Airway epithelial innate host defence in chronic obstructive pulmonary disease

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CHAPTER 4

Antibacterial defense of human airway epithelial cells from chronic obstructive pulmonary disease patients induced by acute exposure to nontypeable *Haemophilus influenzae*: modulation by cigarette smoke.

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ABSTRACT

Antimicrobial proteins and peptides (AMPs) are a central component of the antibacterial activity of airway epithelial cells. It has been proposed that a decrease in antibacterial lung defense contributes to an increased susceptibility to microbial infection of smokers and patients with chronic obstructive pulmonary disease (COPD). Whether reduced AMP expression in the epithelium contributes to this lower defense is however largely unknown. We investigated the bacterial killing activity and expression of AMPs by air-liquid interface cultured primary bronchial epithelial cells from COPD patients and non-COPD (ex)smokers that were stimulated with non-typeable *Haemophilus influenzae* (NTHi). In addition, the effect of cigarette smoke on AMP expression and activation of signaling pathways was determined. COPD cell cultures displayed reduced antibacterial activity, whereas smoke exposure suppressed NTHi-induced expression of AMPs and further increased IL-8 expression in COPD and non-COPD cultures. Moreover, smoke exposure impaired NTHi-induced activation of NF-κB, but not MAP-kinase signaling. Our findings demonstrate that the antibacterial activity of cultured airway epithelial cells induced by acute bacterial exposure is reduced in COPD and suppressed by cigarette smoke, whereas inflammatory responses persisted. These findings help to explain the imbalance between protective antibacterial and destructive inflammatory innate immune responses in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a severe inflammatory lung disorder in which colonization and infections with opportunistic respiratory bacteria are a major disease hallmark (1). It is hypothesized that a vicious circle of attenuated antibacterial lung defense, enhanced bacterial colonization, and induction of lung inflammation and injury, contribute to the disease (2). However, the underlying mechanisms remain incompletely understood. Smoking is the main risk factor for COPD development, and is furthermore associated with an increased susceptibility to respiratory infections (3). Despite the strong association, only a subpopulation of approximately 15-20% of the smokers develops the disease. This suggests that there are differences between susceptible smokers and smokers with a normal lung function that may help to explain disease etiology. These differences may persist in cell culture (4, 5), for that reason, comparison of cell cultures from COPD patients and non-COPD smoker may reveal differences in host defense activities that may help to explain the increased microbial susceptibility.

Airway epithelial cells contribute to innate lung defense by displaying direct antibacterial activity, which is mediated in part by the production of antimicrobial proteins and peptides (AMPs) (6, 7). In addition to AMPs that are expressed at a steady-state basis, others are induced for example upon recognition of microbes or microbial structures by pattern recognition receptors. This response is highly similar amongst species, and murine models have shown that the inducible antibacterial activity of airway epithelial cells provides full protection against various pathogenic microbes without further involvement of immune cells (8). Impairment of this induced activity in COPD might therefore contribute to the increased susceptibility to infection with respiratory pathogens. Observational studies in lung tissue, airway secretions and tracheal washings have shown that the expression of several AMPs is reduced in smokers and patients with COPD, including the microbial-induced antimicrobial peptide human beta-defensin-2 (hBD-2) (9-12). Moreover, it has been demonstrated that cigarette smoke exposure attenuated the antibacterial activity and microbial-induced expression of hBD-2 in cultured airway epithelial cells (12-14). This suggests that the inducible antibacterial activity is affected in airway epithelial cells from smokers and COPD patients. However, it is unclear whether this activity differs between airway epithelial cells from non-COPD smokers and COPD patients. Interestingly, microbial-induced expression of the pro-inflammatory mediator IL-8 is not reduced by cigarette smoke (14, 15). Conflicting to this, cellular signal transduction pathways regulating the expression of AMPs and pro-inflammatory mediators largely overlap (16, 17), and it is unknown whether imbalances in these signaling pathways reflect the alterations in AMP and pro-inflammatory mediator expression.

To gain more insight in the role of the inducible antibacterial defense function of airway epithelial cells in COPD, we examined the antibacterial activity and expression of AMPs following acute bacterial exposure of cultured airway epithelial cells from mild-to-moderate COPD patients and (ex)smokers with a normal lung function (non-COPD). To understand the mechanism underling reduced AMP expression, we determined the effect of cigarette smoke on microbial-induced expression of AMPs, and the regulation of cellular signal transduction pathways.

MATERIALS AND METHODS

Primary bronchial epithelial cell cultures and stimuli

Primary bronchial epithelial cells (PBEC) were isolated from tumor-free resected lung tissue and cultured in an air liquid interface model (further referred to as ALI-PBEC) to obtain mucociliary-differentiated cultures, essentially as previously described (14, 18). PBEC cultures were used from a total of 28 patients, all undergoing lung resection surgery for lung cancer. Clinical history and lung function data were obtained from anonymized patients. Disease status was determined based on lung function according to the Global Initiative for Chronic Obstructive Lung Disease classification (19). The patients included 15 COPD and 13 non-COPD subjects (Tables 1 and 2 for indicated experiments). Both groups included

Table 1: Patient characteristics bacterial killing assay.

	COPD	non-COPD	p-value
N=	5	5	
Gender (Females/Males)	2/3	2/3	
Age, years	66±11	64±7	0.6
FEV_1 , % predicted	68±17	96±14	0.05
FEV_1/FVC %	58±7	78±4	< 0.01

Characteristics of COPD and non-COPD patients used the bacterial killing assay.

Table 2: Patient characteristics NTHi-induced AMP expression.

	COPD	non-COPD	p-value
N=	5	5	
Gender (Females/Males)	2/3	2/3	
Age, years	66±11	64±7	0.6
FEV_1 , % predicted	68±17	96±14	0.05
FEV_1/FVC %	58±7	78±4	< 0.01

Characteristics of COPD and non-COPD patients. Age and lung function are shown as means ± SEM. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test. Abbreviations: COPD = chronic obstructive pulmonary disease, FEV_1= Forced expiratory volume in one second, FVC = Forced vital capacity.

current smokers and ex-smokers. Non-typeable *Haemophilus influenzae* (NTHi) strain D1 (20) was cultured and UV-inactivated as described earlier (14). UV-inactivated NTHi was applied to the apical surface of ALI-PBEC in a volume of 100 µl; PBS was used for dilutions and as negative control. ALI-PBEC were exposed to mainstream cigarette smoke (CS) from 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) using an exposure model previously described (14, 15). In brief, epithelial cultures were placed in air (control) or CS exposure chambers, localized in a tissue incubator at 37°C and 5% CO2. In these exposure chambers, ALI-PBEC were respectively exposed to air or CS from one cigarette (approximately 2 mg of CS particles), which was infused by a mechanical pump with a continuous flow of 1 liter/minute for a period of 4-5 minutes. Residual CS was removed by flushing the chamber

with air derived from the incubator for 10 minutes. After exposure cells were stimulated at the apical surface with UV-inactivated NTHi or PBS (negative control).

Bacterial killing assay

The bacterial killing assay was adapted from Pezzulo *et al.* (21). Instead of golden grids, bacteria were linked to glass coverslips. 6 mm round coverslips were rinsed in ethanol, dried at room temperature and subsequently silanized with 2% (v/v) 3-Aminopropyltriethoxysilane (APTES, $H_2N(CH_2)_3Si(OC_2H_5)_3$, Sigma-Aldrich) solution in acetone for 10 seconds. After rinsing in H2O for 10 minutes and drying at room temperature, coverslips were immersed in 1 mM 11-mercaptoundecanoic acid (MUA, $HS(CH_2)_{10}COOH$, Sigma-Aldrich) for 30 minutes at room temperature. Afterwards, coverslips were incubated in 0.1 M N-hydroxysuccinimide (NHS) and 0.1 M 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC) (1:2 molar ratio) (both Sigma-Aldrich) for 30 minutes, and subsequently coated for 30 minutes with 10 μg/mL streptavidin (Sigma Aldrich) in PBS. Next, the coverslips were washed and immersed with 1 M glycine for 30 minutes. $1*10^8$ CFU/ml (OD_{600}) of log phase growth cultured NTHi was incubated in 0.88 mg/ml mg/mL N-hydroxysulfosuccinimide-biotin (sulfo-NHS-biotin, Thermo Scientific) for 30 minutes on ice. Next, biotin-labeled NTHi was linked to the surface of the streptavidin-coated glass coverslips in PBS for 30 minutes and subsequently washed two times in PBS and finally in 0.01M Phosphate buffer pH 7.4, to remove the unbound bacteria. Bacterial killing activity was determined of ALI-PBEC that were pre-stimulated with UV-inactivated NTHi or PBS (untreated control), which was applied at the apical surface. After 6 hours of incubation, cultures were washed with PBS and incubated for 48 hours. Afterwards, NTHi coated coverslips were placed on the apical surface of ALI-PBEC for 1 minute. Coverslips were subsequently stained for 30 seconds with SYTO 9 and propidium iodide (both Life Technologies, Darmstadt, Germany), to visualize live and dead bacteria respectively. Coverslips were mounted on microscopic slides and covered in Baclight mounting oil (Life Technologies). Digital images were made with a Zeiss Axio Scope A1 fluorescent microscope and Zeiss Axiocam mRc 5 camera (Carl Zeiss Microscopy, Göttingen, Germany). The number of live and dead bacterial was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). The bacterial killing activity was determined by calculating the percentage of dead bacteria.

Microarray gene expression analysis

Primary bronchial epithelial cells from 6 different donors were cultured in submerged conditions as previously described (14). Cells were left unstimulated or triggered with UV-killed NTHi. RNA from these cultures were then isolated at two timepoints: early (6 hour) and late (24 hour) and subsequently subject to whole genome profiling of gene expression by microarray (Affymetrix GeneTitan platform).

