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**Title:** Airway epithelial cell cultures for studying obstructive lung disease effects of IL-13 and cigarette smoke  
**Date:** 2018-05-09
CHAPTER 5

WHOLE CIGARETTE SMOKE EXPOSURE DIFFERENTIALLY ALTERS AIRWAY EPITHELIAL RESPONSES TO NON-TYPEABLE HAEMOPHILUS INFLUENZAE AND RHINOVIRUS

In progress


*These authors contributed equally
ABSTRACT

Increased susceptibility to bacterial and viral respiratory tract infections is a characteristic of smokers with and without chronic obstructive pulmonary disease (COPD), and these infections contribute to acute exacerbations and disease progression in COPD. Cigarette smoke exposure is considered the main risk factor for the development of COPD. To better understand how mainstream whole cigarette smoke exposure (CS) modulates epithelial responses to viral and bacterial exposure, we use air-liquid interface (ALI) cultures of primary human bronchial epithelial cells (PBEC) of control and COPD patients. These cultures were exposed to CS followed by addition of UV-inactivated non-typeable *Haemophilus influenzae* (NTHi) or human rhinovirus 16 (RV16). Exposure of ALI-PBEC to CS was found to inhibit the NTHi-induced expression of the antimicrobial peptide hBD-2 (*DEFB4*) in ALI-PBEC of both COPD patients and controls. In contrast, NTHi-induced expression of *GADD34*, a marker of endoplasmic reticulum stress and of the integrated stress response, was synergistically induced by cigarette smoke, which appeared to be more pronounced in ALI-PBEC from COPD patients compared to controls. CS also reduced RV16-induced expression of antiviral genes in ALI-PBEC of COPD patients, but not in controls with the exception viperin. In conclusion, our results indicate that CS differentially affects viral and bacterial infection responses in airway epithelial cells. The reduced antiviral response of airway epithelial cells from COPD patients, but not from controls, suggests that epigenetic mechanisms are involved in the acquisition of reduced antiviral protection of epithelial cells in COPD as these effects are preserved in culture.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease that is characterized by progressive airflow limitation that is not fully reversible. Both airway disease and parenchymal destruction contribute to airflow limitation in COPD, and the relative contribution of these varies amongst patients. The main risk factor for the development of COPD in Westernized societies is cigarette smoking (1). Despite being the main risk factor, only 25 to 30% of smokers develop COPD, suggesting that other factors contribute, such as genetic background and respiratory infections (2, 3). Furthermore, acute exacerbations that are frequently accompanied by respiratory infections further enhance airflow limitation and accelerate disease progression.

Acute exacerbations in COPD patients are defined by a sustained worsening of the patient’s condition, which is acute in onset and necessitates a change in regular medication (4). Both bacterial and viral respiratory tract infections contribute to acute exacerbations in COPD. Non-typeable *Haemophilus influenzae* (NTHi) colonizes the lower respiratory tract of approximately 30% of COPD patients and is involved in both chronic airway infections and acute exacerbations (5, 6). In addition, also human rhinovirus (RV) contributes to COPD exacerbations. RV causes self-limiting infections in healthy individuals, but RV infections are associated with the majority of virus-related exacerbations in patients with COPD (7, 8).

Airway epithelial cells are actively involved in the protection of the lung against inhaled particles and pathogens. They line the surface of the conducting airway as a pseudostratified epithelial layer and function as a physical and immunological barrier. Cigarette smoke (CS) has been shown to alter various epithelial functions including a reduction in antimicrobial peptide expression, activation of the integrated stress response and increased inflammation (9). We and others have shown that CS impairs antibacterial defenses in *in vitro* cultures of human airway epithelial cells, and that *in vitro* cultured airway epithelial cells from patients with COPD display impaired antibacterial activity and impaired production of antimicrobial peptides (10). Additionally, previous *in vitro* studies on cigarette smoke extract (CSE) treatments showed a reduced antiviral epithelial response to RV16 (11).

To better understand how CS exposure modulates epithelial responses to viral and bacterial infection, we use air-liquid interface (ALI) cultures of primary human bronchial epithelial cells (PBEC) of COPD patients and controls in this study, and exposed these to CS followed by
exposure to RV16 or UV-inactivated NTHi. Additionally, since airway epithelial dysfunction has been described for COPD patients, we also compared epithelial responses of ALI-PBEC from control patients to ALI-PBEC of COPD patients.

