., Eindhoven, The Netherlands) and LAS AF Lite software (Leica Microsystems B.V.).

Antibacterial activity assay

Direct antimicrobial activity was assessed in cultures of ALI-PBEC that were exposed daily to whole cigarette smoke or air controls for 13 days, followed by replacement with antibiotics-free cell culture medium for an additional 48 h period. *Moraxella catarrhalis* strain LUH2760 and *Klebsiella pneumoniae* strain LUH2754 were cultured in Tryptic Soy broth (TSB) while shaking overnight at 37^0 C. Next, the overnight cultures were transferred

into fresh TSB medium (1/50 dilution) and incubated for 4 h at 37°C -while shaking- to obtain mid log-phase-growing bacteria. Bacterial concentrations of log-phase cultures were determined by $OD_{600\ nm}$ measurements, pre-diluted in PBS and final dilution was made in antibiotics-free cell culture medium. Twenty µl of bacterial suspension was added on the apical surface of the cells at a concentration of ~$6x10^5$/ml CFU/ml for *M. catarrhalis* and ~$1x10^4$ CFU/ml for *K. pneumoniae* and incubated at 37°C, 5% CO_2 for 2 h. Hereafter, membranes containing the cells with bacteria were dissected from the inserts and placed into tubes containing sterile glass beads and 1% TSB in PBS. Next cells were disrupted by using a minilys personal homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 times 30 s and kept on ice in between. Serial dilutions of both bacterial suspensions were plated on Tryptic Soy Sheep blood (TSS) agar plates (Biomerieux, Zaltbommel, The Netherlands), and incubated overnight at 37°C to assess surviving bacteria by CFU determination.

ELISA

CXCL8/IL-8 production by ALI-PBEC was determined in the basal medium by use of the CXCL8/IL-8 Duoset kit from R&D (MN, U.S.A.). hBD-1 was measured in the apical wash and in the basal medium using the hBD-1 kit from Peprotech (London, U.K.) and SLPI was measured as described[2].

Trans-epithelial electrical resistance

Epithelial barrier integrity of ALI-PBEC cultures was determined during cell differentiation by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS (Millipore, Bedford, MA). TEER values were shown as Ω*cm^2 and calculated as TEER = (measured value – background value) *surface transwell insert in cm^2.

Transcytosis assay

Transcytosis capacity of the epithelial cultures was assessed in cultures exposed daily to whole cigarette smoke for 13 days, or air as a control. Dimeric IgA was added to the basal compartment of the cell cultures and 24 h thereafter, apical washes (PBS) were collected and stored at -20°C for further analysis. Apical washes were assessed for secretory (S-)IgA levels by sandwich ELISA[3].

***Changes in cell culture media for the experiments described in Fig. 4B, sFig. 8C-D and sFig. 2**

Due to ongoing optimisation of our cell culture methods, several relatively minor changes in cell culture conditions have been made in the cultures that were part of the experiments described in Fig. 4B, Fig. 8C-D and sFig. 2 that are different from our previously published cell culture media [1] that were used for the other experiments that were part of this study. For these experiments, culture media of PBEC cultures (when seeded on inserts) comprised of BEpiCM-b (Bronchial Epithelial Cell Medium-basal, ScienCell, Carlsbad, CA, U.S.A.) diluted 1:1 with DMEM from Stemcell Technologies (Vancouver, Canada) with bronchial epithelial cell growth supplements from ScienCell, further supplemented with 50 nM EC-23 (Tocris, Bio-Techne Ltd. Abingdon, U.K.); EC-23 is a photostable analogue of retinoic acid. The observed effects of chronic CS exposure on airway epithelial HDP expression and differentiation were validated in chronic CS-exposed cells cultured with this new medium composition.