Quantitative real-time PCR (qPCR)

RNA was isolated from ALI-PBEC using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. RNA quantities were determined using the Nanodrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE) and cDNA was synthesized by reverse transcription polymerase chain reaction (PCR) of 1 μg of RNA, using oligo(dT) primers (Qiagen) and Moloney murine leukemia virus

(M-MLV) polymerase (Promega, Leiden, The Netherlands). qPCR was done using IQ SYBR green supermix (Bio-Rad) and a CFX-384 real-time PCR detection system (Bio-Rad). qPCR reactions were performed using the primers shown in Table 3. The housekeeping genes RPL13A and ATP5B were selected using the "Genorm method" (Genorm; Primer Design, Southampton, United Kingdom). Bio-Rad CFX manager 3.0 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

Table 3: qPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
DEFB4	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
CCL20	GCAAGCAACTTTGACTGCTG	TGGGCTATGTCCAATTCCAT
LCN2	CCTCAGACCTGATCCCAGC	CAGGACGGAGGTGACATTGTA
S100A7	ACGTGATGACAAGATTGACAAGC	GCGAGGTAATTTGTGCCCTTT
TLR2	TCTCGCAGTTCCAAACATTCCAC	TTTATCGTCTTCCTGCTTCAAGCC
ATF3	CCTCTGCGCTGGAATCAGTC	TTCTTTCTCGTCGCCTCTTTTT
pri-mir-147b	TTCATGACTGTGGCGGCGGG	GGCGAGGGCTCGTCATTTGGT
A20	TCCTCAGGCTTTGTATTTGAGC	TGTGTATCGGTGCATGGTTTTA
DEFB3	AGCCTAGCAGCTATGAGGATC	CTTCGGCAGCATTTTGCGCCA
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC
SLPI	GAGATGTTGTCCTGACACTTGTG	AGGCTTCCTCCTTGTTGGGT
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
IL8	CAGCCTTCCTGATTTCTG	CACTTCTCCACAACCCTCTGC
IL6	CAGAGCTGTGCAGATGAGTACA	GATGAGTTGTCATGTCCTGCAG
NFKBIA	TGTGCTTCGAGTGACTGACC	TCACCCCACATCACTGAACG
ZC3H12A	GTACGTCTCCCAGGATTGCC	GGGACTGTAGCCCGTGTAAG
NFKBIZ	AGAGGCCCCTTTCAAGGTGT	TCCATCAGACAACGAATCGGG
FOS	CCTAACCGCCACGATGATGT	TCTGCGGGTGAGTGGTAGTA
JUN	TCCTGCCCAGTGTTGTTTGT	GACTTCTCAGTGGGCTGTCC
FOSL1	AACCGGAGGAAGGAACTGAC	CTGCAGCCCAGATTTCTCAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT

ELISA

Secretion of innate immune mediators by ALI-PBEC was determined in the apical surface liquid, collected by washing the apical surface with 100 µl PBS for 15 minutes, and in the basal medium as indicated. The secretion of IL-8 (Sanquin, Amsterdam, The Netherlands) and CCL20 (R&D, Minneapolis, MN, USA) was determined by ELISA following the manufacturer's protocol. Reagent for hBD-2 detection was a generous gift of D. Proud (Calgary, Canada), and the hBD-2 ELISA was conducted as previously described (22). The optical density values were measured with a microplate reader (Bio-Rad).

Trans-epithelial electrical resistance

The epithelial barrier integrity of ALI-PBEC cultures was determined by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS (Millipore, Bedford, MA, USA).

Western blot

ALI-PBEC were washed 3 times with cold PBS and cell lysis was done in lysis buffer consisting of 150 mM NaCl, 50 mM Tris HCl pH 7.4, 0.1% NP-40 (v/v) and EDTA-free protease inhibitor cocktail (Roche, Basel, Switserland) . Samples were subsequently mixed with sample buffer, consisting of 0.5 M Tris pH 6.8, 10% SDS (w/v), 20% Glycerol (v/v), 0.02% Bromophenolblue and 50 mM DTT. Nuclear fractions were isolated using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufactur's protocol. Protein samples were separated by SDS-PAGE gel electrophoresis on 10% glycine-based gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% (w/v) skim-milk (Sigma-Aldrich) in PBS/0.05% (v/v) Tween-20 and stained with primary antibodies in 5% (w/v) BSA PBS/0.05% (v/v) Tween-20 overnight at 4°C. Antibodies were used to detect p- IKKα/β, IKKβ, IκB-α, p- and t-ERK1/2, p- and t-p38, TBP (all Cell Signaling, Beverly, MA, USA), p50, p65 (both Santa Cruz), and β-actin (Leica), Next, membranes were stained with secondary HRP-labelled antibody (Sigma-Aldrich) in 5% (w/v) skim-milk (Sigma-Aldrich) in PBS/0.05% (v/v) Tween-20 for 1 hour. Afterwards, membranes were developed with enhanced chemiluminescence substrate (Thermo Scientific). Intensity of bands were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA)

Immunofluorescence confocal microscopy

ALI-PBEC were fixed in 4% paraformaldehyde for 30 minutes, permeabilized in 100% methanol for 10 minutes at 4°C, and blocked in blocking solution consisting of 5% (w/v) BSA, 0.3% (v/v) Triton X in PBS. In between each step, cells were washed 3 times with PBS. Afterwards, the PBEC-containing filters were cut from the Transwell using a razorblade and subsequently incubated overnight at 4°C with anti-mouse p50 or anti-rabbit p65 primary antibodies (both Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking solution. Next, filters were washed 3 times in PBS and stained with goat anti-rabbit AlexaFluor 568 or goat anti-mouse AlexaFluor 488 (both Life Technologies) respectively, and DAPI as nuclear staining. Secondary antibodies and DAPI were diluted in blocking solution and incubated in the dark for 30 minutes at room temperature. Afterwards, the filters were washed three times with PBS and mounted on coverslips with Vectashield Hard Set Mounting Medium (Vector Lab, Burlingame, CA, USA). Images were made using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems) and processed using the Leica Application Suite Advanced Fluorescence software (LAS AF; Leica Microsystems).

Data analysis

Statistical analysis was performed in GraphPad PRISM 6.0 (GraphPad Software Inc., La Jolla, Ca). Analysis of differences was conducted with a one/two-way repeated measurements ANOVA and Bonferroni post-hoc test and (un)paired Student's t-test as indicated. Differences were considered significant with p-values < 0.05.

RESULTS

Lower NTHi-induced antibacterial activity by COPD airway epithelial cells

We first determined the bacterial killing activity of cultured ALI-PBEC from COPD patients and non-COPD (ex)smokers. ALI-PBEC were first stimulated at the apical surface with UV-inactivated NTHi or PBS (negative control) to investigate microbial exposure-induced and baseline antibacterial activity. 6 hours after stimulation, the apical surface was washed and cells were incubated for an additional 42 hours prior to assessment of the antibacterial activity (Figure 1A). A killing assay was used that allows direct assessment of the antibacterial activity of the airway surface liquid (ASL) of cultured ALI-PBEC by placing NTHi-coated coverslips

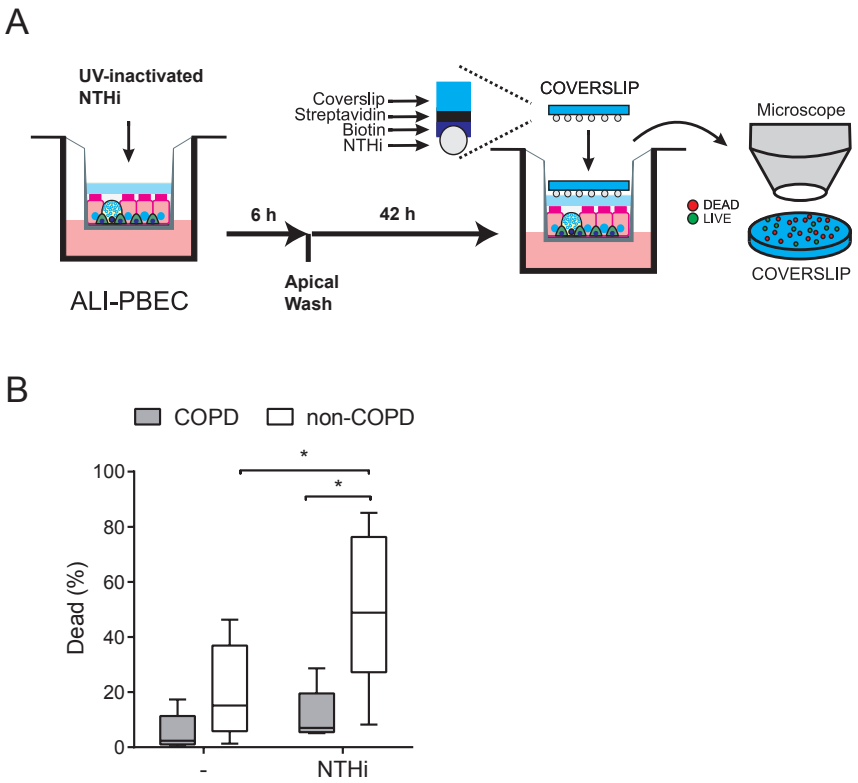

Figure 1. Impaired bacterial killing by COPD ALI-PBEC. (A) Schematic representation of the bacterial killing assay. ALI-PBEC cultures were stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi or PBS as negative control for 6 h, washed at the apical surface, and incubated for 42 h. Next, streptavidin-coated glass coverslips linked to biotin-bound NTHi were placed on the apical surface of ALI-PBEC. Bacterial killing was determined by counting individual live and dead bacteria. (B) Bacterial killing was assessed of cultured ALI-PBEC from COPD patients (grey boxplots, n=5 patients) and non-COPD smokers (white boxplots, n=5 patients), either unstimulated or stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi. Data is shown as % dead bacteria. The killing assay was performed in triplicates. COPD and non-COPD comparison results were depicted as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$.

on the apical surface. Minimal antibacterial activity was seen in control-treated ALI-PBEC from both COPD and non-COPD donors (Figure 1B). Exposure to NTHi significantly increased antibacterial activity of non-COPD cultures. In contrast, this increase was not observed in COPD epithelia. These findings suggest that cultured airway epithelial cells from COPD patients have reduced microbial-induced antibacterial activity compared to cell cultures from non-COPD (ex)smokers.

NTHi-induced expression of hBD-2 and S100A7 is altered in COPD ALI-PBEC

To investigate the role of AMPs in the reduced antibacterial activity of COPD airway epithelial cells, we examined the expression of microbial-induced AMPs in COPD and non-COPD ALI-PBEC exposed to various concentrations of UV-inactivated NTHi for 24 hours. We first focused for this analysis on microbial-induced AMPs that were found to be highly expressed in submerged cultured and undifferentiated PBEC upon exposure to UV-inactivated NTHi, based on a micro-array gene expression analysis (Supplementary Table 1). These genes included *DEFB4*, encoding human β-defensin-2 (hBD-2), the antimicrobial chemokine *CCL20*, lipocalin 2 (*LCN2*), and *S100A7* (23-26). NTHi stimulation induced the expression of all examined genes in both COPD and non-COPD cultures (Figure 2A-D) However, COPD ALI-PBEC displayed lower NTHi-mediated expression of *DEFB4* and *S100A7* compared to non-COPD cultures (Figure 2A,B), whereas *LCN2* and *CCL20* did not reveal statistically significant differences (Figure 2C,D). We also did not observe differences in the expression of other AMPs that were not observed in the micro-array gene expression analysis (LL-37/CAMP, SLPI, β-defensin 1 and 3 [hBD-1 and hBD-3]) (Supplementary Figure 1). We further analyzed the NTHi-induced protein secretion of hBD-2 and CCL20 in apical washes and basal medium of COPD and non-COPD ALI-PBEC 24 hours after stimulation. In contrast to the mRNA expression, we did not detect a difference in hBD-2 peptide release at the apical surface and basal medium between COPD and non-COPD ALI-PBEC (Figure 2E,F). Moreover, no differences were observed in secretion of CCL20. To explain this discrepancy between hBD-2 mRNA expression and peptide levels, we examined whether the difference in mRNA expression was time-dependent. Indeed, in contrast to NTHi-induced mRNA expression at 24 hours, we did not detect differences between COPD and non-COPD at 3 and 12 hours of stimulation (Figure 3A-D). These findings suggest that the attenuated antibacterial defense of COPD airway epithelial cells observed 48 h after microbial stimulation was not accompanied by differences in the early effects of microbial stimuli on AMP expression, but possibly related to lower expression of hBD-2 and *S100A7* in COPD cultures only at later time points.