MATERIALS AND METHODS

**Subjects and cell culture conditions**

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Details on isolation and culture of PBEC were described previously (12). Clinical information on the control (CTRL) and COPD patients is presented in table 1.

Cultured PBEC were used to generate mucociliary differentiated cultures by differentiation at the air-liquid interface (ALI) as described previously (12). Briefly, PBEC at passage 2 were cultured submerged on semipermeable Transwell inserts with 0.4 μm pore size (Corning Costar, Cambridge, USA) that were coated with a mixture of collagen, fibronectin and bovine serum albumin. Once full confluence was reached, apical medium was removed followed by two weeks of ALI differentiation.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CTRL (n = 8)</th>
<th>COPD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yr)</td>
<td>67.1 ± 11.38</td>
<td>70.4 ± 6.2</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>4/4</td>
<td>7/1</td>
</tr>
<tr>
<td>BMI</td>
<td>24.2 ± 2.8</td>
<td>25.7 ± 1.4</td>
</tr>
<tr>
<td>FEV₁ (% pred)</td>
<td>100.5 ± 21.2(*)</td>
<td>65.7 ± 8.9</td>
</tr>
<tr>
<td>Pre-BD FEV₁ (L)</td>
<td>2.8 ± 0.6(*)</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>FVC (% pred)</td>
<td>103.6 ± 16.5</td>
<td>95.9 ± 20.6</td>
</tr>
<tr>
<td>FEV₁₁/FVC (%)</td>
<td>80.0 ± 8.1(*)</td>
<td>54.7 ± 7.7</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of control and COPD patients from whom primary bronchial epithelial cells were obtained. Data is represented as mean ± SD. (*) p < 0.01. BD, bronchodilator; BMI, body mass index; CTRL, control; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity.
**Whole cigarette smoke (CS) exposure**

The apical surface of the cultures was washed with 100 µl and basal culture medium was replaced with infection medium 24 h prior to CS exposure. Infection medium is defined as PBEC culture medium without hydrocortisone, bovine pituitary extract, epidermal growth factor and bovine serum albumin. ALI-PBEC were exposed to CS generated from 3R4F reference cigarettes (University of Kentucky, Lexington, USA) in a CS exposure model adapted from Beisswenger et al. (13, 14). In brief, ALI-cultures were placed into modified hypoxic chambers (Billups Rothenberg, Del Mar, USA), localized inside an incubator at 37°C and 5% CO₂. Whole CS derived from one cigarette, or air as negative control, was guided through the respective exposure chamber using a continuous flow of 1 l/min for a period of 4–5 min, and CS was distributed within the exposure chamber using a small ventilator. After exposure, residual CS inside the exposure chamber was removed by flushing the chambers with air derived from the incubator for a period of 10 min. Bacterial or viral infection was performed directly after air or CS exposure.

**Exposure to UV-inactivated non-typeable Haemophilus influenzae**

A log-phase culture of non-typeable *Haemophilus influenzae* (NTHi) strain D1 (15) was obtained from a single colony as previously described (14). Bacterial cells in log-phase growth were washed and diluted in PBS to a concentration of 1*10⁹ colony forming unit (CFU)/ml. Bacteria were killed by exposure to UV-light for 2 h. ALI-PBEC were exposed to NTHi, by adding 100 µl of 10⁸ CFU/ml of UV-NTHi to the apical surface followed by 3 h incubation at 37 °C with 5% CO₂. For additional controls, ALI-PBEC were exposed for 3 h to TNF-α (20 ng/ml; Peprotech, Rocky Hill, USA) or tunicamycin (Sigma-Aldrich, St. Louis, USA) added to the basal media. Basal media and apical fluid (100 µl PBS ± NTHi) were collected directly after incubation, and stored at -80°C. Cells were lysed using 200 µl of lysis buffer added directly to the cells and stored at -20 °C until RNA extraction.