SUPPLEMENTAL RESULTS

Reduced respiratory host defence protein levels by chronic CS exposure are not a consequence of toxicity

To exclude that possible toxic effects of the chronic CS exposure affected the observations we made, we performed a selection of additional experiments. We assessed trans-epithelial electrical resistance (TEER) of ALI-PBEC exposed to CS or air as a control: results showed that chronic CS-exposed ALI-PBEC displayed a slight but non-significant decrease of TEER in CS-exposed cultures in the first week of exposure, and a similar TEER as the air-exposed controls in the second week of exposure up until day 19 (sFig. 4A). LDH levels in chronic CS-exposed cell cultures were not increased, but rather reduced compared to air-exposed cultures (sFig. 4B). Indirect evidence for absence of marked cytotoxicity was the observation that chronic CS exposure significantly increased secretion of the neutrophil-attracting chemokine IL-8 at 13 days of differentiation in CS-exposed cells compared to air-exposed controls (sFig. 4C). The cell size in chronic CS-exposed cell cultures seemed bigger in some donors, but not all, compared to air-exposed cultures, but no other morphological changes could be detected by microscopic inspection (an example illustrated in sFig. 4D). Together these data show that chronic CS-exposure-mediated loss of specific HDP expression by ALI-PBEC is unlikely to be a result of toxicity. This conclusion is further supported by measurements on the expression of a selection of inducible HDPs**.** We previously reported induction of *RNASE7* mRNA and protein in ALI-PBEC upon acute exposure to one cigarette [1], in line with these findings, chronic CS exposure also caused a progressive increase in *RNASE7* compared to air-exposed controls (sFig. 4E). In addition also increased *CAMP* gene expression (LL-37-coding gene) was detected in chronic CS-exposed cultures (sFig. 4E). In

contrast, we did not observe a significant difference in the expression of *DEFB4* (human β-defensin 2) (sFig. 4E).

SUPPLEMENTAL TABLES

sTable 1. Primer sequences.

Gene	forward sequence (5' to 3')	reverse sequence (5' to 3')
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
B2M	GACCACTTACGTTCATTGACTCC	CAGGGTTTCATCATACAGCCAT
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
SLPI	CCA GGG AAG AAG AGA TGT TG	CCT CCA TAT GGC AGG AAT C
BPIFA1	CTTGGCCTTGTGCAGAGC	CAACAGACTTGCACCGACC
BPIFB1	CAGTGCCATGCGGGAAAAG	GCTGGAGGATGTTAGCTGTGA
PIGR	CTCTCTGGAGGACCACCGT	CAGCCGTGACATTCCCTG
LCN2	CCTCAGACCTGATCCCAGC	CAGGACGGAGGTGACATTGTA
KRT5	AGGAGTTGGACCAGTCAACAT	TGGAGTAGTAGCTTCCACTGC
TP63	CCACCTGGACGTATTCCACTG	TCGAATCAAATGACTAGGAGGGG
KRT8	TCCTCAGGCAGCTATATGAAGAG	GGTTGGCAATATCCTCGTACTGT
FOXJ1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC
SCGB1A1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA
MUC5B	GGGCTTTGACAAGAGAGT	AGGATGGTCGTGTTGATGCG
MUC5AC	CCTTCGACGGACAGAGCTAC	TCTCGGTGACAACACGAAAG
JAG2	TGGGACTGGGACAACGATAC	AGTGGCGCTGTAGTAGTTCTC
DLL1	GACGAACACTACTACGGAGAGG	AGCCAGGGTTGCACACTTT
NOTCH1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
NOTCH2	CCTTCCACTGTGAGTGTCTGA	AGGTAGCATCATTCTGGCAGG
NOTCH3	CGTGGCTTCTTTCTACTGTGC	CGTTCACCGGATTTGTGTCAC
NOTCH4	GATGGGCTGGACACCTACAC	CACACGCAGTGAAAGCTACCA

HES1	CCTGTCATCCCCGTCTACAC	CACATGGAGTCCGCCGTAA
HEY1	ATCTGCTAAGCTAGAAAAAGCCG	GTGCGCGTCAAAGTAACCT
JAG1	GCCGAGGTCCTATACGTTGC	CCGAGTGAGAAGCCTTTTCAA
MAML1	CCCCAGTGAGTCATTTCCTCT	GAGGTTGCTTTGCGATATGGA
MAML3	CTTAGGACCTCCCTCTAGTCCA	GTTTTGGTTGTTAAAGGCTTGGG
RNASE7	CCAAGGGCATGACCTCATCAC	ACCGTTTTGTGTGCTTGTTAATG
DEFB4	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC