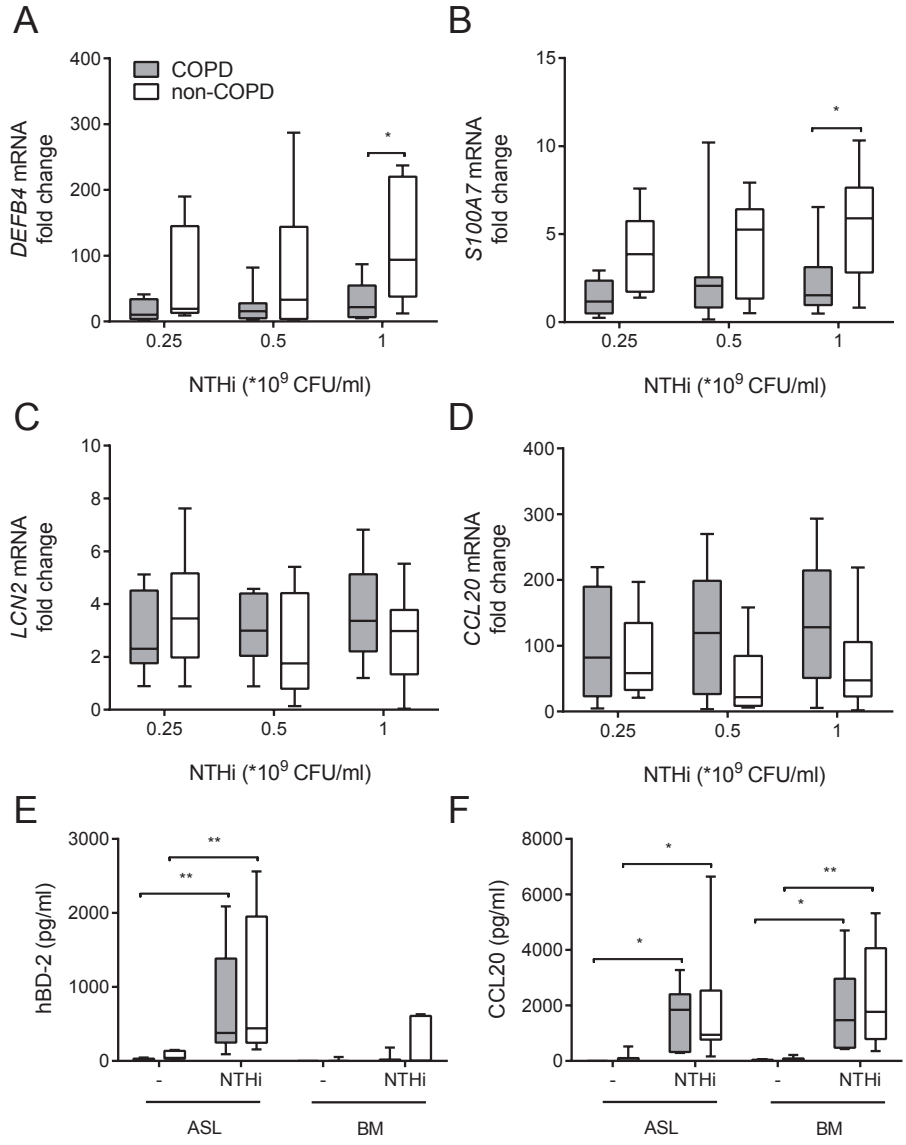

Figure 2. AMPs expression by COPD ALI-PBEC is lower compared to non-COPD. COPD (grey boxplots, n=12 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with different concentrations of UV-inactivated NTHi for 24 hours. mRNA expression of the AMPs: (A) *DEFB4*/hBD-2, (B) *S100A7*, (C) *LCN2* and (D) *CCL20* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Assessment of (E) hBD-2 and (F) CCL20 protein secretion in the apical surface liquid (ASL) and basal medium (BM) of COPD (grey boxplots, n=9) and non-COPD (white boxplots, n=7-8) ALI-PBEC stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 24 hours. Stimulations were performed in duplicates. Results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001.* p<0.05.

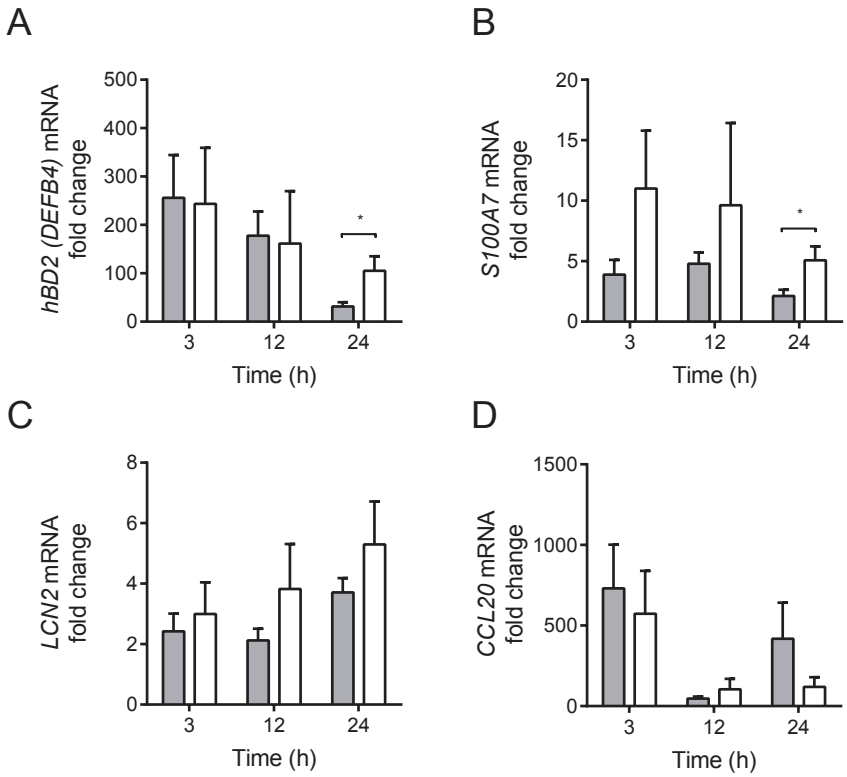

Figure 3. Differences in early- and late-induced transcriptional responses between COPD and non-COPD ALI-PBEC. Time course of NTHi-induced mRNA expression in COPD (grey bars, n=12) and non-COPD AL-PBEC (white bars, n=9). COPD and non-COPD ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3, 12 and 24 hours, afterwards mRNA expression of (A) *DEFB4*/hBD-2, (B) *S100A7*, (C) *LCN2* and (D) *CCL20* was examined by qPCR. Stimulations were performed in duplicates. Data is shown as fold change compared to unstimulated cells. All results are depicted as mean ± SEM. Analysis of differences was conducted with an unpaired t-test.* $p<0.05$.

Cell differentiation, barrier function and expression of regulatory genes do not differ between COPD and non-COPD ALI-PBEC

We further studied the cause of the impaired expression of AMPs in COPD airway epithelial cells. Previous research has demonstrated a reduced host defense function of airway epithelial cells from severe COPD patients due to impaired cell differentiation (5, 27). In addition, it was reported that COPD airway epithelial cells display a reduced barrier function (4). Therefore, we examined cell differentiation and the epithelial barrier integrity of COPD and non-COPD ALI-PBEC. In contrast to previous findings, we did not observed differences in mRNA expression levels of the club cell marker *SCGB1A1*, ciliated cell marker *FOXJ1*, goblet cell marker *MUC5AC*, and basal cell marker *TP63* (Figure 4A-D). Moreover, we did not observed differences in the epithelial barrier function between COPD and non-COPD ALI-PBEC, based on trans-epithelial electrical resistance (TEER) measurements (Figure 4E). COPD

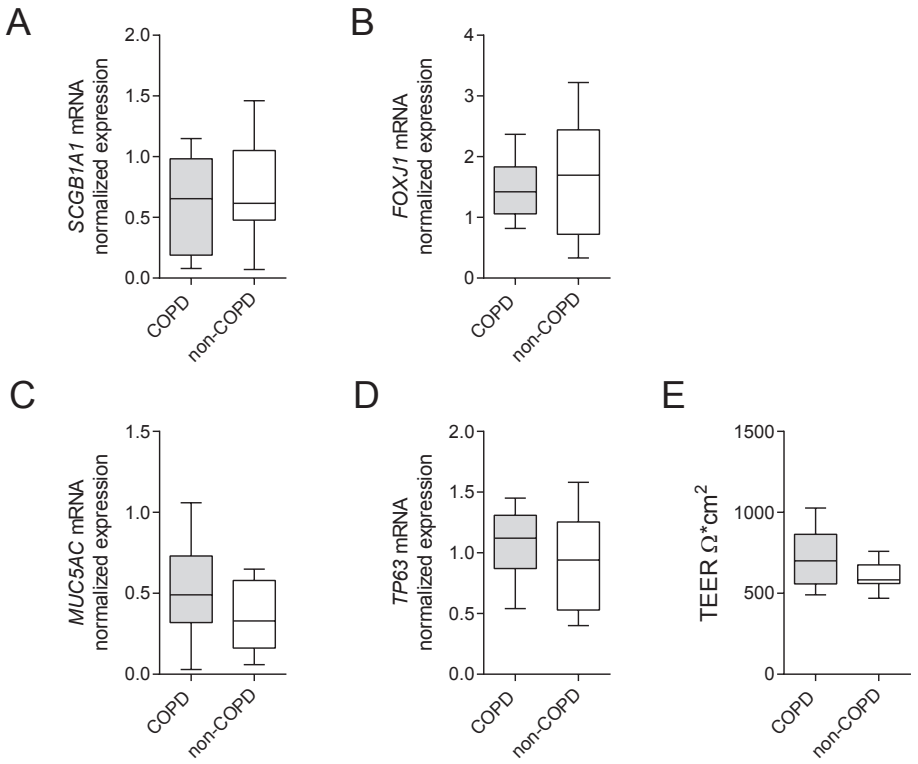

Figure 4. Expression of epithelial differentiation markers and barrier function in COPD and non-COPD ALI-PBEC. Baseline mRNA expression of the cell differentiation markers: (A) *SCGB1A1* (club cell), (B) *FOXJ1* (ciliated cell), (C) *MUC5AC* (goblet cell), and (D) TP63 (basal cell), was determined in differentiated ALI-PBEC from COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC. Data is shown as normalized values. (E) The epithelial barrier integrity of COPD (grey boxplots, n=9 patients) and non-COPD (white boxplots, n=7 patients) ALI-PBEC was determined by measuring the trans-epithelial electrical resistance (TEER). TEER values are shown as Ω*cm2. ALL results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test.