**RV16 infection**

Human rhinovirus type 16 (RV16, VR-283) and H1-HeLa cells (CRL-1958) were purchased from the American Type Culture Collection (ATCC, Rockville, USA). RV16 was propagated in H1-HeLa cells as described previously (16, 17). Prior to CS or air exposure, the cellular content of one insert per donor was trypsinized and counted to obtain the number of cells...
per inserts to calculate a multiplicity of infection (MOI) of 1. RV16 was diluted to achieve a MOI1 by adding the virus in 100 µl of infection media to the apical surface of ALI-PBEC. After 1 h incubation at room temperature with intermittent swirling every 5 min, the apical liquid was removed and washed 3 times with infection medium to remove residual viral particles, followed by 24 h incubation at 37 °C with 5 % CO₂. Basal media and apical fluid (100 µl PBS ± RV16) were collected directly after incubation and stored at -80°C. Cells were lysed using 200 µl of lysis buffer added directly to the cells and stored at -20 °C until RNA extraction.

**RNA isolation, reverse transcription (RT) and qPCR**

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) according to the manufacturer’s instructions and quantified using the Nanodrop ND-1000 UV-visible spectrophotometer (Nanodrop Technologies, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using oligo-(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37°C. Primer sequences are listed in table 2. RPL13A and ATP5B were used as reference genes following selection by the Genorm method (18). All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFAST™ SYBR green (Bioline, Luckenwalde, Germany). Bio-Rad CFX manager 3.1 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.
Whole cigarette smoke exposure differentially alters airway epithelial
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Table 2. Primer sequences with gene names and NCBI gene ID used in present study.

<table>
<thead>
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<th>Gene</th>
<th>Primer Sequence</th>
<th>NCBI Gene ID</th>
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<tbody>
<tr>
<td>DEFB4</td>
<td>F: ATC AGC CAT GAG GGT CTT G R: GCA GCA TTT TGT TCC AGG</td>
<td>1673</td>
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<tr>
<td>GADD34</td>
<td>F: ATG TAT GGT GAG CGA GAG GC R: GCA GTG TCC TTA TCA GAA GGC</td>
<td>23645</td>
</tr>
<tr>
<td>HMOX1</td>
<td>F: AAG ACT GCG TTC CTG CTC AAC R: AAA GCC CTA CAG CAA CTG TCG</td>
<td>3162</td>
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<tr>
<td>RV16</td>
<td>F: ACC CTC AAT ACA TAC GCC AAC T R: TTC CAA GCC ATC CAT TCC A</td>
<td>L24917</td>
</tr>
<tr>
<td>MDA5</td>
<td>F: TCG AAT GGG TAT TCC ACA GAC G R: GTG GCG ACT GTC CTC TGA A</td>
<td>64135</td>
</tr>
<tr>
<td>RIG1</td>
<td>F: CTG GAC CCT ACC TAC ATC CTG R: GGC ATC CAA AAA GCC ACG G</td>
<td>5920</td>
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<tr>
<td>IFNB</td>
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<tr>
<td>ISG15</td>
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<tr>
<td>Viperin</td>
<td>F: GTCA GCC AGA ACA GGT CGT C R: AGT GCT TTG ATC TGT TCC GTC</td>
<td>91543</td>
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<tr>
<td>RPL13A</td>
<td>F: AAG GTG GTG TGA CGC TGT G R: CGG GAA GGG TTG GTG TTC ATC C</td>
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<tr>
<td>ATP5B</td>
<td>F: TCA CCC AGG CTG GTT CAG A R: AGT GGC CAG GGT AGG CTG AT</td>
<td>506</td>
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</table>
**TCID\textsubscript{50} calculation**

Viral titers were assessed on the apical washes of RV16-infected ALI-PBEC using confluent layers of H1-HeLa in 96-well plates. A 10-fold serial dilution of the apical washes was made in DMEM containing 2% FBS (Bodinco, Alkmaar, The Netherlands), 2% (vol/vol) 1 M HEPES (Lonza) and 1% (wt/vol) NaHCO\textsubscript{3} (Gibco). The 50% tissue culture infection dose was evaluated by assessing the cytopathic effect after 5 days of incubation at 37 °C with 5 % CO\textsubscript{2} and expressed as TCID\textsubscript{50}/ml.

**CXCL8 and CXCL10 ELISA**

CXCL8 and CXCL10 was measured in the basal media and assessed using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, USA).