sTable 2. Antibodies used for confocal imaging

Antibody	**Supplier**	**Catalog #**	**species**	**Antibody dilution**
CK-8	Novus Biologicals	NBP2-34266	mouse	1/100
pIgR	R&D Systems	MAB27171	mouse	1/100
p63	Abcam	ab124762	rabbit	1/100
sPLUNC	Hycult Biotech	HM2314	mouse	1/100
SLPI	Hycult Biotech	HM2037	mouse	1/100
Mucin 5AC	Labvision Neomarkers	MS-145-P1	mouse	1/1000
CC16	Hycult Biotech	HM2178	mouse	1/50
Acetylated α-Tubulin	Sigma Aldrich	T6793	mouse	1/100

SUPPLEMENTAL FIGURE LEGENDS

sFigure 1. Details of the cigarette smoke exposure design and procedure.

Approximately 4 h before the cigarette smoke exposure, the apical surface of the cell cultures were washed with PBS and every other day the basal medium was replaced. Next, the cells were placed in the exposure chamber and the lid was removed. The closed exposure chamber was then infused with cigarette smoke from 1 cigarette for 4-5 min, or normal air in the control chamber. Hereafter, the tubing from the cigarette is clamped and vents on the exposure chamber connecting to the space in the incubator are opened and the air is refreshed with air from the incubator for an additional 10 min. The smoke-containing air is removed via separate tubing outside the incubator into a fume hood. After the exposure and refreshing, the chamber is opened, the lid placed back on the cells and the cells are placed back in a separate incubator for 20 h when the procedure is repeated.

sFigure 2. Expression of respiratory host defence proteins in the luminal cell fraction of air-liquid interface-differentiated primary bronchial epithelial cells (PBEC).

PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface (ALI). After 3 weeks of differentiation, luminal and basal cell fractions were separated. Cells were fixed in 1% paraformaldehyde and cytospins were prepared of the luminal cell enriched fraction. Luminal cell cytospins and the basal cell enriched fraction located on the transwell inserts were subsequently stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI, sPLUNC, CC16 and acetylated α-tubulin (all green) and DAPI for nuclear staining (blue). Scale bars equal 50 µm. Images shown are representative for results obtained with cells from 3 different donors.

sFigure 3. Persistence of cigarette smoke-induced changes in airway epithelial HDP expression and cellular composition.

(A) Primary bronchial epithelial cells (PBEC) were cultured at the air-liquid interface (ALI) and exposed during differentiation for 13 consecutive days to whole CS after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: *TP63*, *KRT5* (basal cells) and *KRT8* (intermediate cells), the HDPs: *DEFB1* (human beta-defensin 1) and *LCN2* (lipocalin 2). Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, **** $p<0.0001$. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which the cells were lysed and similar analyzed as in (A). Grey bars: T=0 (day 7), open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B, B2M* and *RPL13A*. n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

sFigure 4. Chronic cigarette smoke exposure of airway epithelial cell cultures does not lead to cell toxicity

Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. (A) Each day trans-epithelial electrical resistance (TEER) measurements were performed ~18 h after the previous CS exposure. Data are expressed as $\Omega*cm^2$. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=8 different donors. Significance was determined using a two-way ANOVA and Bonferroni post-hoc test. (B) At several time-points during differentiation apical washes were collected and assessed for LDH content. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$ between AIR and CS. (C) At day 7 and Day 13 (~18 h after the last CS exposure), IL-8 protein levels were assessed by ELISA in the basal medium of the ALI-PBEC cultures. Open bars are air-exposed controls, grey bars are chronic CS-exposed cultures; n=8 different donors. Statistical differences were tested using a paired t-test. * $p<0.05$. (D) Illustrating phase contrast light microscopy images showing the increasing effects of 13 days of CS exposure (CS) or air as a control (AIR) on cell morphology in some donors. (E) At several time-points during differentiation, cells were lysed and RNA was isolated followed by cDNA synthesis, to assess gene expression of *RNASE7, CAMP* (LL-37) and *DEFB4* (human beta defensin-2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*, n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ between AIR and CS.

References

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