ALI-PBEC furthermore did not display low TEER values as observed in a previous study (4). These findings suggest that the altered NTHi-induced antibacterial defense of COPD airway epithelial cells is not caused by impaired cell differentiation or a reduced barrier function. Another possible explanation for the persistence of this difference after prolonged culture could be the presence of epigenetic modifications in the epithelium from COPD patients. These modifications can lead to a variety of changes that may help to explain the observed differences. First, this differential expression might be caused by altered expression of pattern recognition receptors at later time points. It has been shown that NTHi increases expression of *TLR2* (28), and impairment of this induction in cells from COPD patients can influence late-induced innate immune responses. However, we did not observe differences in *TLR2* mRNA expression between COPD and non-COPD cultures (Supplementary Figure 2). Second, alterations in negative-feedback loop mechanism of the NF-κB signaling pathway may also result in impaired expression of AMPs, such as differential expression of micro-RNA's, like

mir-147b, the deubiquitinating enzyme/ ubiquitin ligase A20, or the transcription factor ATF3 (29-31). However, we furthermore did not observe differences in the expression of the primary mir-147b transcript, and expression of *A20* and *ATF3* mRNA (Supplementary Figure 2). This suggest that other mechanism underlie the altered microbial-induced expression of AMPs in COPD airway epithelial cultures.

Cigarette smoke differentially affects the induction of AMPs and pro-inflammatory mediators in ALI-PBEC

We next set out to investigate the effect of cigarette smoke (CS) exposure on the expression of AMPs and pro-inflammatory mediators. This was done using a whole CS exposure model in which ALI-PBEC are exposed to mainstream smoke derived from one cigarette or room air (AIR) as negative control. The exposure is done for a period of 15 minutes, after which the cells are stimulated with UV-inactivated NTHi for 3, 12, and 24 hours. After the indicated time points mRNA expression was measured by qPCR. Corresponding with earlier reports (12-14), CS exposure significantly reduced the NTHi-mediated expression of *DEFB4* at 3 and 12 hours after exposure (Figure 5A). This effect was no longer observed at 24 hours after exposure. CS also attenuated the NTHi-induced expression of *LCN2*, *S100A7* and *CCL20* after 3 hours (Figure 5B-D). In accordance with preceding studies (14, 15), we observed a further increase in IL-8 and IL-6 mRNA expression upon CS and NTHi co-stimulation (Figure 5E,F). We also found decreased NTHi-induced hBD-2 and CCL20 protein secretion in CS exposed cultures (Figure 5G,H), whereas IL-8 secretion was not attenuated and further enhanced (Figure 5I). Overall, this data demonstrates that CS exposure differentially modulates microbial-induced innate immunity, impairing induction of AMPs while increasing pro-inflammatory mediators.

Cigarette smoke impairs NTHi-induced expression of AMPs in both COPD and non-COPD ALI-PBEC

Next, we determined the effect of CS on COPD and non-COPD ALI-PBEC. As we observed an acute effect of CS exposure, which was primarily seen at 3 hours after exposure, we studied mRNA expression levels of AMPs at this particular time point. AMP expression was reduced by smoke in both COPD and non-COPD cultures, thus suggesting that attenuation of AMP expression in our CS exposure model occurs independent of disease status (Figure 6A-D). We furthermore did not observe a significant difference in IL-8 mRNA expression between CS and NTHi exposed COPD and non-COPD ALI-PBEC (Figure 6E). This suggests that modulation of the expression of AMPs and pro-inflammatory mediators by CS occurs in a similar extent in COPD and non-COPD ALI-PBEC cultures.

Cigarette smoke inhibits NTHi-mediated activation of NF-κB but not MAPK signaling in ALI-PBEC

We subsequently examined the underlying mechanism of the differential regulation of AMPs and pro-inflammatory mediators by CS. Microbial- and cytokine-mediated expression of AMPs and pro-inflammatory mediators are regulated in particular by the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (16, 17). Therefore, we examined the effect of CS exposure on NF-κB and MAPK signal transduction in ALI-PBEC. Epithelial cultures were exposed to CS or air as control and subsequently stimulated with UV-

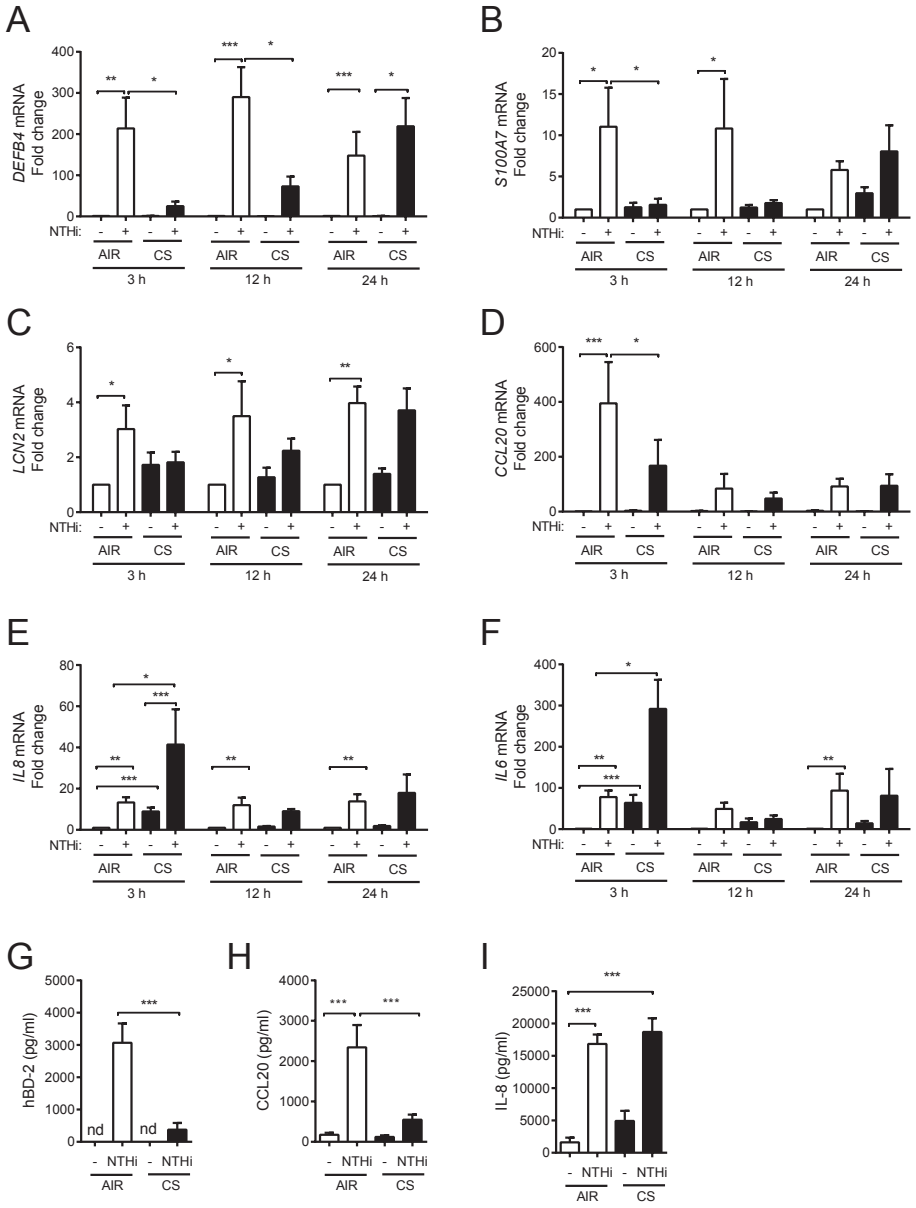

Figure 5. Cigarette smoke differentially modulates innate immune gene expression. ALI-PBEC (n=7) were exposure to AIR or CS and subsequently stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 3, 12, and 24 hours. mRNA expression of (A) *DEFB4*, (B) *S100A7*, (C) *LCN2*, (D) *CCL20*, (E) *IL8* and (F) *IL6* was measured by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Assessment of (G) hBD-2 secretion in the apical surface liquid and (H) CCL20 and (I) IL-8 secretion in the basal medium of AIR/CS exposed ALI-PBEC (n=7) stimulated with 1*10^9 CFU/ml UV-inactivated NTHi. Stimulations were performed in duplicates. All results are shown as mean ± SEM. Analysis of differences was conducted with a one-way ANOVA and Bonferroni post-hoc test (A-F), and paired t-test (G-I). * p<0.05, ** p<0.01, *** p<0.001.