**Statistics**

Within group (control and COPD) results were evaluated using one-way ANOVA for repeated measures with Bonferroni as post-hoc test with GraphPad Prism 6. Differences between control and COPD patients were compared with a non-parametric t-test for independent samples (Mann-Whitney). Differences were considered statistically significant at *p<0.05, **p<0.01 and ***p<0.001.
RESULTS

_Innate immune responses, integrated stress response and oxidative stress responses in airway epithelial cells of control and COPD patients cultured at air-liquid interface_

To evaluate difference in the cellular response of ALI-PBEC derived from COPD patients and controls, we exposed control and COPD derived ALI-PBEC to TNF-α (20 ng/ml) or tunicamycin (20 ng/ml) for 3 h, or to whole cigarette smoke (CS) exposure (4-5 min exposure to one cigarette, followed by 10 min removal of smoke), followed by 3 h incubation. We measured gene expression patterns of _DEFB4, GADD34_ and _HMOX1_ to evaluate the innate immune response, integrated stress response and oxidative stress response respectively. Both ALI-PBEC of control and COPD patients showed increased expression of _DEFB4_ by TNF-α, _GADD34_ by tunicamycin and _HMOX1_ by CS. Furthermore, ALI-PBEC from COPD patients showed a significantly higher induction of _DEFB4_ expression following TNF-alpha exposure compared to ALI-PBEC from controls (Fig. 1). Together these results suggest that both ALI-PBEC of control and COPD patients are able to elicit a physiologic response to various stimuli, but this response may vary in ALI-PBEC from COPD patients compared to controls.
Figure 1. Analysis of antimicrobial peptide expression (innate immunity; \textit{DEFB4}), activation of the integrated stress response (\textit{GADD34}) and oxidative stress response (\textit{HMOX1}) in ALI-PBEC from COPD patients and controls (CTRL). COPD and CTRL ALI-PBEC were exposed to CS, TNF-α (20 ng/ml) or tunicamycin (20 ng/ml) for 3 h. \textit{DEFB4}, \textit{GADD34} and \textit{HMOX1} gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to incubator control with n = 8 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; PBEC, primary bronchial epithelial cells; TM, tunicamycin.
**Differential airway epithelial responses to bacterial infection following cigarette exposure in ALI-PBEC of control and COPD patients**

After evaluating whether ALI-PBEC from both control and COPD patients were able to elicit a physiologic response to various stimuli, we next evaluated whether whole cigarette smoke exposure (CS) alters epithelial responses to bacterial exposure, and whether this response differed between COPD patients and controls. Analysis of *DEFB4A* (innate immune response), *GADD34* (Integrated stress response) and *HMOX1* (oxidative stress response) expression was assessed on ALI-PBEC from control and COPD donors. These ALI-PBECs were exposed to air or CS, and next incubated for 3 h with or without UV-inactivated NTHi (10⁹ CFU/ml) apically. CS exposure significantly inhibited the NTHi-induced *DEFB4A* expression in both control and COPD ALI-PBEC. Additionally, unexpectedly NTHi-induced *DEFB4* expression was higher in ALI-PBEC from COPD patients compared to controls (p = 0.0353). Together these data suggest that patient status can affect the intensity of the response to CS exposure followed by bacterial infection. *GADD34* expression showed a non-significant increase after CS exposure in both control and COPD ALI-PBEC. However, *GADD34* expression was significantly increased in CS treated ALI-PBEC following NTHi exposure, but not in air-exposed control and COPD ALI-PBEC. Furthermore, combined CS and NTHi exposure appears to synergistically increase *GADD34* expression in both control and COPD ALI-PBEC, suggesting that CS exposure exacerbates the NTHi-induced integrated stress response. CS alone significantly induced *HMOX1* mRNA in both donor groups, whereas NTHi exposure alone did not. However, HMOX1 induction by CS exposure appears to be lower in COPD ALI-PBEC compared to controls. No significant differences were observed in *HMOX1* expression between COPD and controls, suggesting that control and COPD ALI-PBEC have a similar capacity to induce an oxidative stress response following CS exposure which is unaffected by NTHi exposure (Fig. 2).
Figure 2. Whole cigarette smoke exposure differentially alters responses of COPD ALI-PBEC to NTHi. ALI-PBEC from COPD and CTRL donors were exposed to air or CS, followed by 3 h exposure to UV-inactivated NTHi (10^9 CFU/ml). *DEFB4, GADD34 and HMOX1* gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to untreated control with n = 8 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; NTHi, non-typeable *Haemophilus influenzae*, PBEC, primary bronchial epithelial cells.
Differential airway epithelial responses to viral infection following cigarette exposure in ALI-PBEC of control and COPD patients