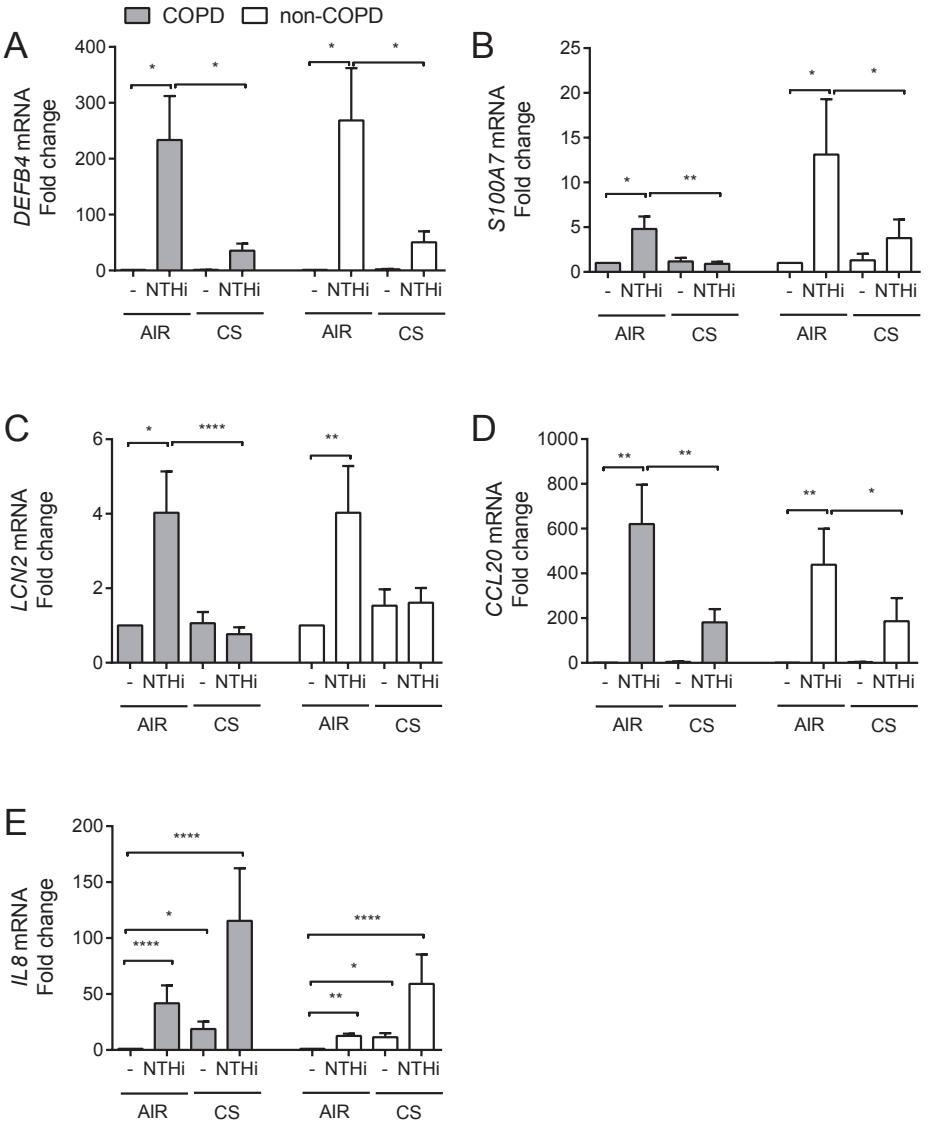

Figure 6. Suppression of AMPs by CS in both COPD and non-COPD ALI-PBEC. COPD (grey bars, n=12 patients) and non-COPD (white bars, n=8 patients) ALI-PBEC were exposure to AIR or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 hours. mRNA expression of (A) *DEFB4*, (B) *S100A7,* (C) *LCN2*, (D) *CCL20*, and (E) *IL8* was determined by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells and depicted as mean ± SEM. Analysis of differences was conducted with a paired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

inactivated NTHi at the apical surface for 30 minutes. Activation of NF-κB was determined by assessing phosphorylation of the upstream NF-κB signaling kinases IKKα/β and degradation of the NF-κB inhibitor IκB-α. MAPK signaling was evaluated by assessing phosphorylation of p38 and ERK1/2. NTHi stimulation of air-exposed cultures resulted in enhanced activation of NF-κB signal transduction based on phosphorylation of IKKα/β and degradation of the NFκB inhibitor IκB-α (Figure 7A-C). In contrast, CS exposure suppressed IKKα/β phosphorylation and IκB-α degradation. Both NTHi and CS induced phosphorylation of the MAPKs ERK1/2 and p38, and CS exposure did not attenuate the NTHi-mediated phosphorylation of ERK1/2 and p38 (Figure 7A,D,E). These findings demonstrate that CS alters NF-κB and MAPK signaling, which corresponds with attenuated expression of AMPs and enhanced expression of pro-inflammatory mediators.

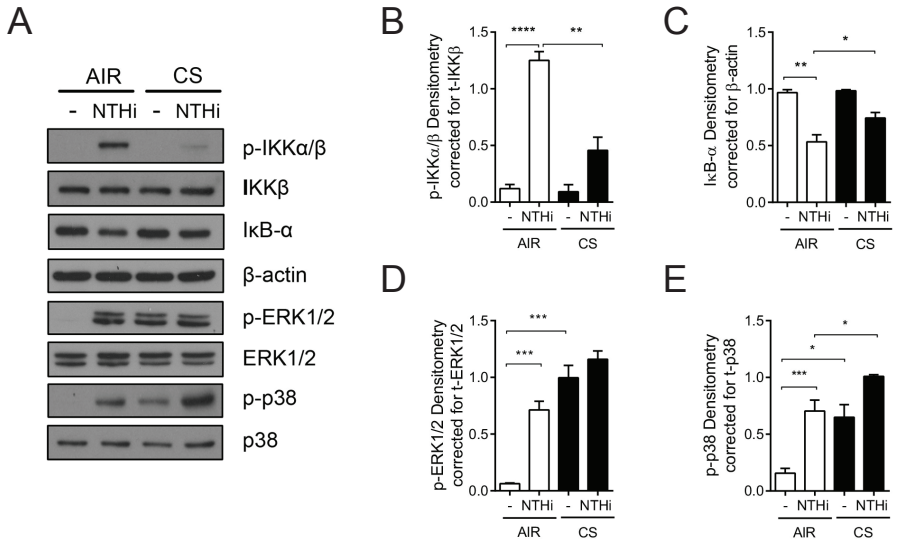

Figure 7. CS impairs NTHi-induced NF-κB but not MAPK signal transduction in ALI-PBEC. (A) ALI-PBEC (n=4-8) were exposed to AIR/CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 30 minutes. NF-κB activation was assessed by measuring phosphorylation of IKKα/β, and degradation of IκB-α. MAPK signaling was assessed by determining phosphorylation of ERK1/2 and p38. (B) Analysis of data by densitometry. Results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Cigarette smoke suppresses NF-κB-mediated transcriptional activity

To further assess whether alterations in NF-κB signaling corresponds with reduced AMPs expression we investigated nuclear translocation of NF-κB and transcription of early induced target genes. In agreement with impaired IκB-α degradation, CS inhibited nuclear translocation of the p50 and p65 NF-κB subunit, as shown by immunofluorescence staining and analysis of isolated nuclear fractions (Figure 8A-C). To validate whether this reduced nuclear localization impaired NF-κB-mediated transcription, we examined the expression of early induced NF-κB-target genes, including the negative feedback regulators *NFKBIA* and *ZC3H12A* (32), and *NFKBIZ*, which represent an essential co-transcription factor required

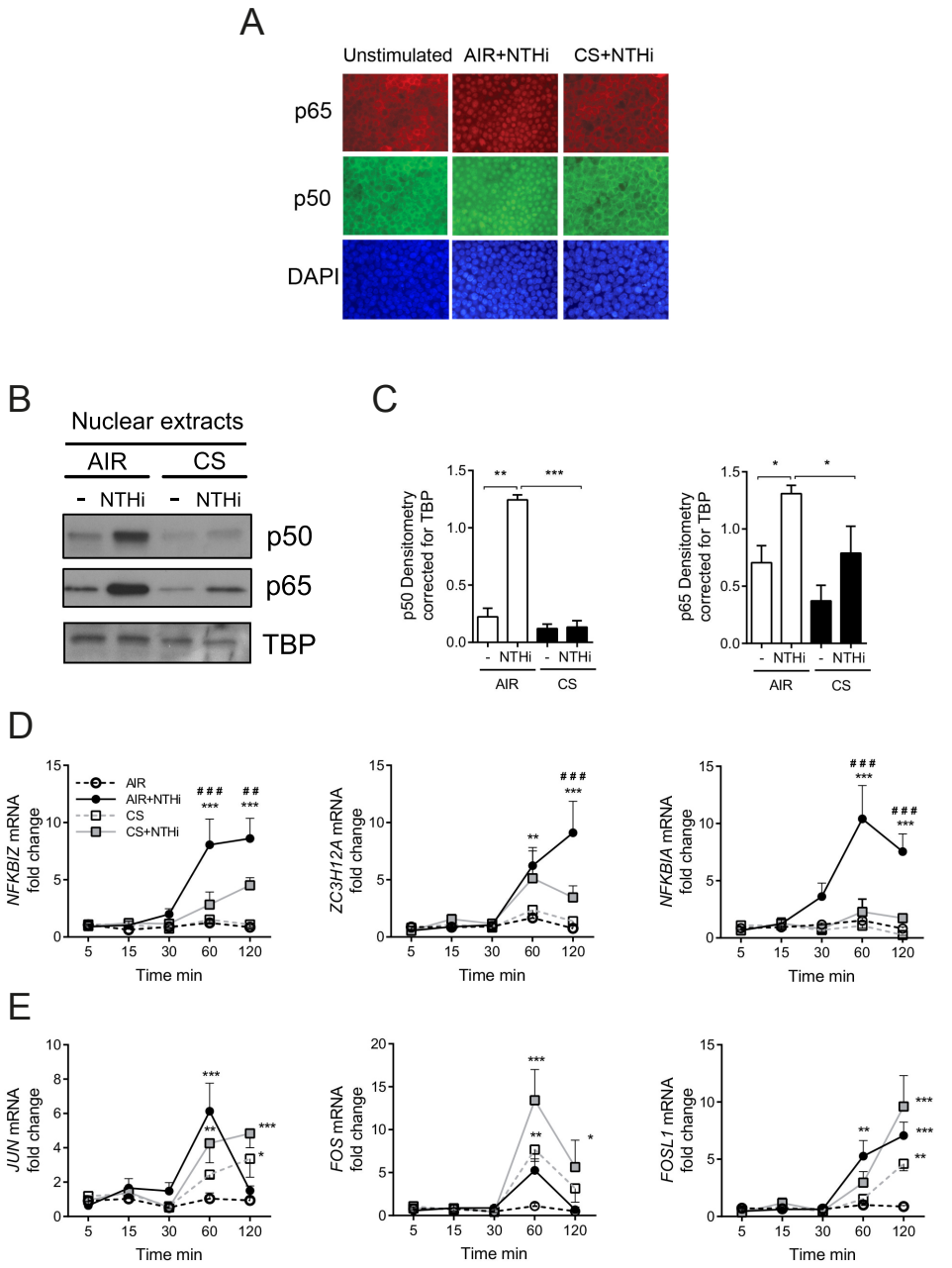

Figure 8. CS impairs NF-κB transcriptional activity by ALI-PBEC. ALI-PBEC were left untreated or exposed to AIR or CS and stimulated with UV-inactivated NTHi for 1 h. (A) Cellular localization of the NF-κB subunits p50 and p65 was determined by immunofluorescence microscopy. Data shown represent n=3 independent donors. (B) ALI-PBEC (n=4) were exposed to AIR or CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 1 hour. Protein expression of p50 and p65 was measured in isolated nuclear extracts. (C) Data was quantified by densitometry. ALI-PBEC were unstimulated or exposed to AIR or CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi

for 1 hour. ALI-PBEC (n=4) were exposure to AIR or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 5, 15, 30, 60 and 120 minutes. mRNA expression of (D) early induced NF-κB-target genes: *NFKBIA*, *ZC3H12A* and *NFKBIZ*, and (E) early induced MAPK-target genes *FOS*, *JUN* and *FOSL1*, was determined by qPCR. Data is shown as fold change in mRNA compared to air exposed cells. All results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test (C) and two-way ANOVA with Bonferroni post-hoc test (D,E). Significant different compared to air: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Significant different compared to CS+NTHi: ## p<0.01, ### p<0.001.

for AMP expression (33). Moreover, induction of MAPK-regulated early induced target genes and AP-1 transcription factors: *FOS*, *JUN* and *FOSL1* (34) was determined. CS exposure attenuated the NTHi-induced expression of *NFKBIZ* and *NFKBIA* at 60 and 120 minutes after stimulation, whereas induction of *ZC3H12A* was impaired at 120 minutes (Figure 8D). In contrast, CS exposure further increased the induction of FOS at 60 and 120 minutes, and *JUN* and *FOSL1* expression after 120 minutes of stimulation (Figure 8E). Overall, these observations demonstrate selective attenuation of NF-κB-mediated transcriptional activity by CS, which reflects impaired expression of AMPs.

DISCUSSION

In this study we provide evidence that the antibacterial defense of airway epithelial cells from COPD patients is decreased compared to non-COPD (ex)smokers. In addition, we show that the impairment of microbial-induced expression of AMPs by CS exposure may be related to inhibition of NF-κB activation. The persistence of pro-inflammatory responses to microbial stimulation in presence of CS may be explained by enhanced MAPK signaling. In contrast to the impaired antibacterial activity of cystic fibrosis airway epithelial cells at steady state conditions (35, 36), COPD cultures only display an attenuated antibacterial activity upon microbial-stimulation, suggesting that the induction of antibacterial factors is impaired. Indeed, a previous study reported lower hBD-2 expression in airway epithelial tissues from COPD patients compared to non-COPD (ex)smokers (10). In line with this study, we detected lowered NTHi-mediated expression of hBD-2/*DEFB4* mRNA expression in cultured COPD airway epithelia. In addition, we also observed lower NTHi-induced *S100A7* mRNA by COPD epithelial cultures. In contrast to hBD-2, *S100A7* displays antibacterial activity by impairing bacterial acquisition of zinc (26), which suggests that expression of AMPs with different modes of action is affected in COPD. In contrast to the differential regulation of *DEFB4* at 24 hours after NTHi stimulation, we did not observe differences in mRNA levels at earlier time points and protein secretion at 24 hours. This suggests that COPD and non-COPD airway epithelial cells differ in late-induced mRNA expression.

It has been shown that ALI-PBEC cultures from severe COPD patients display impaired epithelial differentiation and epithelial barrier integrity, which causes impaired host defense (4, 5, 27). However, we did not observed differences in the mRNA expression of cell differentiation markers and the epithelial barrier function. It is therefore unlikely that altered epithelial differentiation is the underlying cause for the impaired antibacterial activity of COPD cultures observed in this study. In contrast to the airway epithelial cell cultures from mild-to-moderate COPD patients used in this study, impaired epithelial differentiation

in severe COPD may have an additional impact on antibacterial host defense. Impaired differentiation may for example lead to a loss of CFTR expression, which in turn may result in impaired activity of AMPs in the airway surface liquid (37).

We further hypothesized that the differences in late-induced mRNA expression between COPD and non-COPD airway epithelial cells might be caused by the differential expression of second messengers, post-transcriptional and/or post-translational mechanisms influencing these late-induced responses. TLR-2, pri-mir-147b, A20, and ATF3, are known regulators of microbial-induced innate immune responses that might be affected in COPD cultures and thereby explain the impaired antibacterial defense. Despite of this, we did not observe differences in the NTHi-induced mRNA expression of these regulators.

Differences in the antibacterial activity between COPD and non-COPD cell cultures were determined with an assay previously used to assess the antibacterial activity of cystic fibrosis airway epithelial cell cultures (21, 36). Conventional killing assays rely on collection of apical washes or inoculation of bacteria to the apical surface that may result respectively in selective collection of antimicrobial substances and dilution or modulation of the physiological conditions of the apical surface liquid. This may have a major influence on determining the antibacterial activity of epithelial cells, and the assay used in the present study is not affected by these factors.

Although it is tempting to speculate that a reduced antibacterial activity of COPD airway epithelial cells is due to a reduced expression of *DEFB4* and *S100A7*, a clear link between these findings is missing. Indeed, other antibacterial defense mechanisms may also be affected in the epithelium of COPD patients, and contribute to the reduced antibacterial activity. In addition to various AMPs, also antimicrobial lipids and reactive oxygen species contribute to the bacterial killing activity of the airway epithelium (38, 39). Moreover, lower antimicrobial activity might also result from other alterations in the airway surface liquid, such as reduced pH, and the presence of mucins, F-actin and proteoglycans (21, 40-44). Therefore we cannot exclude that changes in the presence or activity of other antibacterial mediators and changes in the composition of the airway surface liquid also contributed to the reduced antibacterial activity of the COPD airway epithelial cells.

In line with previous studies demonstrating reduced antibacterial activity and inhibition of hBD-2 expression caused by cigarette smoke (12-14), we showed that the expression of *S100A7*, *LCN2* and *CCL20* was also inhibited in cigarette smoke-exposed COPD and non-COPD cultures. In contrast to AMPs, we showed increased NTHi-induced expression of IL-8 and IL-6 upon CS exposure. This further demonstrates that AMPs and pro-inflammatory mediators are differentially regulated in airway epithelial cells. The modulation of innate immune responses by CS was primarily observed 3 h after exposure, which is in line with the transient effect of the acute exposure (14).

Our study has several limitations. Our findings are limited to the acute effects of microbial- and cigarette smoke on airway epithelial innate immune responses, and chronic exposures of airway epithelial cells to both NTHi and CS may cause different effects. Moreover, differences in smoking status of both COPD and non-COPD subjects may have an influence on our

findings. Current and ex-smokers were both included in the COPD and non-COPD group, however we only have limited information about i.e. pack years smoked. Further research regarding the influence of smoking status is therefore needed.

To assess whether alterations in AMPs and pro-inflammatory mediator expression are reflected by changes in cellular signal transduction pathways, we examined the effect of CS on NF-κB and MAPK signaling. Whereas CS increased MAPK signaling, it reduced NTHi-induced NF-κB signal transduction. NTHi-induced activation of MAPK and NF-κB is mediated by the common upstream signaling kinase TAK1, which directly phosphorylates IKKα/β (45). Therefore, we speculate that CS causes selective suppression of NF-κB signaling by directly modulating IKKα/β phosphorylation, rather than affecting TAK1 or more upstream signaling components (Supplementary Figure 3). This appears in contrast to some studies that have reported increased NF-κB activation in COPD lung tissue (46-48). However, this is not observed in all studies and it has been suggested that NF-κB signaling may not contribute to COPD pathogenesis (49). Furthermore, our results are also in line with results of a study showing that long-term passive cigarette smoke exposure inhibits UVB-induced NF-κB signaling in skin (50), and with earlier reports showing impaired IKKα/β phosphorylation and kinase activity caused by post-translational modifications, mediated for instance by oxidative stress (51, 52). Previous studies demonstrated the importance of NF-κB in both microbial-induced hBD-2 and IL-8 by airway epithelial cells. However, our findings suggest a more fundamental role of NF-κB in the antibacterial defense, whereas induction of IL-8 and IL-6 persisted independent of NF-κB. Restoration of NF-κB signaling may therefore improve the airway epithelial antibacterial defense in smokers. However, further research is required to study this.

As also discussed in the previous section, the role of NF-κB in COPD remains a matter of debate since inflammation in COPD may occur independent of NF-κB (49). Based on our findings, it can be speculated that MAPK signaling has a more prominent role in CS-induced airway epithelial inflammatory responses than NF-κB. This is furthermore supported by findings from a recent study examining ozone-induced pro-inflammatory responses by differentiated airway epithelial cells, revealing MAPK-dependent and NF-κB-independent induction of inflammatory mediators (53).

Our study focused on airway epithelial responses to NTHi, because of the critical role of this microbe in COPD pathogenesis (54). However, often multiple respiratory pathogens are isolated from the airways of COPD patients and these micro-organisms may affect epithelial cell function in a different way than NTHi. In recent years there is an increased awareness of the role of the microbiome in COPD development and progression (55). Cigarette smoke-induced changes in airway epithelial defense may affect the composition of this microbiome, and its altered composition may contribute to COPD development and progression. We reported in a previous study that cigarette smoke exposure increased expression of the antimicrobial protein RNase 7 in ALI-PBEC cultures (14). This raises the possibility that, in addition to a selective down-regulation of microbial-induced AMPs, expression of other AMPs might be increased by smoke exposure. These and other changes in airway epithelial defense may contribute to changes in the microbiome. However, further research is needed to study the role of AMPs in regulating the airway microbiome in COPD.

In summary, our findings demonstrate that cultured airway epithelial cells from COPD patients have reduced antibacterial activity. Moreover, we observed an imbalance between the protective antibacterial defense and destructive inflammatory innate immune response by airway epithelial cells from COPD patients and in response to CS. This imbalance explains in part the enhanced bacterial burden and increased lung inflammation observed in COPD development and progression. Therefore, application of exogenous AMPs to compensate for the loss of AMPs might have therapeutic potential in COPD (56). Furthermore, the differential regulation of AMPs and pro-inflammatory mediators at the signal transduction level suggest that selective therapeutic targeting of airway inflammation, without affecting the beneficial antibacterial defense, might be possible.