ALI-PBEC cultures from COPD patients show altered responses to bacterial infection following whole cigarette smoke exposure (CS). Next we evaluated if whole cigarette smoke exposure also alters the viral response in ALI-PBEC of COPD patients and whether these responses are different compared to ALI-PBEC from control patients. ALI-PBEC from 8 control and 8 COPD patients were exposed to air or CS and then infected with RV16 at a MOI of 1 for 1 h followed by 24 h incubation. Analysis of total viral RNA of RV16 showed a higher viral RNA load after CS exposure compared to air-exposed controls; this effect however did not reach statistical significance (Fig. 3A). Furthermore, shedding of infectious particles, indicated by the TCID₅₀, showed non-significant differences between COPD and control cultures, and prior exposure to CS had opposite effects on cultures derived from both groups. The CS-induced increase in viral particle levels in cultures from COPD patients nearly reached statistical significance (Fig. 3B). This apparent difference between shedding of infectious RV16 particles and viral load assessment by qPCR requires further investigation.

RV16 exposure significantly induced expression of the cytoplasmic viral RNA sensors RIG-1 and MDA5 in COPD-derived ALI-PBEC cultures, but not in CTRL-derived ALI-PBEC. This effect was unaffected by CS exposure (Fig. 3C). RV16 exposure induced a non-significant increase in IFNβ expression, which followed a similar trend as RIG-I and MDA5 expression (Fig. 3C). The expression of interferon-stimulated genes Viperin and ISG15 was increased by RV16. This effect was unaffected by CS exposure (Fig. 3C). Taken together, these results suggest that CS exposure mainly affects RV16-induced responses in COPD-derived ALI-PBEC.
Figure 3. Whole cigarette smoke exposure differentially alters the response of COPD ALI-PBEC to RV16. ALI-PBEC from COPD and CTRL donors were exposed to air or CS, followed by 1 h infection with RV16 (MOI1) and 24 h incubation. (A) RV16 gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM normalized expression compared to incubator control with n = 8-9 independent donors. (B) Viral titration was performed on the apical wash and reported as TCID50/ml. (C) IFNß, Viperin, RIG-I, MDA5 and ISG15 gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to incubator control with n = 8 independent donors.*p < 0.05, **p < 0.01. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; MOI, multiplicity of infection; PBEC, primary bronchial epithelial cells; RV16, rhinovirus 16; TCID50, 50% tissue culture infective dose.
Whole cigarette smoke exposure differentially alters cytokine secretion of ALI-PBEC following RV16 exposure

Whole cigarette smoke (CS) exposure mainly affected RV16-induced responses in COPD-derived ALI-PBEC compared to ALI-PBEC from controls. To investigate whether this effect was also observed when investigating cytokine protein release, ALI-PBEC from 8 control and 8 COPD donors were exposed TNF-α for 24 h or to air or CS followed by infection with 1 MOI of RV16 for 1 h followed by 24 h incubation. CXCL8 and CXCL10 release following TNF-α stimulation was similar in ALI-PBEC from control and COPD patients (Fig. 4A). CXCL8 was increased by CS exposure, but remained unaffected by RV16 exposure in ALI-PBEC of both control and COPD patients. Baseline secretion of CXCL8 appeared to be lower in ALI-PBEC of COPD patients compared to controls, but this effect was not significant (Fig. 4B). CXCL10 was detected only in RV-16 exposed ALI-PBEC. CS exposure appeared to lower RV16-induced CXCL10 secretion in both control and COPD ALI-PBEC, but this did not reach statistical significance (Fig. 4B). Overall, CXCL8 and CXCL10 secretion did not appear to be differentially regulated in ALI-PBEC of COPD patients compared to controls.
Figure 4. Effect of RV16 infection and whole cigarette smoke exposure on secretion of CXCL8/IL-8 and CXCL10/IP10 protein secretion of ALI-PBEC from COPD patients and controls. (A) ALI-PBEC from COPD and control donors were exposed to TNF-α for 24 h or (B) air or CS, followed by 1 h infection with RV16 (MOI1) followed by 24 h incubation. Protein quantification of CXCL8 and CXCL10 was performed by ELISA. Statistical significance is indicated as *p<0.05, **p<0.01 and ***p<0.001. ALI, air-liquid interface; CS, whole cigarette smoke; CTRL, control; MOI, multiplicity of infection; ND, none detected; PBEC, primary bronchial epithelial cells; RV16, rhinovirus 16.
DISCUSSION