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REFERENCES

1. Sethi S. Infection as a comorbidity of COPD. European Respiratory Journal. 2010;35(6):1209-15.
2. Sethi S, Mallia P, Johnston SL. New Paradigms in the Pathogenesis of Chronic Obstructive Pulmonary Disease II. Proceedings of the American Thoracic Society; 9/15/2009: American Thoracic Society - PATS; 2009. p. 532-4.
3. Arcavi L, Benowitz NL. Cigarette smoking and infection. Archives of Internal Medicine. 2004;164(20):2206-16.
4. Heijink IH, Noordhoek JA, Timens W, van Oosterhout AJM, Postma DS. Abnormalities in Airway Epithelial Junction Formation in Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine; 6/1/2014: American Thoracic Society - AJRCCM; 2014. p. 1439-42.
5. Gohy ST, Detry BR, Lecocq Mn, Bouzin C, Weynand BA, Amatngalim GD, et al. Polymeric Immunoglobulin Receptor Downregulation in COPD: Persistence in the Cultured Epithelium and Role of Transforming Growth Factor-b. American Journal of Respiratory and Critical Care Medicine; 7/31/2014: American Thoracic Society - AJRCCM; 2014.
6. Bals R, Hiemstra PS. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. European Respiratory Journal. 2004;23(2):327-33.
7. Ganz T. Antimicrobial polypeptides in host defense of the respiratory tract. The Journal of Clinical Investigation. 2002;109(6):693-7.
8. Evans SE, Xu Y, Tuvim MJ, Dickey BF. Inducible Innate Resistance of Lung Epithelium to Infection. Annual Review of Physiology; 2/11/2010: Annual Reviews; 2010. p. 413-35.
9. Parameswaran GI, Sethi S, Murphy TF. Effects of Bacterial Infection on Airway Antimicrobial Peptides and Proteins in COPD. Chest. 2011;140(3):611-7.
10. Pace E, Ferraro M, Minervini MI, Vitulo P, Pipitone L, Chiappara G, et al. Beta Defensin-2 Is Reduced in Central but Not in Distal Airways of Smoker COPD Patients. PLoS ONE. 2012;7(3):e33601.
11. Mallia P, Footitt J, Sotero R, Jepson A, Contoli M, Trujillo-Torralbo MB, et al. Rhinovirus Infection Induces Degradation of Antimicrobial Peptides and Secondary Bacterial Infection in Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine; 12/1/2012: American Thoracic Society - AJRCCM; 2012. p. 1117-24.
12. Herr C, Beisswenger C, Hess C, Kandler K, Suttorp N, Welte T, et al. Suppression of pulmonary innate host defence in smokers. Thorax. 2009;64(2):144-9.
13. Zhang W, Case S, Bowler RP, Martin RJ, Jiang D, Hu HW. Cigarette smoke modulates PGE2 and host defence against Moraxella catarrhalis infection in human airway epithelial cells. Respirology. 2011;16(3):508-16.
14. Amatngalim GD, van Wijck Y, de Mooij-Eijk Y, Verhoosel RM, Harder J, Lekkerkerker AN, et al. Basal Cells Contribute to Innate Immunity of the Airway Epithelium through Production of the Antimicrobial Protein RNase 7. The Journal of Immunology. 2015;194(7):3340-50.
15. Beisswenger C, Platz J, Seifart C, Vogelmeier C, Bals R. Exposure of Differentiated Airway Epithelial Cells to Volatile Smoke in vitro. Respiration. 2004;71(4):402-9.
16. Jang BC, Lim KJ, Paik JH, Kwon YK, Shin SW, Kim SC, et al. Up-regulation of human b-defensin 2 by interleukin-1b in A549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-kB. Biochemical and Biophysical Research Communications. 2004;320(3):1026-33.
17. Bezzerri V, Borgatti M, Finotti A, Tamanini A, Gambari R, Cabrini G. Mapping the Transcriptional Machinery of the IL-8 Gene in Human Bronchial Epithelial Cells. The Journal of Immunology. 2011;187(11):6069-81.
18. van Wetering S, Zuyderduyn S, Ninaber DK, van Sterkenburg MAJA, Rabe KF, Hiemstra PS. Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13. Molecular Immunology. 2007;44(5):803-11.
19. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and

Critical Care Medicine; 2/15/2013: American Thoracic Society - AJRCCM; 2013. p. 347-65.
20. Groeneveld K, van Alphen L, Eijk PP, Visschers G, Jansen HM, Zanen HC. Endogenous and Exogenous Reinfections by Haemophilus influenzae in Patients with Chronic Obstructive Pulmonary Disease: The Effect of Antibiotic Treatment on Persistence. Journal of Infectious Diseases. 1990;161(3):512-7.
21. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature. 2012;487(7405):109-13.
22. Proud D, Sanders SP, Wiehler S. Human Rhinovirus Infection Induces Airway Epithelial Cell Production of Human b-Defensin 2 Both In Vitro and In Vivo. The Journal of Immunology. 2004;172(7):4637-45.
23. Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, et al. Production of b-defensins by human airway epithelia. Proceedings of the National Academy of Sciences. 1998;95(25):14961-6.
24. Starner TD, Barker CK, Jia HP, Kang Y, McCray PB. CCL20 Is an Inducible Product of Human Airway Epithelia with Innate Immune Properties. American Journal of Respiratory Cell and Molecular Biology; 11/1/2003: American Thoracic Society - AJRCMB; 2003. p. 627-33.
25. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature. 2004;432(7019):917-21.
26. Glaser R, Harder J, Lange H, Bartels J, Christophers E, Schroder JM. Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. Nat Immunol. 2005;6(1):57-64.
27. Gohy ST, Hupin C, Fregimilicka C, Detry BR, Bouzin C, Gaide Chevronay H, et al. Imprinting of the COPD airway epithelium for dedifferentiation and mesenchymal transition. European Respiratory Journal. 2015.
28. Mikami F, Lim JH, Ishinaga H, Ha UH, Gu H, Koga T, et al. The transforming growth factor-beta-Smad3/4 signaling pathway acts as a positive regulator for TLR2 induction by bacteria via a dual mechanism involving functional cooperation with NF-kappaB and MAPK phosphatase 1-dependent negative cross-talk with p38 MAPK. J Biol Chem. 2006;281(31):22397-408.
29. Liu G, Friggeri A, Yang Y, Park YJ, Tsuruta Y, Abraham E. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. Proc Natl Acad Sci U S A. 2009;106(37):15819-24.
30. Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, et al. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol. 2004;5(10):1052-60.
31. Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC, et al. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature. 2006;441(7090):173-8.
32. Iwasaki H, Takeuchi O, Teraguchi S, Matsushita K, Uehata T, Kuniyoshi K, et al. The I[kappa]B kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. Nat Immunol. 2011;12(12):1167-75.
33. Kao CY, Kim C, Huang F, Wu R. Requirements for Two Proximal NF-kB Binding Sites and I-kB-z in IL-17A-induced Human b-Defensin 2 Expression by Conducting Airway Epithelium. Journal of Biological Chemistry. 2008;283(22):15309-18.
34. Karin M. The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases. Journal of Biological Chemistry. 1995;270(28):16483-6.
35. Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic Fibrosis Airway Epithelia Fail to Kill Bacteria Because of Abnormal Airway Surface Fluid. Cell. 1996;85(2):229-36.
36. Shah VS, Meyerholz DK, Tang XX, Reznikov L, Abou Alaiwa M, Ernst SE, et al. Airway acidification initiates host defense abnormalities in cystic fibrosis mice. Science. 2016;351(6272):503-7.
37. Puchelle E, Zahm JM, Tournier JM, Coraux C. Airway Epithelial Repair, Regeneration, and Remodeling after Injury in Chronic Obstructive Pulmonary Disease. Proceedings of the American Thoracic Society; 11/1/2006: American Thoracic Society - PATS; 2006. p. 726-33.
38. Do TQ, Moshkani S, Castillo P, Anunta S, Pogosyan A, Cheung A, et al. Lipids Including Cholesteryl Linoleate

and Cholesteryl Arachidonate Contribute to the Inherent Antibacterial Activity of Human Nasal Fluid. The Journal of Immunology. 2008;181(6):4177-87.
39. Moskwa P, Lorentzen D, Excoffon KJDA, Zabner J, McCray PB, Nauseef WM, et al. A Novel Host Defense System of Airways Is Defective in Cystic Fibrosis. Am J Respir Crit Care Med. 2007;175(2):174-83.
40. Abou Alaiwa MH, Reznikov LR, Gansemer ND, Sheets KA, Horswill AR, Stoltz DA, et al. pH modulates the activity and synergism of the airway surface liquid antimicrobials b-defensin-3 and LL-37. Proceedings of the National Academy of Sciences. 2014;111(52):18703-8.
41. Felgentreff K, Beisswenger C, Griese M, Gulder T, Bringmann G, Bals R. The antimicrobial peptide cathelicidin interacts with airway mucus. Peptides. 2006;27(12):3100-6.
42. Weiner DJ, Bucki R, Janmey PA. The Antimicrobial Activity of the Cathelicidin LL37 Is Inhibited by F-actin Bundles and Restored by Gelsolin. American Journal of Respiratory Cell and Molecular Biology; 6/1/2003: American Thoracic Society - AJRCMB; 2003. p. 738-45.
43. Baranska-Rybak W, Sonesson A, Nowicki R, Schmidtchen A. Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids. Journal of Antimicrobial Chemotherapy. 2006;57(2):260-5.
44. Bergsson G, Reeves EP, McNally P, Chotirmall SH, Greene CM, Greally P, et al. LL-37 Complexation with Glycosaminoglycans in Cystic Fibrosis Lungs Inhibits Antimicrobial Activity, Which Can Be Restored by Hypertonic Saline. The Journal of Immunology. 2009;183(1):543-51.
45. Shuto T, Xu H, Wang B, Han J, Kai H, Gu XX, et al. Activation of NF-KB by Nontypeable Hemophilus Influenzae Is Mediated by Toll-Like Receptor 2-TAK1-Dependent NIK-IKKa/b-IkBa and MKK3/6-P38MAP Kinase Signaling Pathways in Epithelial Cells. Proceedings of the National Academy of Sciences. 2001;98(15):8774-9.
46. Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, et al. Increased expression of nuclear factor-kB in bronchial biopsies from smokers and patients with COPD. European Respiratory Journal. 2002;20(3):556-63.
47. Szulakowski P, Crowther AJL, Jiménez LA, Donaldson K, Mayer R, Leonard TB, et al. The Effect of Smoking on the Transcriptional Regulation of Lung Inflammation in Patients with Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine; 7/1/2006: American Thoracic Society - AJRCCM; 2006. p. 41-50.
48. Yagi O, Aoshiba K, Nagai A. Activation of Nuclear Factor-kB in Airway Epithelial Cells in Patients with Chronic Obstructive Pulmonary Disease. Respiration. 2006;73(5):610-6.
49. Rastrick JMD, Stevenson CS, Eltom S, Grace M, Davies M, Kilty I, et al. Cigarette Smoke Induced Airway Inflammation Is Independent of NF-kB Signalling. PLoS ONE. 2013;8(1):e54128.
50. Gottipati KR, Poulsen H, Starcher B. Passive cigarette smoke exposure inhibits ultraviolet light B-induced skin tumors in SKH-1 hairless mice by blocking the nuclear factor kappa B signalling pathway. Experimental Dermatology. 2008;17(9):780-7.
51. Korn SH, Wouters EFM, Vos N, Janssen-Heininger YMW. Cytokine-induced Activation of Nuclear Factor-kB Is Inhibited by Hydrogen Peroxide through Oxidative Inactivation of IkB Kinase. Journal of Biological Chemistry. 2001;276(38):35693-700.
52. Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EFM, et al. Nitric oxide represses inhibitory kB kinase through S-nitrosylation. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(24):8945-50.
53. McCullough SD, Duncan KE, Swanton SM, Dailey LA, Diaz-Sanchez D, Devlin RB. Ozone Induces a Proinflammatory Response in Primary Human Bronchial Epithelial Cells through Mitogen-Activated Protein Kinase Activation Without Nuclear Factor-kB Activation. American Journal of Respiratory Cell and Molecular Biology; 4/2/2014: American Thoracic Society - AJRCMB; 2014. p. 426-35.
54. Murphy TF, Brauer AL, Schiffmacher AT, Sethi S. Persistent Colonization by Haemophilus influenzae in Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine; 8/1/2004: American Thoracic Society - AJRCCM; 2004. p. 266-72.

55. Mammen MJ, Sethi S. COPD and the microbiome. Respirology. 2016;21(4):590-9.
56. Hiemstra PS, Amatngalim GD, van der Does AM, Taube C. Antimicrobial Peptides and Innate Lung Defenses: Role in Infectious and Noninfectious Lung Diseases and Therapeutic Applications. Chest. 2016;149(2):545-51.

SUPPLEMENTARY TABLES

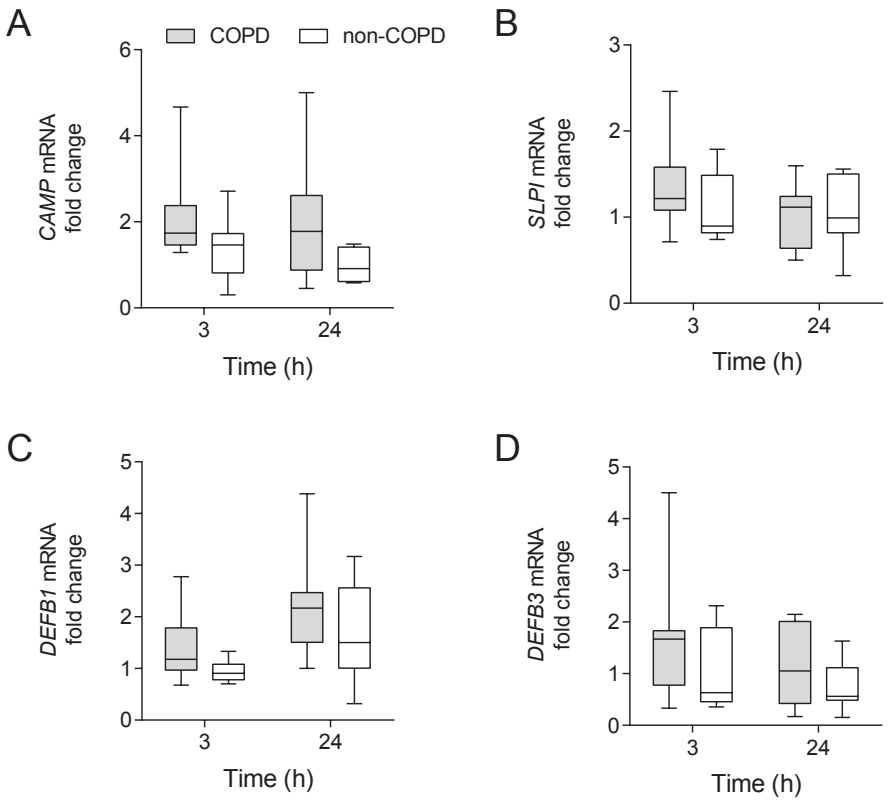
Supplementary Table 1: Top 25 most differentially expressed genes in NTHi stimulated PBEC

Gene Symbol	FC NTHi vs UNST (6 h)
DEFB4	*60.3*
CCL20	*33.0*
PLAT	21.3
BCL2A1	20.0
IL8	18.3
SPRR2C	16.2
LCE3D	15.9
CYP1A1	15.7
IL1R2	12.6
SPRR2G	12.1
C15orf48	11.9
MRGPX3	10.5
MMP9	8.5
TLR2	8.3
IL1F9	8.1
SOD2	7.5
CXCL3	7.0
IL13RA2	6.7
MMP1	6.3
LCN2	6.0
MMP10	5.9
ADH7	-5.3
S100A7	5.2
G0S2	5.0
APOBEC3A	5.0

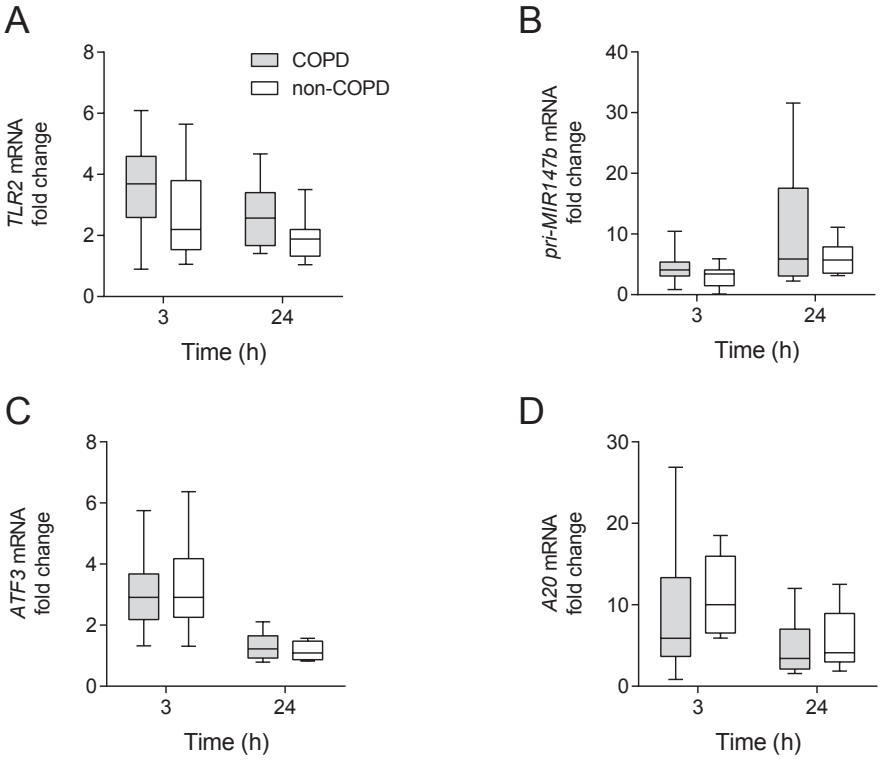
Gene Symbol	FC NTHi vs UNST (24 h)
DEFB4	110.6
IL1F9	0.3
SPRR2G	37.0
SPRR2C	31.6
IL8	17.2
RNASE7	16.1
IL1R2	14.7
CCL20	34
CYP4B1	-12.8
ADH7	-12.6
LCN2	11.9
IL1RL1	11.1
LCE3D	10.8
KRT15	-10.7
DAPL1	-10.7
IL13RA2	10.6
MRGPX3	10.0
XDH	9.9
WFDC12	9.8
PPIF	9.7
MMP1	9.6
MMP9	8.2
HMOX1	8.2
SLC16A9	-8.1
C15orf48	8.1

Gene expression analysis by microarray of unstimulated (UNST) and UV-inactivated NTHi stimulated PBEC. Fold change values are shown for the top 25 most differentially expressed genes at 6 h (left table) and 24 h (right table) after stimulation. Selected AMPs that are further analysed are highlighted in bold and grey boxes. PBEC from 6 independent donors were included in the analysis. The criteria for differential expression was an absolute fold change > 2 and $p < 0.05$, corrected for multiple testing.

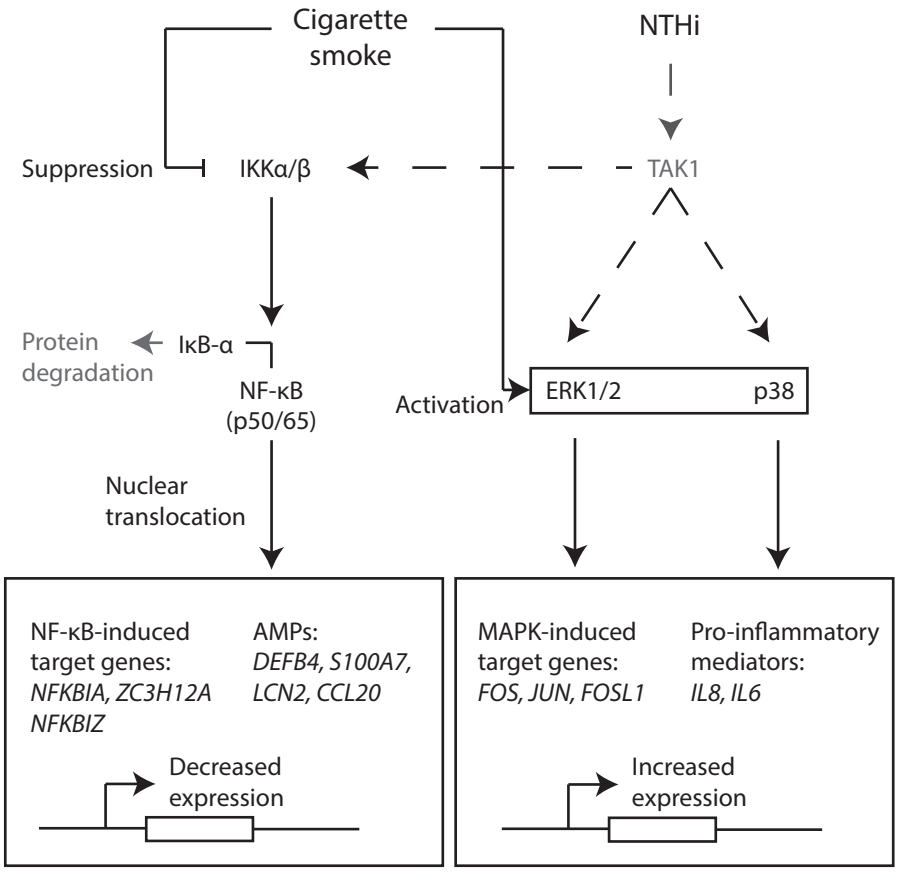
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Expression of LL-37/*CAMP, SLPI*, *DEFB1* and *DEFB3* in COPD and non-COPD ALI-PBEC. COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 and 24 hours. mRNA expression of (A) LL-37/*CAMP,* (B) *SLPI,* (C) *DEFB1* and (D) *DEFB3* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni *post-hoc* test.



Supplementary Figure 2. Expression of NTHi-induced innate immune regulators by COPD and non-COPD airway epithelial cells. COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 and 24 hours. Afterwards, mRNA expression of (A) *TLR2*, (B) the primary microRNA 147b (pri-mir-147b) transcript, (C) *ATF3* and (D) *A20* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. All results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni *post-hoc* test.



Supplementary Figure 3. Hypothetical model of the effect of CS on airway epithelial cell innate immunity. NTHi activates both the NF-κB and MAPK signal transduction through the kinase TAK1. Cigarette smoke suppresses IKKα/β phosphorylation, which results in impaired NF-κB transcriptional activity reflected by impaired expression of early-induced target genes (*NFKBIA*, *ZC3H12A*, and *NFKBIZ*) and AMPs. In contrast, CS further enhances activation of the MAP-kinases ERK1/2 and p38, promoting expression of AP-1 transcription factor (*FOS*, *JUN*, and *FOSL1*) and pro-inflammatory mediators.