In this study we aimed to compare responses to bacterial exposure and rhinovirus infection between primary bronchial epithelial cells (PBEC) from COPD patients cultured at air-liquid interface (ALI). Cigarette smoke (CS) exposure reduced NTHi-induced expression of the antimicrobial peptide *DEFB4* and decreased the viral titer in the supernatant of rhinovirus (RV) infected cells. Moreover, ALI-PBEC cultures from COPD patients and controls showed no significant differences in their responses to NTHi exposure or RV16 infection.

Cigarette smoking is considered the main risk factor for the development of COPD. Cigarette smoking has been shown to affect various epithelial cell functions through a range of different mechanisms. Cigarette smoke causes oxidative stress and we previously showed the cigarette smoke-mediated induction of *HMOX1* in cells from control donors cultured at the air-liquid interface (19). Here we observed a lower induction of *HMOX1* in COPD compared to control donors, which was not statistically significant. Cigarette smoke can also activate toll-like receptors through endotoxin contamination present in tobacco (20, 21). Furthermore, PBEC from active smokers have impaired antiviral responses (22). In a previous *in vitro* study from our group, NTHi stimulation of COPD patients resulted in a lower antimicrobial activity and a lower expression of *DEFB4A* compared to control patients at 24 h after treatment with NTHi (10). In the same study, exposure to cigarette smoke impaired the NTHi-induced *DEFB4A* expression at different time points and hBD2 release at 24 h after treatment with bacteria. In line with these findings, our results indicate that cigarette smoke dampens the innate immune response to NTHi, as suggested by the reduced *DEFB4* expression at 3 h after bacteria-treatment. In contrast, we observed a higher expression of *DEFB4A* in COPD compared to control patients after NTHi stimulation and this difference was significantly higher after TNFα treatment. Difference between ours and previous findings on the NTHi-induced expression of *DEFB4A* in control and COPD patients might be explained by the different time of incubation after stimulation. *DEFB4* expression has previously been shown to be lower in patients with smoking history during acute pneumonia (23), which is in line with our results *in vitro* using whole cigarette smoke exposure. Cigarette smoke synergistically increased the integrated stress response induced by bacterial stimulation in both ALI-PBEC of control and COPD patients. Since we only investigated expression of *GADD34*, we cannot rule out that its expression was part of the unfolded protein response to endoplasmic reticulum stress, instead of the integrated stress response. Interestingly, in a model of whole exposure to diesel exhaust followed by NTHi treatment, ALI-PBEC of
control and COPD patients showed similar responses to those induced by cigarette smoke (24). Diesel exhaust also reduced the NTHi-induced expression of \textit{DEFB4A} but this inhibition was only significant in cultures from COPD patients. Both diesel exhaust and cigarette smoke increased the NTHi-induced expression of \textit{GADD34} (24). Cigarette smoke has been shown to increase adhesion of \textit{Haemophilus influenzae} to airway epithelial cells, which may help to explain the synergistic effect on the integrated stress response following bacterial infection (25). Overall, these data suggest that cigarette smoke may alter epithelial responses to bacterial infection.

Bronchial epithelial cells are the primary site for RV infection, indicating an important role for bronchial epithelial cells and their innate immune response against inhaled respiratory viruses (26). After entering the cell, viral RNA is recognized by cytoplasmic RIG-I-like receptors that recognize viral double-stranded RNA which subsequently trigger antiviral innate immune responses and induce type I interferon production (27). Important RIG-I-like receptors are RIG-1 and MDA5, which induce type I and type III interferons following viral infection. Interferons can then activate the expression of multiple interferon-responsive genes, including \textit{CXCL10, ISG15} and \textit{Viperin} (28, 29). Our results showed no obvious effects of CS exposure on RV16-induced expression of \textit{MDA5}, \textit{RIG1} and their downstream effectors \textit{IFN\textbeta}, \textit{ISG15} and \textit{Viperin}. It has been shown that PBEC from COPD patients have impaired induction of type I IFN, resulting from reduced protein kinase R (PKR) and decreased PKR-mediated stress granules, leading to increased viral replication after viral infection (30). Viperin, an interferon-responsive gene, can be induced by human rhinoviruses and has been shown to inhibit viral replication by inhibiting viral proteins that are required for viral assembly and maturation (29, 31). Whole cigarette smoke exposure did not inhibit \textit{Viperin} expression 24 h after infection. Results by Proud and colleagues showed that \textit{Viperin} expression is continuously increased even 48 h after infection, suggesting that we may have to investigate Viperin expression at later time points. Taken together, our results are not in line with current reported findings that show increased susceptibility to viral infections in COPD patients (7, 32-34). Nonetheless, we did observe a decreased viral titer following CS exposure in RV infected cells. Our \textit{in vitro} smoke exposure setup may require further adaptations to mimic \textit{in vivo} exposure more closely. Alternatively, our antiviral data is mostly limited to gene expression data that may not reflect actual protein expression.

Total viral RNA of RV16 showed a non-significant increase after CS exposure compared to air-exposed control ALI-PBEC. Primary bronchial epithelial cells of COPD patients have previously
been shown to have increased expression of antiviral and pro-inflammatory genes following human rhinovirus compared to healthy controls (35, 36). Various factors may contribute to the observed differences in the results. Various human rhinovirus serotypes exist and have been used for research, which may elicit different cellular responses. Additionally, our controls may have a smoking background whereas the aforementioned studies used non-smoking controls. Furthermore, Baines and colleagues used submerged cultures of primary bronchial epithelial cells, which do not form a pseudostratified epithelial layer. Submerged epithelial layers consist mainly of dividing basal cells, whereas a pseudostratified epithelial layer consists of multiple epithelial subtypes, which resembles the in vivo epithelium more closely (37). Moreover, basal cells have been shown to be more susceptible to RV infection (38). Overall, our data suggests that the reduced antiviral response in airway epithelial cells of COPD patients, but not in controls, may result from epigenetic mechanisms as these effects are preserved in culture.

The airway epithelium of COPD patients is exposed to persistent airway inflammation with increased numbers of inflammatory cells, despite smoke cessation in many patients (39). Various reports have indicated differences in epithelial characteristics between control and COPD patients. Airway epithelial cells of COPD patients have been shown to release more CXCL8 compared to smoking controls (40). This cytokine plays an important role in neutrophil recruitment to the airways. Additionally, rhinovirus induced-CXCL10 production in airway epithelial cells was shown to be reduced by cigarette smoke exposure (41-43). Our results do not recapitulate these findings for increased CXCL8 release in airway epithelial cells of COPD patients and reduced CXCL10 expression following RV16 infection after smoke exposure. This may be explained by differences in culture methods and the use of whole cigarette smoke exposure rather than an aqueous extract of cigarette smoke. Furthermore, since most of our donors were (ex-)smokers, the observed effects may be attributed to the effect of cigarette smoking rather than patient status.

Strengths of our study are the use of primary epithelial cells derived from multiple donors with and without COPD. Additionally, we used ALI differentiated cells to allow mucociliary differentiation, rather than using cell lines that do not differentiate and display other abnormalities. Finally, we used freshly prepared mainstream whole cigarette smoke containing both the gaseous and particulate components instead of the widely used aqueous extracts of cigarette smoke. Aqueous extracts of cigarette smoke fail to capture the complexity of whole cigarette smoke and also lack the presence of volatile compounds.
Nevertheless, this study also has some limitations. Bronchial epithelial cells were derived from macroscopically normal resected tissue obtained during surgery for lung cancer from patients that were largely (ex-)smokers. Furthermore, epithelial cells from healthy non-smoking controls were not available to further evaluate the effect of whole cigarette smoke on bacterial and viral infection in ALI-PBEC.

In conclusion, our results indicate that cigarette smoke differentially affects bacterial exposure responses in airway epithelial cells, but these effects do not appear to differ in airway epithelial cells from COPD patients compared to controls. The observation that cigarette smoke synergistically increases the integrated stress response by *Haemophilus influenzae* implies this pathway as a possible therapeutic target in COPD patients. Our study however could not confirm existing reports on reduced antiviral responses in airway epithelial cells from COPD patients. Collectively, our results provide novel insight in the interaction between cigarette smoke exposure and subsequent bacterial or viral infection that is relevant for COPD pathogenesis.
REFERENCES

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